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Insect antiviral innate immunity: pathways, effectors, and connections

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Abstract

Insects are infected by a wide array of viruses some of which are insect-restricted and pathogenic, and some of which are transmitted by biting insects to vertebrates. The medical and economic importance of these viruses heightens the need to understand the interaction between the infecting pathogen and the insect immune system in order to develop transmission interventions. The interaction of the virus with the insect host innate immune system plays a critical role in the outcome of infection. The major mechanism of antiviral defense is the siRNA pathway that responds through the detection of virus-derived dsRNA to suppress virus replication. However, other innate antimicrobial pathways such as Imd, Toll, Jak-STAT, and the autophagy pathway have also been shown to play important roles in antiviral immunity. In this review we provide an overview of the current understanding of the main insect antiviral pathways and examine recent findings that further our understanding of the roles of these pathways in facilitating a systemic and specific response to infecting viruses.

Introduction

Viruses are obligate intracellular pathogens with a limited coding capacity mandating the sequestration of cellular resources to promote their replication. Viruses that infect insects have huge consequences economically and medically. Insect transmitted arboviruses, such as the dengue viruses (DENV) and yellow fever virus (YFV), place billions of people across the globe at risk of life-threatening diseases. Obtaining a deeper understanding of the biology of the virus within the insect host and the host response to the virus provides the potential for the development of novel transmission interventions.

In insects, the innate response plays the major role in the control and clearance of pathogens following infection, although there is some evidence for an immune response that resembles the vertebrate adaptive response^{1; 2}. The innate immune system is characterized by the activation of pattern recognition receptors (PRRs) capable of binding pathogen-associated

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molecular patterns (PAMPs), molecules present in the pathogen but not found in the host. Binding of PAMPs leads to the activation of signaling pathways resulting in the production of effector molecules capable of suppressing pathogen replication. This system provides the first line of defense against invading pathogens. In insects, the innate response is robust and may function to clear infection in the case of true insect pathogens; however, in the case of arbovirus infection, the insect innate response limits pathogenesis but does not clear the infection allowing transmission of the virus to a vertebrate host. In fact, it can be seen that arbovirus infection of insects in which the innate immune system has been compromised can result in increased viral load, morbidity or mortality suggesting that the innate immune system is engaged and necessary for vector survival ^{3; 4; 5; 6; 7; 8; 9}.

When challenged with viruses, the most robust insect response is through the RNAi pathway that utilizes virus generated double-stranded RNA (dsRNA) to produce small, interfering RNAs (siRNA) that function to target viral RNA for degradation and hence inhibit replication ^{9; 10; 11; 12}. Additionally, signal transduction pathways resulting in changes in cellular gene expression are an important component of the antiviral innate immune system. Nf- κ B pathways, Toll and Imd, have been well characterized as essential in the immune response to bacteria and fungi (reviewed in Lemaitre *et al.* ¹³). Evidence from studies in *Drosophila* and mosquitoes indicates that these pathways also play a role in antiviral defenses ^{14; 15; 16; 17}. There is also growing evidence that the Jak-STAT pathway may be functionally analogous to the mammalian interferon system ¹⁸. This pathway is typically activated in uninfected bystander cells resulting in alterations in cellular transcription and downstream antiviral activity ⁴.

In vertebrates, the innate immune system signals for an immediate response to infection that potentiates a systemic and specific adaptive response resulting in immune memory. While insects lack an orthologous adaptive response it is becoming apparent that innate immune pathways are connected and give rise to a systemic antiviral immune response that is specific and has the potential to last beyond the duration of a given viral infection. In this review we will provide an overview of the current understanding of these antiviral pathways, and examine evidence for the connectivity of pathways and a systemic, specific antiviral response.

RNAi Antiviral Response

RNA interference in insects plays a significant role in limiting and controlling virus infection ^{9; 10; 11; 12}. There are currently three well characterized RNAi-related pathways (reviewed in Kim *et al.* ¹⁹): (i) small interfering RNA pathway in which siRNAs are generated from dsRNA derived either from exogenous sources such as virus infection or encoded by the cell genome, (ii) micro-RNA pathway in which miRNAs are generated from cell-encoded transcripts and ultimately function to regulate gene expression generally at the level of translation, and (iii) PIWI-interacting RNA pathway in which piRNAs are transcribed from the cellular genome and do not require processing to function in the epigenetic control of genomic elements in the germ line. The pathway predominantly responsible for antiviral activity in insects is the siRNA pathway and this will be the main focus of the following section.

siRNA Pathway Components

Virus-derived siRNAs are generated through the recognition and processing of double-stranded RNAs (dsRNA) produced during virus infection (reviewed in Ding *et al.* ²⁰). The dsRNA can occur as a replication intermediate for single-stranded RNA viruses, due to secondary structure in viral RNAs, or as a consequence of base pairing of convergent transcripts. Recognition of the dsRNA by Dicer proteins (members of the RNaseIII family of endoribonucleases) results in the production of siRNA (Figure 1). These are loaded into the pre-RNA induced silencing complex (RISC). Unwinding of the duplex occurs along with guide strand selection that defines target specificity on the basis of complementarity. Targeted RNA is degraded through the RNase activity of Argonaute ²¹.

