Chapter 12

Antiviral Immunity in the Fruit Fly, *Drosophila melanogaster*

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Abstract

The fruit fly, *Drosophila melanogaster*, is an extremely useful model to study innate immunity mechanisms. A fundamental understanding of these mechanisms as they relate to various pathogens has come to light over the past 30 years. The discovery of Toll-like receptors and their recognition of shared molecules (pathogen-associated molecular patterns or PAMPs) among pathogenic bacteria were the first detailed set of receptors to be described that act in innate immunity. The immune deficiency pathway (Imd) described in *D. melanogaster* functions in a very similar way to the Toll pathway in recognizing PAMPs primarily from Gram-negative bacteria. The discovery of small interfering RNAs (RNAi) provided a means by which antiviral immunity was accomplished in invertebrates. Another related pathway, the JAK/STAT pathway, functions in a similar manner to the interferon pathways described in vertebrates, also providing antiviral defense. Recently, autophagy was also shown to function as a protective pathway against virus infection in *D. melanogaster*. At least three of these pathways (Imd, JAK/STAT, and RNAi) show signal integration in response to viral infection, demonstrating a coordinated immune response against viral infection. The number of pathways and the integration of them reflect the diversity of pathogens to which innate immune mechanisms must be able to respond. The viral pathogens that infect invertebrates have developed countermeasures to some of these pathways, in particular to RNAi. The evolutionary arms race of pathogen vs. host is ever ongoing.

Keywords: antiviral immunity, autophagy, innate immunity, RNAi, virus

1. Introduction

1.1. The fruit fly, *Drosophila melanogaster*, as a model for innate immunity

Immunity is a vital component in understanding host-pathogen relationships. It is composed of two responses: innate and adaptive. Innate immunity recognizes morphological characteristics of pathogens for immediate antimicrobial and antiviral defense [1]. Adaptive immunity develops during infection to produce immunological memory against pathogens. This memory provides an immediate pathogen-specific defense against future infections of the same pathogen [2]. Most vertebrate organisms utilize both immune responses for pathogen defense. However, the fruit fly, *Drosophila melanogaster,* does not have an adaptive immune response and relies solely on an innate immune response [3]. This provides a powerful model system to better understand the interaction between innate immunity and pathogenic infections.

Innate immunity is composed of various pathways that target bacteria, fungi, and viruses. These pathways include the immune deficiency pathway (Imd), Toll-Dorsal pathway (Toll), Janus kinase/signal transducer and activator of transcription pathway (JAK/STAT), autophagy, and RNA interference (RNAi) [3–6]. The Imd and Toll pathways contribute to the antibacterial and antifungal defense. However, their function in antiviral defense is not fully understood [7, 8]. The JAK/STAT, autophagy, and RNAi pathways contribute to antiviral defense, with RNAi as the main contributor for antiviral defense.

1.2. *Drosophila* viruses

As a model organism, *D. melanogaster* is used to study host immunity to pathogen interactions. Most research is focused on the interaction between bacteria, fungi, and the *D. melanogaster* innate immune response, but viruses are a subject of current interest. Populations of *Drosophila* have naturally occurring infections of RNA viruses, such as Nora virus, Sigma virus (DmelSV), Drosophila C virus (DCV), and Drosophila X virus (DXV). In addition, the first naturally occurring DNA virus, Kallithea virus, is found in *D. melanogaster* (**Table 1**) [9–13].

Nora virus is a recently discovered picorna-like *D. melanogaster* virus. The virus is sequenced and has a 12 kilobase (kb), single-stranded, positive-sense RNA genome. Viral particles measure 30 nm in diameter and are non-enveloped [9]. It establishes a persistent infection in natural and laboratory populations of *D. melanogaster* with no known effect of viral load and no display of pathology on the fly. The virus is transmitted horizontally through the fecal-oral route with infection localizing to the intestinal tract [14]. The genome is organized into four open reading frames (ORFs), unlike other picorna-like viruses such as DCV, which has two ORFs [15]. ORF1–3 partially overlaps, suggesting ribosomal frame shifting events during translation. However, an 88 nucleotide region is found between ORF3 and ORF4, suggesting that an independent initiation translation event is occurring [16]. ORF1 encodes a highly charged protein, which is a suppressor of RNAi [17]. ORF2 encodes a picorna-like replicative cassette, which consists of a helicase, protease, and RNA-dependent RNA polymerase [9]. The hypothesized major capsid proteins of Nora virus are products of ORF3 and ORF4 at the 3′ end of the genome. ORF3 encodes VP3, which is crucial for the stability of Nora virus virions [18]. ORF3 is not fully