Most of the characterization of the virus-derived siRNA pathway in insects has been performed in *Drosophila*. Flies possess two Dicer proteins; Dicer-1 is required for the production of miRNA, whereas Dicer-2 is necessary for processing dsRNA in order to generate siRNA ^{22; 23}. Dicer-2 functions as the activating PRR for the siRNA pathway binding to dsRNA and cleaving it to small 21 nt siRNAs with 2 nt 3' overhangs. For exogenous dsRNA (such as virus-derived dsRNA) processing by Dicer-2 requires R2D2 and is independent of Loquacious function, whereas Dicer-2 processing of endogenously produced dsRNA requires both of these dsRNA binding proteins^{24; 25}. Dicer-2 also functions as a PRR for the activation of the Jak-STAT pathway in *Cx. quinquefasciatus* cells ¹⁸. Recognition of virus-derived dsRNA by Dicer-2 leads to the secretion from infected cells of a Jak-STAT activating ligand leading to the establishment of an antiviral state in uninfected, responsive cells. This function provides a direct connection between the siRNA pathway and the Jak-STAT pathway implying a deliberate coordination of these responses.

R2D2 and Dicer-2 are the key components of the RISC-loading complex (RLC) that along with Argonaute-2 (Ago-2), the catalytic component of the RISC, selects the guide strand of the siRNA and loads it into the RISC. This guide strand serves to specify the RNA substrate for Ago-2 mediated cleavage resulting in silencing ^{21; 26}.

Flies with null or loss of function mutations of *dcr-2* or *ago-2* fail to control virus replication and develop disease far more readily than wild-type counterparts ^{9; 10; 11; 12}. Virus-derived siRNAs have been detected in *Aedes spp.* mosquito cells during infection with arboviruses ^{7; 27; 28; 29}. Inhibition of the RNAi pathway results in greater virus accumulation and increased pathogenesis in infected mosquitoes. These lines of evidence clearly demonstrate the importance of this pathway in the antiviral immune response. Mutations in the pathway do not significantly affect anti-bacterial or anti-fungal immunity, emphasizing the specificity of this response to viruses ²⁰.

Viral Suppressors of RNAi

Perhaps the strongest evidence for the importance of the RNAi pathway in controlling virus infection is the encoding of RNAi suppressor proteins by viral pathogens of insects. The B2 and 1A proteins produced by members of the *Nodaviridae* and *Dicistroviridae* respectively have been shown to suppress the RNAi response during infection ^{12; 20; 30; 31}. The absence of these proteins results in reduced virus yield or spread, and clearance of the infection by

the insect; however, viruses lacking the RNAi suppressors can productively infect animals deficient in components of the RNAi machinery such as *dcr-2* and *ago-2*.

The Flock House virus (FHV) B2 protein functions as a dimer and inhibits the RNAi pathway by binding to dsRNA thus preventing cleavage of dsRNA by Dicer-2^{32;33} (Figure 1). Additionally, should dsRNA be cleaved by Dicer-2, B2 binding to short dsRNA molecules can block loading into the RISC complex³³. Members of the *Dicistroviridae* express a suppressor of RNAi, protein 1A. This protein functions differently for different members of the family³¹. *Drosophila* C virus (DCV) 1A is functionally analogous to FHV B2, binding to dsRNA and preventing processing. In contrast the 1A protein of cricket paralysis virus (CrPV) interacts with Ago-2 inhibiting its activity. CrPV 1A is a drastically more potent RNAi suppressor, causing a decrease in insect survivability; whereas, DCV 1A suppresses the RNAi pathway to a lesser extent allowing for survival and the development of a persistent infection³¹. Thus closely related viruses have evolved different mechanisms of overcoming the antiviral activity of the RNAi pathway.

While it is apparent that viral pathogens of insects encode suppressors of RNAi, the situation with arboviruses is less clear. Arbovirus infection of insects results in a persistent infection with little associated pathogenesis. It is apparent from *in vivo* and cell culture systems that, while infection is controlled by the RNAi response, it is not cleared^{7;27;28}. Thus it appears that arboviruses have evolved mechanisms of evading the full effect of the RNAi response in order to maintain a persistent infection. One hypothesis is that the replication intermediates are sequestered in compartments that are inaccessible to the RNAi machinery. In this scenario RNA generated by the replication factories and released into the cytoplasm is susceptible to recognition and silencing by the RNAi machinery; the required templates for generating new virus are compartmentalized and hence protected.

However, Schnettler *et al.* have provided direct evidence for an arbovirus encoded suppressor of RNAi³⁴. A subgenomic RNA (sfRNA) corresponding to the 3'UTR of the flavivirus genome functions to counter the RNAi response in both mammalian and insect cells. This RNA inhibits the processing of dsRNAs by human Dicer *in vitro*, suggesting that it directly disrupts the function of Dicer in cells to suppress the RNAi response. Interestingly while this study found no RNAi suppressor function associated with West Nile virus (WNV) proteins, a subsequent study has reported that the NS4B protein of another flavivirus, DENV-2, functions to suppress miRNA and siRNA pathways in mammalian and insect (Sf21) cells³⁵.

Systemic RNAi response

Over the past decade evidence has accumulated to support the idea of a systemic RNAi response to virus infection in insects (reviewed in detail in Karlikow *et al.*³⁶). Initial evidence was obtained from distant silencing of endogenous genes following *in vivo* dsRNA uptake. This was first observed in *Tribolium castaneum* (flour beetle) following injection of dsRNA into the haemocoel of adult beetles³⁷. The effect was transferred to offspring, demonstrating transfer of the signal across cell boundaries. The idea of RNAi spread was further bolstered by observations that infection of *Drosophila* with Sindbis virus (SINV) expressing a non-viral gene (GFP) could suppress expression of the gene at sites distant

from infection³⁸. These lines of evidence point to a systemic RNAi silencing pathway. The nature of the transmitted molecules, the mechanism by which they are passed from cell to cell and a presumed mechanism by which the signal is amplified are currently unknown; however, evidence related to some of these issues is emerging.

Studies using cultured *Drosophila* cells indicate that long dsRNAs, but not siRNAs, are internalized when simply added to culture medium^{39; 40}. This uptake is dependent on the cell surface receptors Sr-CI and Eater, and clathrin-dependent endocytosis⁴⁰; however, these receptors do not possess obvious nucleic acid binding motifs. This suggests that the RNA may be associated with other molecules in the form of a vesicle and/or ribonucleoprotein (RNP) complex to protect the dsRNA signal and to promote uptake into naïve cells. Exosomes represent a mechanism of intercellular molecular transport and are an attractive candidate as the mode of dissemination of the dsRNA-based immune signal⁴¹. They are found in numerous extracellular fluids and carry various cargo including components of the RNAi silencing machinery⁴². Another hypothesis is that infected cells secrete RNPs that comprised of components necessary for RNA silencing. Evidence of circulating RNPs containing miRNA and Ago in human plasma raises the possibility of secreted RNPs being largely responsible for the transmission of the immune signal⁴³.

If a dsRNA (or similar) signal is to be passed from an infected cell to a naïve cell the problem of signal dilution must be overcome. Unlike plants and nematodes, insects do not possess an RNA-dependent RNA polymerase to amplify an incoming dsRNA²⁰; however, recently Goic *et al.* have reported a mechanism whereby virus-derived RNA is reverse transcribed into dsDNA and integrated into retrotransposable elements in the genome of *Drosophila* cells⁴⁴. This observation was consistent with the previous finding in mosquito system that sequences from flavivirus genome were present in the genomes of both cultured cells and wild type mosquito strains⁴⁵. The DNA copies of virus sequence can give rise to dsRNAs that are utilized in siRNA-based immunity. This was reported to occur in cells infected with pathogenic *Drosophila* viruses as well as SINV and led to virus control and the establishment of a persistent infection. It is tempting to speculate that if dsRNA is released from infected cells and can be taken up by naïve cells then it could in turn be reverse transcribed and stabilized in the cell genome providing a mechanism of resistance to virus infection. Thus a dsRNA signal is effectively amplified through the integration of a DNA copy into the genome followed by continuous transcription of dsRNA.

While evidence for the cell-to-cell transmission of RNAi is mounting in *Drosophila*, this may not be the case for mosquito species. Arbovirus spread within an infected mosquito is necessary for continued transmission. In addition to spread to salivary glands certain viruses can be transmitted transovarially by mosquitoes. This would indicate that there are differences between insect species in terms of systemic spread of RNAi, or that certain tissues are more capable of participating in such a systemic response^{46; 47; 48; 49}.

Antiviral effects of other small RNA pathways

The role of miRNAs and piRNAs in insect antiviral immunity is not clear. The role of miRNAs in viral pathogenesis has been more deeply studied in mammalian systems in which virus induction of cellular miRNAs and production of virus encoded miRNAs have

been shown to influence the outcome of virus infection and regulation of the cellular response^{50; 51; 52}. The production of an miRNA from a subgenomic RNA corresponding to the 3' untranslated region of WNV has been demonstrated in *Ae. albopictus* cells, but not vertebrate cells. This miRNA regulates the synthesis of GATA4 by the cell resulting in a proviral effect⁵³. It is not yet apparent whether this miRNA-like activity is functionally related to the suppression of RNAi activity also assigned to this subgenomic RNA.

piRNAs of viral origin have been observed in mosquito cells infected with a number of different arboviruses^{54; 55}. The role of these RNAs remains unresolved, however they may be involved in regulation of the antiviral response as their presence precedes the siRNA response then wanes as the siRNA response increases suggesting a regulatory role. Schnettler *et al.* have demonstrated an antiviral role for the piRNA pathway in response to Semliki Forest virus (SFV)⁵⁶. Knocking down components of the piRNA pathway resulted in increased levels of virus replication and accumulation, particularly in the absence of a Dicer-2 mediated siRNA response⁵⁷. How directly piRNAs interfere with virus replication is not clear.

Jak-STAT pathway

In addition to the RNAi pathway, several signaling pathways also contribute to the antiviral response in insects. Initially characterized for its role in development and hemocyte proliferation, the Jak-STAT pathway has been shown to also respond to bacterial and viral infections by regulating the production of downstream effector molecules including antimicrobial peptides (AMPs). This pathway is activated in a paracrine fashion through the binding of secreted ligands. Activation appears to be consequent to initial recognition of infection by PRRs that also feed into other pathways. The mechanism by which the Jak-STAT pathway exerts its antiviral effect is not known, but it is apparent that the response is complex, specific and versatile.