Virus	Family	Genome nucleic acid	Mode of transmission in *D. melanogaster*	Effects of infection
Nora virus	*Picornavirales*	(+) ssRNA	Horizontal	No documented pathology, slight effect on longevity [14]
Sigma virus (DmelSV)	*Rhabdoviridae*	(−) ssRNA	Vertical	Anoxia sensitivity [10]
Drosophila C virus (DCV)	*Dicistroviridae*	(+) ssRNA	Horizontal	Intestinal obstruction [21] and increased female fecundity and reduced developmental timing [73]
Drosophila X virus (DXV)	*Birnaviridae*	dsRNA	Horizontal	Anoxia sensitivity [12]
Cricket paralysis virus (CrPV)	*Dicistroviridae*	(+) ssRNA	Horizontal	No documented pathology
Flock house virus (FHV)	*Nodaviridae*	(+) ssRNA	Horizontal	High mortality [34]
Sindbis virus (SINV)	*Togaviridae*	(+) ssRNA	Vertical	No documented pathology
Vesicular stomatitis virus(VSV)	*Rhabdoviridae*	(−) ssRNA	Horizontal	Anoxia sensitivity [30, 40]
Kallithea virus	*Nudiviridae*	dsDNA	Horizontal	No documented pathology
Invertebrate iridescent virus 6 (IIV-6)	*Iridoviridae*	dsDNA	Horizontal	Low mortality rate [31]

Table 1. Characteristics of a set of *Drosophila* viruses.

characterized, but certain aspects of its protein products were predicted using bioinformatics. It has a predicted alpha-helical domain as a key structural motif [9]. ORF4 is processed into three major proteins, VP4A, VP4B, and VP4C. VP4A and VP4B are predicted to form jelly roll folds, which are also found in other capsid proteins of *Picornavirales*. The third protein, VP4C, has a predicted alpha-helical structure and is also a structural component of the virus [16].

Another virus naturally occur in *D. melanogaster* is Sigma virus. Sigma virus belongs to the family *Rhabdoviridae* [10]. It is composed of a negative-sense, single-stranded RNA genome that consists of five genes: *N, P, M, G,* and *L.* The gene *N* is a nucleoprotein, *P* is the polymerase-associated protein, *M* is the matrix protein, *G* is the glycoprotein, and *L* is the polymerase [19]. A sixth gene, *X*, exists between *P* and *M,* but its current function is not fully understood [20]. In natural infections, the virus causes paralysis or death if flies are exposed to CO_2. It is passed through vertical transmission through the sperm or eggs and is the only known host-specific pathogen of *D. melanogaster* [10, 20].

Drosophila C virus is in the family *Dicistroviridae* [21]. The virus particle measures 30 nm in dm with a 9264 kb, positive-sense, single-stranded RNA genome [22]. The genome consists of two ORFs separated by 191 nucleotides. ORF1 encodes an RNA-dependent RNA polymerase, helicase domain, and protease domain [15]. Also, an RNAi suppressor, DCV-1A, is encoded at the N-terminus of ORF1. The suppressor binds long dsRNA, which inhibits Dicer-2 (Dcr-2) processing [23]. ORF2 encodes the structural proteins VP0, VP1, VP2, VP3, and VP4. VP0 is a precursor for VP3 and VP4, which combine to form the capsid [24]. The capsid proteins are encoded in a different reading frame and initiated independently from ORF1 [15]. In addition, DCV is a naturally occurring pathogen found within *D. melanogaster* and spread through horizontal transmission by infected flies or contaminated food. Viral infection can be lethal if injected, but naturally infected flies display decreased pathogenicity [11].

Drosophila X virus is a double-stranded RNA virus, which belongs to the family *Birnaviridae*. It was discovered in a study involving *D. melanogaster* and Sigma virus. Like Sigma virus, DXV is pathogenic, induces CO_2 sensitivity, and is lethal [12]. The virus displays a non-enveloped capsid and a bi-segmented dsRNA genome. Segment A encodes a polyprotein, which forms the capsid. The capsid consists of VP1, preVP2, VP2, VP3, and VP4. Segment B encodes VP1, an RNA-dependent RNA polymerase [25].

Recently, a DNA virus was discovered in wild populations of *Drosophila*. By using a metagenomic approach, the Kallithea virus was identified. The virus is closely related to *D. innubila* and the beetle *Orcytes rhinoceros* Nudiviruses. In addition, this is the first DNA virus found naturally occurring in *D. melanogaster*. However, the virus has not been characterized in *D. melanogaster* with recent research using other *Drosophila* species [13]. In wild *D. innubila*, Nudivirus infection is associated with greatly reduced survival and offspring production. In wild *D. falleni*, infection resulted in greatly reduced offspring production. Additionally, infection is highly pathogenic and mediated through the fecal-oral route [26]. Further research with naturally occurring *Drosophila* viruses is important because not many of these viruses exist or have been discovered.