Jak-STAT pathway components

The Jak-STAT pathway is a highly conserved insect innate immune signaling pathway that has been characterized primarily in *Drosophila*. The well-characterized pathway is comprised of one signal transducer and activator of transcription (STAT92E; hereafter referred to as STAT), a Janus kinase (Hopscotch), a receptor (Domeless) and 3 Unpaired-related ligands^{58; 59; 60; 61; 62} (Figure 2). Binding of Upd induces dimerization of the receptor Domeless (dome), allowing transphosphorylation of the Hopscotch (hop) kinases. This is followed by the recruitment and trans-phosphorylation of the conserved tyrosine residue in the STAT transcription factors. Phosphorylated STATs dimerize and are transported to the nucleus to regulate the expression of downstream effector genes.

In addition to these characterized pathway components, several splice variants have been predicted for STAT, hop, and dome in the *Drosophila* genome and two alternatively spliced STAT isoforms have been identified in *Spodoptera frugiperda* (army worm) cells⁶³. In the nucleus, the STAT DNA-binding domain binds the canonical TTCNNNGAA binding site, where C3 and G7 are the critical residues; however, this binding site has been shown to have flexibility in the 5' Ts, 3' As, and/or the linker region⁶². Both *S. frugiperda* STAT

isoforms show a weak binding affinity to the canonical STAT DNA binding site, but it is unclear whether these isoforms lead to differential expression of downstream effectors⁶³. Mammalian STATs have additional modifications including serine phosphorylation, acetylation, and glycosylation that alter their transcriptional activity and interactions with co-activators and other transcription factors (reviewed in Schindler *et al.*⁶⁴). The modifications of the arthropod STAT orthologs are not yet known.

A mosquito STAT ortholog has been characterized in *An. gambiae* and is most similar to *Drosophila* STAT and mammalian STAT-5 with between 37.6–51.2% protein sequence identity in the conserved central region containing the DNA binding region, the SH2 phosphotyrosine-binding region and a conserved tyrosine known to be phosphorylated by hop⁶⁵. In addition, STAT orthologs have also been identified in *Ae. albopictus* and *Cx. tritaeniorhynchus* and contain the conserved tyrosine and high levels of protein sequence identity in the DNA-binding and SH2 domains⁶⁶. While there is divergence in the C-terminal tail activation region of all mosquito STATs, the *Ae.albopictus* and *Cx.tritaeniorhynchus* STATs have significantly longer C-terminal activation domains, although the possible significance of this has not yet been determined. Orthologs of hop, dome, and STAT have been predicted in other mosquito species including *Ae. aegypti*, *Cx. quinquefasciatus*, and several *Anopheles* species.

The *An.gambiae*, *Ae.albopictus*, and *Cx.tritaeniorhynchus* mosquito STAT orthologs respond to bacterial infection through nuclear localization, DNA-binding, and transcriptional activation of a luciferase reporter gene, thereby confirming a conserved Jak-STAT pathway activation and immune function in mosquitoes^{65; 66}.

Jak-STAT pathway ligands

The *Drosophila* genome encodes three Upd-like ligands; however, the Upd amino acid sequence is divergent among *Drosophila* species and has not been found in other insect genomes⁶⁷. All three ligands are glycosylated proteins secreted by hemocytes and signal non-autonomously by binding to the dome receptor of fat body cells to activate the Jak-STAT pathway. Of the three Upd-like ligands, Upd and Upd2 are involved in development, segmentation, and hemocyte proliferation and differentiation⁶⁸. Although Upd and Upd2 are thought to be semi-redundant, Upd is associated with the extracellular matrix while Upd2 is freely diffusible^{59; 69}. Upd3 is freely diffusible and plays a role in responding to septic injury and Gram-negative bacterial infection^{68; 69}. Upd has been shown to be a more potent activator of a STAT-responsive promoter (6×2xDraLuc) than Upd3 or Upd2, respectively⁶⁹; however, as will be discussed, not all STAT-responsive genes are activated equally by a given stimulus.

Recently, a novel ligand of the Jak-STAT pathway has been identified. Deddouche *et al.* first identified *vago* mRNA as upregulated by DCV and SINV infection and coding for a protein with a single von Willebrand factor type C motif in *Drosophila*⁷⁰. Paradkar *et al.* characterized Vago as a secreted protein that activates the Jak-STAT pathway during WNV infection in *Cx. quinquefasciatus* cells¹⁸ (Figure 2). Interestingly, the protein does not bind the dome receptor. An alternative receptor-ligand pair contributes a new level of complexity to our understanding of the Jak-STAT pathway in insects. Additionally, *vago* mRNA

expression is dependent on Dicer-2 but no other RNAi pathway components⁷⁰. This suggests an RNAi-independent signaling mechanism for Dicer-2 and a link between the RNAi and Jak-STAT pathways. Interestingly the Dicer-2-dependent activation of *vago* expression also appears to be virus specific. In the study by Paradkar *et al.* only WNV dsRNA was able to stimulate *vago* production, poly I:C and bluetongue virus dsRNA did not cause *vago* expression to increase. While the mechanism of discrimination of these RNA species is not clear, it implies a tailoring of the immune response for specific viruses¹⁸.

Regulation of the Jak-STAT pathway and responsive genes

The pathway can be regulated by a variety of methods including dephosphorylation, nuclear export, or negative feedback from two known inhibitors of hop or STAT, Suppressor of Cytokine Signaling 36E (SOCS36E) and Drosophila Protein Inhibitor of Activated STAT (dPIAS) respectively^{71; 72} (Figure 2). Arboviruses can also regulate the pathway by disrupting phosphorylation or nuclear localization of STATs in vertebrates^{73; 74; 75; 76; 77}; however, the interaction between the viruses and the Jak-STAT pathway is not clear in arthropods. Interestingly, during Japanese encephalitis virus (JEV) infection of mosquito cells (*Ae. albopictus*, C6/36), there was a decrease in STAT tyrosine phosphorylation, indicating the inhibition of the STAT pathway during JEV infections although no viral inhibitors of the pathway have been identified⁶⁶. This could be explained due to the lack of Dicer-2 in the C6/36 cell line, further supporting the Dicer-dependent activation of the pathway through Vago^{28; 78}. The absence of Dicer-2 siRNA associated activity in this cell line may make it of use when trying to determine the connection between the RNAi and Jak-STAT pathways.

Several downstream effector genes have been identified as responsive to the Jak-STAT pathway. Although septic injury and bacterial and viral infections can activate the Jak-STAT pathway, studies using a variety of viruses and model organisms show distinct gene expression profiles associated with virus infections. During septic injury, the Jak-STAT pathway induces the expression of *turandot A* (*totA*) in *Drosophila*⁶⁸. The expression of *totA* is activated in the fat body and dependent on both dome and Upd3⁶⁸. Additional members of the tot family, particularly *totC* and *totM*, are also thought to be regulated by the Jak-STAT pathway and respond to stress⁷⁹, although the specific function of these proteins is not known. Tep1, a thioester-containing protein similar to mammalian complement C3/alpha2 macroglobulin was also shown to be upregulated in response to septic injury and bacterial challenge in *An. gambiae*. Mutational analysis indicates that Tep1 is involved in promoting phagocytosis⁸⁰. Additionally, *D-raf*, a known component of the Ras/Raf pathway, was shown to be regulated by Jak-STAT in *Drosophila* during septic injury and involved in hemocyte proliferation; however, its function, if any, during viral infection is unclear. It does, however, suggest a connection between the Jak-STAT and Ras/Raf signaling pathways⁸¹.

Jak-STAT response to virus infection

Many studies have contributed data involving the Jak-STAT response with a variety of viruses using cells, tissues, and whole organism approaches. Jak-STAT is activated by both pathogenic viruses (e.g. DCV, FHV) and arboviruses, which show little pathogenesis (e.g.

SINV, DENV). Jak-STAT was first shown to be involved in the antiviral response to a pathogenic DCV infection in flies. Dostert *et al.* demonstrated that the Jak-STAT pathway was activated and resulted in changes in the transcription profile of DCV infected flies⁸². Genes possessing upstream STAT-binding sites such as *vir-1*, *CG9080*, and *CG12780* were upregulated due to DCV infection. In addition, expression of these genes was significantly decreased in *hop* mutant flies. The specific functions of these gene products are unknown; however, their expression is specific to virus infection⁸². Further studies show that *vir-1* is not upregulated by UV-inactivated DCV or viral dsRNA alone, indicating that viral replication is required; although, *vir-1* does not appear to directly inhibit virus replication or production⁸³. Since these initial findings *vir-1* expression has often been used as a read-out for Jak-STAT activation.

In regards to other pathogenic *Drosophila* viruses, Kemp *et al.* found that *hop* mutant flies (M38/msv1) were more sensitive to DCV and CrPV than wildtype flies⁸⁴. Survival significantly decreased and viral titers significantly increased in the virus-injected *hop* mutant flies as compared to wildtype. In terms of pathway activation, *upd2* and *upd3* mRNA levels were significantly higher in DCV and CrPV injected flies. Other pathogenic viruses, such as FHV and invertebrate iridescent virus 6 (IIV-6) did not significantly affect the survival of *hop* mutant flies⁸⁴.

Immune responses to arboviruses have also been studied. In DENV-infected mosquitos, virus titers increased in RNAi knockdowns of STAT pathway components or silenced STAT inhibitors indicating an anti-viral role for the pathway⁴. Eighteen genes were identified as Jak-STAT-regulated and DENV-responsive and two of these were characterized as DENV response factors due to their increased expression in response to infection in *Ae. aegypti* mosquitoes (DVRF1 and DVRF2⁴). In addition, Behura *et al.* showed increased mRNA expression of genes associated with the Jak-STAT pathway, including *jak* and *stat*, in both susceptible and refractory *Ae. aegypti* strains during DENV infection⁸⁵.

Similarly, SINV-replicon mRNA levels also increase in a *stat* mutant in *Drosophila*¹⁴. Previously identified targets, *vir-1*, *tep1*, and *D-raf* were not found to be sensitive to SINV-replicon. When examining downstream Jak-STAT effectors, 61 SINV-sensitive genes had STAT binding sites and a subset was confirmed as STAT-responsive through STAT mutant analysis³. Of these SINV-sensitive, STAT-regulated genes, the gene coding for an antimicrobial peptide, Attacin-C, was further characterized and found to have anti-viral function. Although the Jak-STAT pathway appears to be active during SINV-replicon replication and SINV infection^{3; 14}, Kemp *et al.* determined that the survival of *hop* mutant flies was not significantly different from wildtype flies⁸⁴. The survival of *hop* mutant flies also did not significantly decrease during other virus infections including VSV, FHV, DXV, and IIV-6, although this does not necessarily indicate lack of pathway activity⁸⁴. Jak-STAT activation in these experiments was determined by *vir-1* mRNA expression.