1.3. Non-*Drosophila* viruses

Laboratory populations of *D. melanogaster* can be experimentally inoculated with RNA viruses, such as Cricket paralysis virus (CrPV), Flock House virus (FHV), Sindbis virus (SINV), and Vesicular stomatitis virus (VSV). Also, the DNA virus, Invertebrate iridescent virus 6 (IIV-6), can be experimentally inoculated into flies (**Table 1**) [27–31]. Artificial infections of *D. melanogaster* allow for a better understanding and novel insights of host-pathogen interactions.

Cricket paralysis virus is a positive-sense, single-stranded RNA virus closely related to DCV. It belongs to the family *Dicistroviridae* and was first discovered in field crickets, *Teleogryllus oceanicus* and *T. commodus* [32]. The crickets displayed rapid paralysis and significant mortality [27]. The viral RNA genome consists of two ORFs, ORF1 and 2. To initiate translation, each ORF requires an internal ribosome entry site (IRES) region. ORF1 encodes non-structural replication proteins, and ORF2 encodes structural proteins, which form the viral capsid. In addition, this virus encodes a suppressor of RNAi, CrPV-1A, which binds to Argonaute-2 (AGO2) inhibiting RNA-induced silencing complex (RISC) activity [32].

Flock house virus contains two positive-sense, single-stranded RNAs within a non-enveloped virion. This virus belongs to the *Nodaviridae* family and was first discovered in the grass grub, *Costelytra zealandica* [28, 33]. Viral inoculation kills *D. melanogaster*, and the virus propagates in *D. melanogaster* cell lines [34]. The bipartite genome consists of *RNA1* and *RNA2*. *RNA1* encodes protein A, an RNA-dependent RNA polymerase, whereas *RNA2* encodes the precursor protein for production of the mature capsid protein. For viral replication, both RNAs must be present within the cell or replication will not occur [35]. A subgenomic RNA, *RNA3*, is produced by *RNA1* and encodes an RNAi suppressor protein B2. The protein binds viral dsRNA to protect it from cleavage by Dcr-2 and to inhibit loading of viral siRNAs into the RISC complex [34, 36, 37].

Sindbis virus is a single-stranded, positive-sense RNA virus, belongs to the *Togaviridae* family, and is transmitted vertically in *Drosophila*. In other hosts, it is transmitted horizontally. The viral genome mimics cellular mRNA as the viral mRNA possesses a 5′ methylguanylate cap and a 3′ poly(A) tail. The 5′ region encodes nonstructural proteins, and the 3′ region encodes viral structural proteins [38]. Most Sindbis virus research with invertebrates is conducted with mosquitoes because they are a natural vector for SINV. However, *D. melanogaster* S2 (Schneider 2) cells are successfully infected establishing an additional invertebrate model system to examine the host-pathogen interaction with SINV [29].

Vesicular stomatitis virus is a single-stranded, negative-sense RNA virus that belongs to the Rhabdoviridae family [30]. It belongs to the same family as Sigma virus, which naturally occurs in *Drosophila* [39]. The genome is composed of the structural proteins (G, N, and M), the minor protein (NS), the partially glycosylated G precursor (G_1), and the L chain. Insects infected with VSV become paralyzed after exposure to CO_2. However, VSV has no observable pathogenic effects in infected insect cells [30, 40].

Invertebrate iridescent virus 6, also known as Chilo iridescent virus, is a large and complex double-stranded DNA virus that belongs to the *Iridoviridae* family. The virus is composed of a capsid, an intermediate lipid layer, and a viral genome composed of linear double-stranded DNA [41]. The viral genome size is approximately 212.5 kb, circular, and encodes 211 ORFs along both strands [31]. Several important ORFs encode a DNA-dependent RNA polymerase II, a helicase, and major capsid proteins [42]. IIV-6 has a broad host range and can be used to experimentally infect *D. melanogaster*. Infections in *D. melanogaster* produce high and stable viral titers exhibiting a low mortality rate [31]. Artificial infections of *D. melanogaster* are important because they provide a valuable model of understanding interactions between virus and host immunity.

2. RNA interference (RNAi) and the immune response

2.1. Antiviral RNAi in *D. melanogaster*

RNAi is the major antiviral immune response pathway for *D. melanogaster* (**Figure 1**). The general pathway occurs in two steps, initiation and execution. To initiate RNAi, dsRNA must be introduced, such as with viral infection. If dsRNAs are greater than 23 bp in length, it is

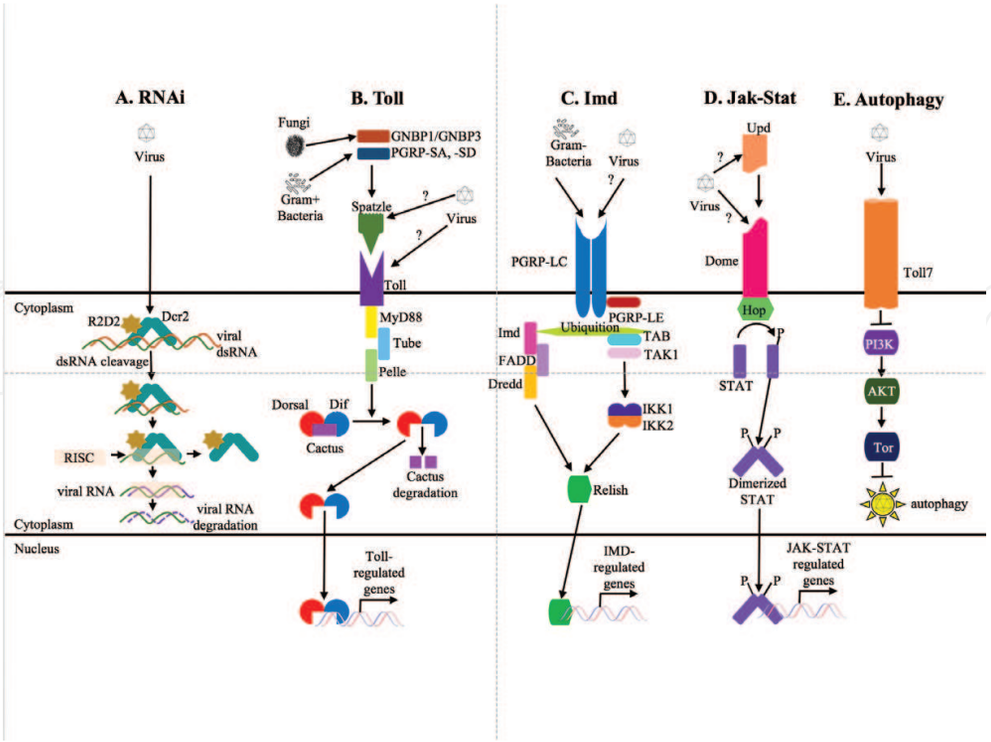

Figure 1. The major virus defense pathways of the fruit fly, *Drosophila melanogaster*. (A) RNA interference (RNAi) is the primary defense mechanism against viruses in invertebrate species. Virus replication results in the production of dsRNA replication intermediates that activate the pathway. R2D2 has two binding sites for dsRNA and in conjunction with the RNaseIII-like enzyme, Dicer-2 (Dcr2), will cleave large dsRNAs into small interfering RNAs (siRNA). The Dcr2/R2D2 siRNA complex subsequently interacts with Argonaut-2 protein, a key component of the RNA-induced silencing complex (RISC), and transfers the siRNA component to it. The siRNA acts by targeting viral RNA via base pairing, allowing the targeted viral RNA to be degraded by the nuclease action of RISC. (B) The Toll pathway is activated primarily by pathogen-associated molecular patterns (PAMPs) associated with fungi and Gram-positive cell wall components. The PAMPs are recognized by cytoplasmic receptors, such as Gram-negative bacteria binding proteins (GNBP-1/-3) and peptidoglycan recognition proteins (PGRP-SA, -SD). These receptors are referred to collectively as pattern recognition receptors (PRRs). Once the PRRs are engaged by their specific PAMPs, they now activate proteases that cleave full-length Spatzle to an active form that now can be bound by the Toll receptor. With virus activation of this pathway, it is unclear whether virions can directly interact with Toll or must also activate Spatzle. The binding of the Spatzle ligand to the Toll receptor results in signal transduction through the cytoplasmic adaptor protein, MyD88. This ultimately leads to the proteolytic degradation of Cactus, the inhibitor of the NF-κB-like transcription factors Dorsal and Dif. With the degradation of Cactus, the Dorsal-Dif heterodimer is now able to be transported to the nucleus where it acts to activate the transcription of Toll-regulated genes. (C) The Imd pathway is activated by PAMPs from Gram-negative bacteria and potentially directly by virions. A transmembrane peptidoglycan receptor protein (PGRP-LC) binds the PAMPs and transduces a signal to the cytoplasmic adaptor proteins Imd and FADD, which results in the activation of the caspase-8 like protease, Dredd. Dredd cleaves the NF-κB-like transcription factor, Relish, which removes an IκB-like C-terminal domain that masks a nuclear localization signal. In addition, Dredd also cleaves Imd, which now allows it to become ubiquitinated. This attracts the Tab2/Tak1 complex that activates the IKK1/IKK2 proteins via phosphorylation. These activated kinases now phosphorylate Relish at multiple sites, especially S528 and S529, which are essential to RNA polymerase II recruitment to Imd-regulated genes. (D) The JAK/STAT pathway is activated by the interaction of the ligand unpaired (Upd) with the receptor Dome. In the *Drosophila* immune response, it appears that Upd3, secreted by activated hemocytes, is the preferred ligand for Dome. Most likely, virions are detected by these cells, which in turn secrete Upd3, although direct interaction of virions with Dome has not been ruled out. Once Dome has engaged Upd, it activates, via signal transduction, the Janus kinase Hop, which now is capable of phosphorylating the STAT transcription factors. Phosphorylation of the STAT proteins results in their dimerization and subsequent translocation to the nucleus where they activate the transcription of JAK/STAT regulated genes. (E) Autophagy can also act as a viral defense pathway. In the absence of a ligand for Toll-like receptor 7 (Toll7), the signal transduction pathway involving phosphatidylinositol-3 kinase (PI3K), Akt kinase, and Tor (target of rapamycin) kinase is active and autophagy is inhibited. However, if the Toll-7 receptor is engaged by its ligand, in this case a virion component, this results in the inhibition of PI3K, which ultimately results in the inhibition of Tor, which now relieves inhibition of the autophagy pathway, resulting in the destruction of the cell.