Activation of the pathway by another alphavirus, SFV, was measured using a 6x*drafc* reporter in *Ae. albopictus* U4.4 cell lines⁷⁴. The cells did not show up-regulation of the *D-raf* reporter, but as we come to understand the complexity of the pathway, it is clear that single genes are not representative of activation in every scenario. This is clearly shown by

studies that have examined and compared the post-infection transcriptomes of several virus infections. Kemp *et al.* compared the transcriptomes of DCV, FHV, and SINV at various time points and found that genes that were regulated differently between infections outnumbered those in common⁸⁴. Particularly, genes regulated by the Jak-STAT pathway were regulated in a different manner during infections with different viruses. Expression of *vir-1* and *totM* mRNA was examined during DCV, CrPV, FHV, SINV, Vesicular stomatitis virus (VSV), DXV, and IIV-6 infections. *vir-1* mRNA was upregulated during DCV, CrPV, and FHV infections, while *totM* was upregulated in SINV, VSV, DXV. It is intriguing that these two Jak-STAT markers do not overlap in any of the infections; however, it should be taken into account that other pathways may be involved such as MEKK1 activation of *totM*⁸⁴.

Interaction of Jak-STAT with Nf- κ B pathways

STATs have been typically characterized as transcriptional activators; however, there is evidence of repressor activity. *Drosophila* STAT has been shown to form a complex with another transcription factor (dAP-1) and chromatin modifying proteins (Dsp1 and HDAC) to compete for Relish binding sites, thereby regulating Nf- κ B immune responses⁸⁶.

On a similar note, Souza-Neto *et al.* found that a Toll ligand (spätzle), four cecropins, and one defensin were down-regulated when the Jak-STAT inhibitor dPIAS was depleted during DENV infection in *Ae. aegypti* midguts. This finding was confirmed by Colpitts *et al.* who also found the same four cecropins and defensin were down-regulated when they examined the transcriptome of *Ae. aegypti* after infection of flaviviruses, DENV, WNV, or YFV. While the mechanism is unclear, this suggests a connection between Jak-STAT activation and inhibition of Toll-regulated genes⁴.

Nf- κ B Innate Immune Pathways

The Imd and Toll pathways are Nf- κ B -related pathways involved in innate immunity in arthropods and are activated upon bacterial and fungal infection in *Drosophila*. In *Drosophila* the Imd and Toll pathways activate cellular transcription mediated by two distinct orthologs of the Nf- κ B transcription factor. Relish is the terminal transcription factor and Nf- κ B ortholog for the Imd pathway, while Dorsal and Dorsal-related immune factor (Dif) function in the Toll pathway. Between the two Toll transcription factors, Dorsal plays a crucial role in early embryogenesis while Dif is involved in antimicrobial immunity⁸⁷.

Imd pathway components and signaling

In the Imd pathway, the diaminopimelic-containing peptidoglycan of Gram-negative bacteria and *Bacillus spp.* is sensed both extra- and intra-cellularly by PRRs, PGRP-LC and PGRP-LE, followed by the activation of the adaptor molecule IMD^{88; 89; 90; 91} (Figure 3A). IMD is essential for the activation of Relish via a two-branched signaling pathway. The first branch phosphorylates Relish through the IKK complex^{92; 93; 94}, and the other branch cleaves the phosphorylated Relish by a caspase, Dredd. After phosphorylation followed by cleavage, the N-terminal DNA binding domain of Relish translocates to the nucleus and thus regulates transcription of effector genes^{93; 95; 96; 97}.

Toll pathway components and signaling

The antimicrobial responses of the Toll pathway are initiated by two detection methods (Figure 3B). The first method involves the PRRs, PGRP-SA, PGRP-SD and GNBP-1, which detect cell wall components of Gram-positive bacteria, as well as GNBP-3 that detects β -glucan on fungal cell wall^{88; 98; 99; 100; 101; 102}. The second method involves the protease Persephone (Psh) that senses foreign proteolytic activity including proteases secreted by fungi^{102; 103}. The signals from PRRs are integrated into a modular serine protease ModSP, which leads to the activation of another set of serine proteases including Grass, spirit, Spheroid, and Sphinx1/2^{103; 104; 105}. Prior to this, the Toll pathway ligand, Spätzle, is inactive with its Toll recognition site masked by a prodomain. Signals from the serine proteases and Psh can activate the Spätzle-processing enzyme. Processing of Spätzle removes the prodomain allowing binding to Toll receptors¹⁰⁶. Signal transduction through the pathway degrades a homolog of the mammalian I κ B, Cactus, allowing the nuclear translocation of Dif and subsequent regulation of effector genes¹⁰⁷.

Nf- κ B effectors in anti-bacterial and -fungal immunity

Both Relish and Dif regulate the transcription of a number of AMPs upon translocation. Relish has been shown to bind well to upstream sequences of AMPs, GGGGATCCCC and GGGGATTCCC, while Dif binds well to GGGAAAACCC. However, these binding sites are not highly conserved and may have great variations¹⁰⁸. The AMPs are secreted into the hemolymph by fat body cells, a counterpart of human liver. Knocking down AMP production leads to enhanced pathogenesis in flies during bacterial and fungal infection¹⁰⁹. Pathway specific AMPs like Drosomycin (Toll) and Diptericin (Imd) also serve as indicators of pathway activation.

Nf- κ B pathways in mosquitoes

Genome sequences of *An. gambiae*, *Ae. aegypti* and *Cx. quinquefasciatus* indicate that mosquito species share a conserved antimicrobial immune pathway with *Drosophila*. Mosquitoes have two Nf- κ B orthologs: Rel1 (*An. gambiae*) or Rel1A and Rel1B (*Ae. aegypti*) orthologous to Dorsal, and Rel2 (*An. gambiae* and *Ae. aegypti*) orthologous to Relish. No Dif orthologs have been identified in mosquitoes^{110; 111; 112}. Both the Dorsal orthologs and the Relish ortholog synergistically mediate the anti-bacterial response in *An. gambiae* and anti-fungal responses in *Ae. aegypti*^{113; 114}. The intracellular signaling module of the Toll and Imd pathways in mosquitoes is very similar to that in *Drosophila*, although genes involved in pathogen detection and effector molecules show some diversification, which is expected due to different selection pressures¹¹².

Imd and Toll pathways in antiviral immunity: pathogenic viruses

Both pathways have been shown to play a role in the antiviral immunity against arthropod pathogens. Knocking down Imd signaling components in *Drosophila* increased their mortality due to CrPV infection¹¹⁵. Likewise, there were increased transcription levels of Imd components *PGRP-SB1* and *PGRP-SD*, as well as downstream antimicrobial peptides in flies infected with a vertically transmitted arthropod pathogen, Sigma virus (SIGMAV)¹¹⁶. Contradictory results finding no induction of Imd- or Toll- regulated genes

were seen in another study examining SIGMAV infection using a different strain of flies ¹¹⁷. Recently, microarray analysis of *Drosophila* persistently infected with Nora virus, showed significant alteration of a gene involved in JNK signaling, an alternative Imd signaling branch ^{118; 119}. Significantly less is known about the Toll pathway responses to pathogenic viruses in arthropods; although, Zamboni *et al.* showed that flies with reduced levels of Dif, are more sensitive to infection with DXV as measured by survival ¹²⁰. It should be noted that Dicer-2 mutations in *Drosophila* have little effect on both Nora virus and DXV infections implying that other pathways such as Nf- κ B play a significant role in the control of these viruses ^{11; 121}.

Imd and Toll pathways in antiviral immunity: arboviruses

The Imd and Toll pathways have also been shown to play a role in antiviral immunity against arboviruses. Flies harboring a SINV replicon demonstrated increased levels of genomic viral RNA replication when components of the Imd signaling pathway were mutated (Rel, FADD, DREDD, IMD, Ird5, Key) ¹²². A follow-up study showed that knocking down Imd-regulated AMP, DptB, resulted in developmental defects in flies harboring SINV replicon as well as enhanced viral replication and production when mutant flies were injected with SINV ³. SFV infection did not activate either Imd or Toll pathway as measured by reporter constructs of *attA* and *drosomycin*, respectively; however, pretreating U4.4 mosquito cells with heat-inactivated *E. coli*, thus activating the Imd pathway prior to SFV infection showed a decrease in the expression of both viral genome and subgenome suggesting that the Imd pathway has an antiviral effect early in SFV infection ¹²³. Additionally, flies homozygous negative for *rel* demonstrated increased levels of virus replication when infected with SINV, SFV and Ross River virus (Avadhanula and Hardy, unpublished data).

In recent years, global transcriptome analysis has facilitated the studies with vectored viruses in mosquitoes, which lack the abundant genetic tools available in *Drosophila*. Microarray analysis of DENV infected *Ae. aegypti* showed increased expression of Toll pathway associated genes. Likewise, activating the Toll pathway in *Ae. aegypti* cells by silencing its negative regulator, *cactus*, resulted in lower DENV titers, while inactivating the pathway led to high viral titers ¹²⁴. This antiviral effect of the Toll pathway was consistent with different DENV serotypes in both lab and field mosquitoes ¹²⁵. In another study, the midgut transcription profiles of *Ae. aegypti* mosquitoes fed with SINV blood meal showed an induction of *dif* mRNA early in infection; this returned to uninfected levels later in infection ¹²⁶. In *An. gambiae* infected with O'nyong-nyong virus, the Imd positive regulator *Ikkb* was inhibited while its downstream effector molecules *CEC3* and *dpt* were upregulated; however, the antiviral role of the Imd pathway is still unclear as knocking down *rel2* did not affect viral load. ¹²⁷

Autophagy

In recent years, autophagy has been proposed as an alternative antiviral mechanism in insects that is independent of the Imd, Toll or JAK-STAT pathways ^{128; 129}. Autophagy is the process by which *de novo* synthesized membranes encompass large cytoplasmic components including damaged organelles or protein aggregates, elongate and form closed

double membrane vesicles named autophagosomes. These vesicles then fuse with lysosomes and degrade the engulfed materials. Under nutrient-deprived conditions, autophagy helps recycle nutrients and maintain cellular homeostasis^{130; 131}. The signaling of autophagy is mediated through the phosphoinositide 3-kinase (PI3K)-Akt pathway. Activation of the PI3K-Akt pathway increases the level of TOR, the negative regulator of autophagy, and thus inhibits this process¹³².

Autophagy in innate immunity

Autophagy also plays a role in innate immunity and targets intracellular parasites or bacteria that enter the cytosol^{129; 133; 134; 135}. The antiviral effect of autophagy has been previously demonstrated in plants and mammals^{136; 137; 138}. Using a *Drosophila* model of human degenerative disease, Nezis *et al.* demonstrated that *ref(2)P* was associated with protein aggregates involved in autophagy^{139; 140}. Several studies have also found that *ref(2)P* determines the permissivity for SIGMA virus (*Rhabdoviridae*) infection in *Drosophila*^{141; 142; 143}.