processed into 21–23 bp dsRNA fragments with 3′ overhanging ends by Dcr-1 or Dcr-2 [5]. Dcr-2 produces small interfering RNAs (siRNAs), and Dcr-1 recognizes precursors of micro RNAs (miRNAs). The siRNA products are recruited by AGO2 into the RISC. Once loaded, one of the siRNA strands is degraded in an AGO2-dependent process involving an endoribonuclease, component 3 promoter of RISC (C3PO) [43]. The single strand in the RISC complex is called the guide strand. It acts as a targeting mechanism for locating complementary mRNA. Matching of the guide strand to the targeted mRNA results in either degradation or inhibition of translation. Degradation occurs if the guide strand completely matches the target mRNA. However, inhibition of translation occurs if there is a small mismatching of base pairs (2–3 bp) [5]. Additionally, RNAi is incorporated in two alternative pathways: the miRNA or piwi RNA (piRNA) pathways. In the miRNA pathway, miRNA and Argonaute-1 (AGO1) regulate cellular gene expression through different mechanisms, such as cleavage or translational inhibition [44]. The piRNA pathway is involved as a transposon regulatory control mechanism in *D. melanogaster* testes [45]. However, the siRNA pathway is the major contributor to the RNAi antiviral defense pathway in *D. melanogaster*.

2.2. Viral suppression of RNAi

RNAi is an effective antiviral mechanism, but viruses have developed strategies to counteract it using virus-encoded suppressors of RNAi (VSRs). RNAi suppression depends on the mechanism the VSR uses to target RNAi components and can vary with each virus [16]. For example, Nora virus VP1, the protein product of ORF1, can suppress RNAi. It inhibits slicer activity of mature RISC by hindering targeted catalytic cleavage by AGO2 [46]. In FHV, *RNA1* produces a subgenomic *RNA3*, which encodes B2, an RNAi suppressor protein. B2 has dual functions for suppression. It binds to long dsRNA to inhibit siRNA production and to siRNA to prevent siRNA assembly into RISC [47]. In CrPV, the N-terminal region of ORF1 encodes the RNAi suppressor protein, CrPV-1A. It directly interacts with AGO2, which suppresses the catalytic activity of the RISC complex [32]. In the DNA virus IIV-6, *ORF340R* encodes a dsRNA-binding domain (dsRBD), which binds dsRNA. For evasion and suppression, the dsRBD binds to long dsRNA shielding it from Dcr-2 processing and inhibiting siRNA loading into the RISC complex, respectively [48]. Viral suppression of RNAi creates an ongoing arms race between viruses and the RNAi pathway. As the RNAi pathway adapts to evade viral infections, viruses counter adapt to evade viral antagonists, which leads to further adaptions of the RNAi pathway [16]. However, RNAi does not clear all viral infections in *D. melanogaster* suggesting that other alternative antiviral mechanisms must exist.

2.3. Vago acts as an RNAi-independent antiviral mechanism

During viral infection of *D. melanogaster*, genes are triggered and expressed. One gene of interest is *Vago,* a 160-amino acid protein, with a signal peptide and eight cysteine residues. The signal peptide contains a single von Willebrand factor type C (VWC) motif. Proteins containing a single VWC domain typically respond to environmental changes and nutritional status, such as viral infection [49]. In *D. melanogaster*, *Vago* functions in response to viral infection [50]. During DCV infection, Vago proteins are important in controlling viral load in the fat body of *D. melanogaster*, which suggests that it may have a tissue-specific role. Also, Vago may act as either an antiviral molecule targeting virions or as a cytokine affecting neighboring cells

by triggering an antiviral state [51]. Another gene of interest is *virus-induced RNA 1* (*vir-1*). This gene is a marker of viral regulation that is regulated by the JAK/STAT pathway [52]. A potential mechanism is suggested including both genes. Viral infection triggers the induction of a cytokine, Vago, which activates the JAK/STAT pathway (**Figure 2**). Once activated, virus-related gene expression is induced, which includes *vir-1* [51].

Currently, the pathway for activation of Vago begins with induction of RNAi. First, viral infection is detected by Dcr-2. Dcr-2 is a viral sensor, which activates the RNAi pathway and *Vago* expression for antiviral defense (**Figure 2**). For *Vago*, viral RNA interacts with the DExD/H-box helicase domain on Dcr-2 activating an inducible antiviral response. This domain is located at the carboxy-terminal end of the gene and acts as a cytoplasmic sensor of viral RNA [51]. The DExD/H-box helicase domain also belongs to the same family as the retinoic acid-inducible gene 1-like (RIG-I) receptors in mammals, which function as pattern recognition receptors for

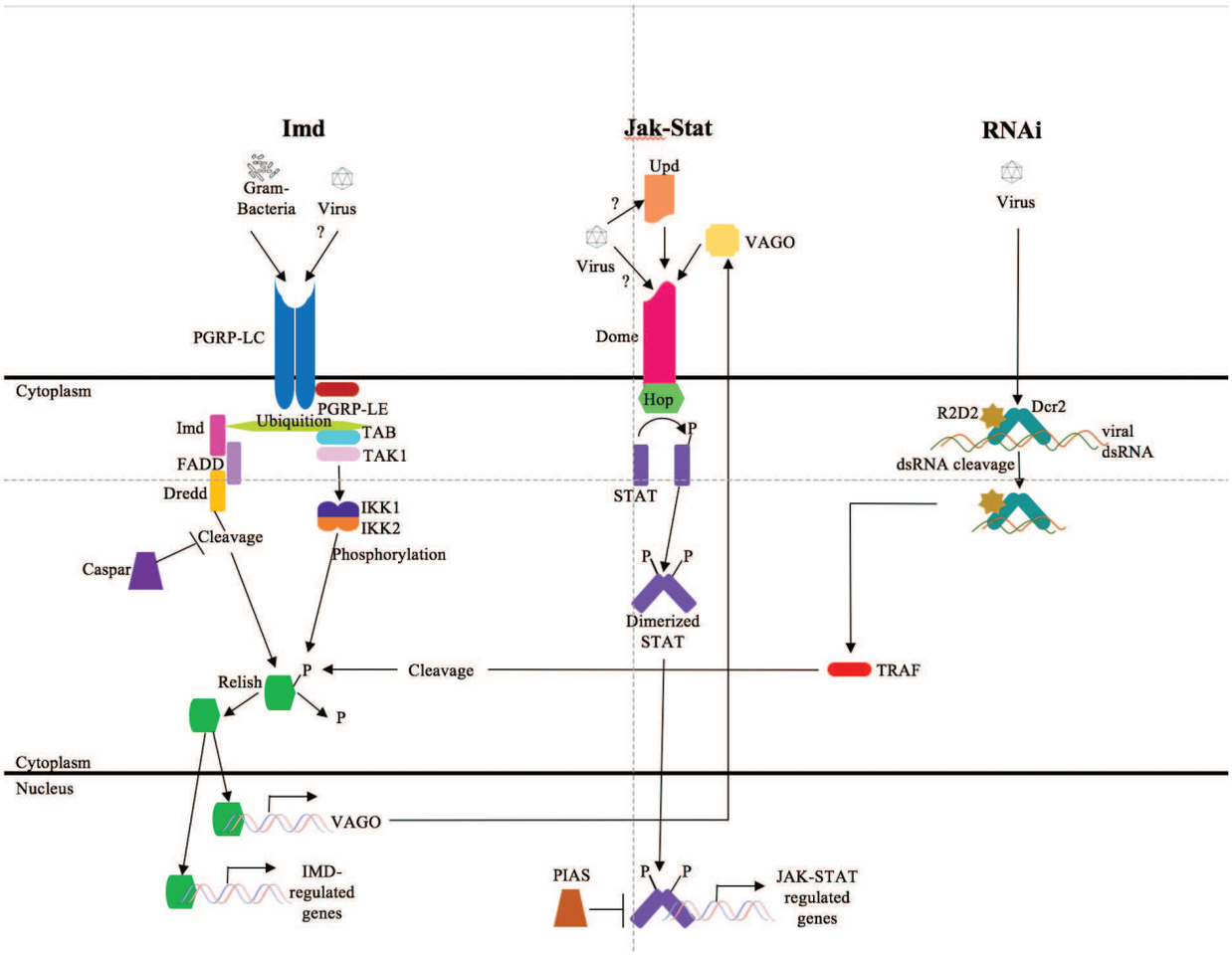

Figure 2. Innate immune signaling among several pathways is integrated. The Imd, JAK/STAT, and RNAi virus defense pathways exhibit coordinate expression of anti-viral genes in *Culex* mosquitoes. The RNAi pathway (see **Figure 1**) through the sensing of dsRNA by Dicer-2 activates tumor necrosis factor (TNF) receptor-associated factor (TRAF). TRAF now interacts with the Imd pathway via driving proteolytic cleavage of the N-terminal region of Relish, allowing the C-terminal region of Relish to be transported into the nucleus where it acts as a transcription factor on IMD-regulated genes. One of these genes is *Vago*, which specifies a small secretory cytokine-like molecule. Vago is able to engage the JAK/STAT pathway via the Dome receptor (see **Figure 1**), leading to the expression of JAK/STAT regulated genes.

intracellular dsRNA during viral infection [53]. In addition, other innate immunity pathways are analyzed to determine their role in the induction of *Vago.* However, Toll, Imd, and JAK/STAT were unable to induce *Vago* expression [51]. Currently, the mode of antiviral action of the protein Vago and its role in the RNAi pathway are not fully understood.

Recently, *Vago* was further investigated in the mosquito, *Culex quinquefasciatus.* The orthologue gene, *CxVago*, contributes to antiviral defense during West Nile virus (WNV) infection. In *C. quinquefasciatus,* Dcr-2 is also required for induction and up-regulation of *Cx*Vago. The study suggests that *Cx*Vago is a stable, secreted cytokine that stimulates an antiviral response in insects by activating the JAK/STAT pathway (**Figure 2**). In addition, *Cx*Vago induces expression of the *Culex* orthologue of the *D. melanogaster* gene *vir-1* during viral infection [49]. Another study was not able to establish a relationship between DCV-stimulated *Vago* and induction of *vir-1* in *D. melanogaster* [51]. However, *Vago* may induce vir-1 during viral infection, but in its absence, other unidentified cytokines may also induce vir-1 expression [49].

A mechanism for the activation of *Cx*Vago was proposed (**Figure 2**). First, Dcr-2 senses a viral infection and activates tumor necrosis factor receptor-associated factor (TRAF). This process activates Relish 2 (Rel2) by dephosphorylation, which allows translocation of the molecule from the cytoplasm into the nucleus. Rel2 is a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor and induces gene expression of *CxVago* [54]. However, *DmVago* is not induced in *D. melanogaster* by members of the NF-κB family. This may indicate that regulation of *DmVago* occurs through a similar or alternative mechanism [51]. The induction of *Vago* is similar to the RIG-I/TRAF-6/NF-κB-mediated interferon pathway, which is triggered by a viral infection in mammals [54]. Further analysis of the proposed *Cx*Vago pathway in *D. melanogaster* is required to discover the mechanism for antiviral defense. Vago and its associated pathway might be a simplistic interferon response pathway but requires an in-depth investigation to determine its role in antiviral defense.

3. Autophagy

Autophagy was first characterized in yeast following starvation, as a process by which cells can degrade long-lived proteins, organelles, and bulk cytoplasm for recycling [55]. Induction of autophagy is both developmentally and nutritionally regulated. When nutrients are sufficient, class I phosphatidylinositol-3-kinases (PI3Ks) and the target of rapamycin (TOR) complex act as inhibitors of autophagy. However, under starvation conditions, class III PI3Ks act to stimulate the production of autophagy-related proteins and induce the autophagy pathway [55] (**Figure 1**).

Following induction, a double-membrane vesicle, the autophagosome, is formed that can sequester cytoplasmic components. Sequestering of the cytoplasmic components is highly regulated by GTPases, phosphatidylinositol kinases, and other various phosphatases. The autophagosome then fuses with the lysosome for the breakdown of the membrane and its contents [56]. In addition, induction of autophagy can occur as an antiviral response during viral infection.

3.1. Antiviral autophagy

Autophagy also plays a direct antiviral role against vesicular stomatitis virus (VSV). *D. melanogaster* has homologs of 11 yeast autophagy-related genes and is confirmed for autophagy during development or starvation [57]. *D. melanogaster* encodes nine Toll receptors. Eight of the Toll receptors are not fully understood but may have roles in innate immunity. Activation of the autophagy pathway requires the interaction of Toll receptor 7, which detects VSV G protein. Once G protein is detected, two toll-7 receptors dimerize transmitting a signal through their toll-interleukin-1 receptor (TFR-1) domain [6]. The signal transduction is regulated by the Tor kinase, which leads to the induction of autophagy [56]. Autophagy can be induced under starvation conditions or high stress (i.e., viral infection) conditions. This becomes apparent when *D. melanogaster* S2 cells are infected with VSV and monitored using fluorescent microscopy for autophagy. Cells with mutant autophagy genes have a significantly higher viral titer than those that contain wild-type autophagy genes [57]. This indicates that autophagy not only plays a critical role in recycling of organelles and proteins during times of starvation, but it may also have an antiviral role as well.

4. Other antiviral response pathways

The Toll pathway controls the dorsal-ventral patterning within the *D. melanogaster* embryo and is activated during fungal and Gram-positive bacterial infections (**Figure 1**). During fungal and bacterial infection, pathogen recognition proteins (PRRs) recognize common molecules from each pathogen called pathogen-associated molecular patterns (PAMPs). Fungi are detected by their glucans by PRR glucan-binding protein 3 (GNBP3). Gram-positive bacteria are detected by their cell wall components that contain lysine-containing peptidoglycan. Recognition requires a combination of different proteins, including peptidoglycan recognition proteins (PGRP)-SA, PRGP-SD, and GNBP1 [58]. After recognition, the protein creates a complex, which activates the Toll pathway. PGRP-SD is not involved in the complex but is required for detection of certain strains of Gram-positive bacteria. Activation of Toll initiates a protease cascade activating Spätzle (Spz) [59, 60]. Spz is a protein ligand of the Toll receptor. Once activated, Spz induces conformational changes within the receptor to facilitate the recruitment of *Drosophila* Myd88, Tube, and Pelle, a protein kinase. This leads to the phosphorylation and degradation of Cactus and NF-κB-like transcription factors, which allows Dif (Dorsal-related immunity factor) to translocate to the nucleus. Dif mediates Toll-dependent gene expression of certain antimicrobial peptides (AMPs) [61]. There are seven specific AMPs identified in *D. melanogaster:* Drosomycin, Metchnikowin, Diptericin, Drosocin, Cecropin, Defensin, and Attacin [62]. Cecropin, Diptericin, Drosocin, Attacin, and Defensin are involved during bacterial infection, whereas Drosomycin and Cecropin are involved during fungal infection. Metchnikowin is involved in both forms of infection [63, 64]. These peptides are secreted into the hemolymph for antibacterial and antifungal defense.

Recently, Toll was found to elicit an antiviral response (**Figure 1**). A Dif^{1} fly mutant, which did not have a functional Toll pathway, developed higher DXV viral titers and higher mortality

when compared to wild-type flies. A gain-of-function Toll mutant, *Tl1°b*, developed a reduced DXV viral titer [65]. These results indicate that Toll may be involved in reducing viral replication of DXV and potentially other viral pathogens and warrants further characterization.

Another pathway involved in antibacterial defense is Imd. Imd has a similar mechanism as Toll but targets Gram-negative bacteria (**Figure 1**). The PAMPs for Gram-negative bacteria are diaminopimelic-containing peptidoglycan (DAP-type PGN), which are recognized by the PRRs, PGRP-LC, and PGRP-LE. This triggers the Imd intracellular signaling cascade [58]. The signaling cascade activates an NF-kB-like factor, Relish (Rel). The Rel domain of Relish translocates to the nucleus, binds to the kB site, and induces transcription of AMPs, regulating expression [3]. Imd and Toll share the same target genes but are activated by different pathogens. In addition, Toll and Imd interact with each other to regulate a coordinated and effective immune response.

The Imd pathway is implicated in an antiviral response in *D. melanogaster* (**Figure 1**). Loss-of-function mutant flies were created for different Imd pathways genes, such as *Rel* and *PGRP-LC*. These flies displayed increased sensitivity to CrPV and had higher viral loads than the controls [8]. The results indicate that Imd signaling may be involved in antiviral innate immune responses during CrPV infection and requires further research.

The JAK/STAT pathway is also involved in the *D. melanogaster* immune response (**Figure 1**). This pathway contributes to a systemic immune response, antiviral response, and regeneration of gut epithelium [52, 66, 67]. JAK/STAT consists of cytokine-like molecules Unpaired (Upd) and the Upd receptor Domeless (Dome), Hopscotch (Hop), the *D. melanogaster* homolog of vertebrate JAK, the signal transducer and activator of transcription protein at 92E (STAT92E), and suppressors of cytokine signaling (SOC3S6E) [4, 68–72]. For activation of the JAK/STAT pathway, Upd binds to Dome. This binding causes Hop to phosphorylate itself and the cytoplasmic tail of Dome [68, 72]. Phosphorylation of Dome allows for the binding of STAT92E proteins. STAT92E is phosphorylated, dimerized, and translocated to the nucleus where it binds and activates transcription. SOCS36E is a negative regulator of the JAK/STAT pathway. It inhibits activation by binding the JAK complex, preventing autophosphorylation [69, 71]. JAK/STAT is also implicated in antibacterial and/or antifungal defense, but its role in antiviral defense needs further investigation.

5. Conclusion

Viral pathogens infect all organisms, including insects. For successful infection, viruses must be able to replicate and evade host immunity. *D. melanogaster* must rely on innate immunity to combat infection. Viral infections are easily controlled and can develop a persistent infection with no apparent pathogenesis. However, this regulation of infection is poorly understood. An uncharacterized antiviral mechanism must exist, which may include *Vago*, but further research is needed. A better understanding of antiviral immunity is important because many of the factors and pathways are conserved among species. Further research with viruses, especially new viruses, will help promote a better understanding of host immunity to pathogen interactions.

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