More direct studies on the antiviral role of autophagy were conducted in a *Drosophila* model successfully infected with VSV, another member of the *Rhabdoviridae*. Shelly *et al.* found that dsRNA knockdown of autophagy-related genes resulted in higher VSV production both in cultured S2 cells and in flies. Depleting one of these autophagy-related genes, *Atg5*, prevented the formation of virus-induced autophagosomes. The induction of autophagy has been shown to occur during virus entry as both UV-inactivated virus and replication-competent virus led to similar number of autophagosomes. Moreover, VSV glycoprotein (VSV-G) was sufficient for this induction. Therefore, rather than the viral replication complex or RNA replication intermediates, the PAMP triggering autophagy in this case is the VSV glycoprotein¹⁴⁴. Later, the same group proposed that one of the *Drosophila* TLR orthologs, Toll-7, was responsible for sensing VSV on the cell surface. Toll-7 signaling was activated upon VSV infection and dsRNA knockdown of Toll-7 led to higher viral protein level *in vitro* and greater pathogenesis *in vivo*¹²⁸. The signaling of this antiviral response is also through the PI3K-Akt pathway. Overexpression of a phosphatase blocking PI3K signaling, PTEN, led to lower viral protein production. Similarly, loss of Akt by dsRNA silencing in flies resulted in decreased viral protein level¹⁴⁴.

Interestingly, in other virus-host systems, viruses subvert autophagy to play a proviral role. In mammals, autophagy facilitates viral RNA replication and virion maturation of HCV, DENV, and poliovirus^{145; 146; 147}. In *Ae. albopictus* cells and *Drosophila* SINV replication complex activated PI3K-Akt signaling and enhanced cap-dependent translation including viral RNA translation¹⁴⁸. One explanation for the contrast in the role for components of the autophagy pathway during VSV and SINV infection is that natural virus-host pairs have co-evolved and the virus has developed certain mechanisms of antagonizing or masking host defense responses¹⁴⁹. More studies with other viruses in both *Drosophila* and mosquitoes would be helpful to elucidate a universal or virus-specific role for autophagy in antiviral immunity.

Concluding remarks

Over the past two decades amazing progress has been made in understanding the innate immune system of insects. The discovery of Toll as an innate immune response receptor and the recognition of the importance of RNAi in response to virus infection have been major leaps forward^{9; 10; 11; 12; 87}. In recent years, the picture of the antiviral response in insects has become richer and more complex. Moving from a view of signaling pathways as isolated, independent responses it is now apparent that these pathways are integrated and responsive to one another, providing a pathogen-specific response.

The importance of dsRNA as a major PAMP triggering the innate immune response has been clearly demonstrated. Dicer proteins, particularly Dicer-2 in *Drosophila* and mosquitoes, are recognized as the main PRR involved in recognition of virus-derived dsRNA and initiating not only the RNAi response, but also the Jak-STAT response^{18; 20; 70}. This discovery is particularly significant in that the recognition of the dsRNA led to a specific response; only WNV dsRNA triggered the Dicer-2 dependent production and secretion of the Vago ligand by infected cells. This implies the ability for Dicer-2 to differentiate between dsRNA of different sources and consequently initiate an appropriate response. The question of how this is achieved warrants further attention, as does the question of whether Dicer-2 functions as a proximal PRR for other signaling pathways.

The activation of the Jak-STAT pathway by a previously unrecognized ligand also opens up new lines of investigation to identify the cellular receptor for Vago, and examine the possibility that other ligand-receptor pairs feed into the Jak-STAT pathway. This increased level of complexity may help explain the different STAT-dependent responses seen for different viruses and allow a greater level of versatility in the Jak-STAT response⁸⁴. In addition to the obvious connection between the siRNA pathway and Jak-STAT, it is apparent that the Jak-STAT pathway regulates the activity of the Nf- κ B pathways at the level of transcriptional repression of Nf- κ B-responsive genes^{4; 86}. These observations highlight the need for greater understanding of the connectivity of all the innate immune response pathways in order to elucidate the mechanisms by which pathogen-specific responses are elicited.

While the mechanism by which the siRNA response exerts its antiviral effect is well understood, far less is known about the effector molecules produced by the Jak-STAT and Nf- κ B pathways. AMPs have been identified as being up-regulated during viral infections³. AMPs have been traditionally associated with antibacterial and antifungal activity, however a human AMP, alpha defensin 5, has been found to stabilize the viral capsid, preventing uncoating and thus inhibiting viral infection^{150; 151; 152}. No antiviral molecular mechanism has yet been uncovered for arthropod AMPs, though Dipteracin B and Attacin C have been implicated to have antiviral effect³.

The intriguing idea that insects can mount a systemic immune response using the spread of an RNA-based signal to facilitate siRNA antiviral immunity is gaining more traction as evidence mounts in its support. Distant dsRNA-mediated gene silencing in conjunction with the remarkable discovery that reverse transcribed DNA copies of viral RNA are

incorporated into the genome of the host cell and transcribed to participate in an siRNA response, changes the way we must think about this type of immunity^{37; 38; 44}. These findings imply a long-lasting, widespread and specific response to virus infection, capable of modulating pathogenesis, and possibly required for the establishment of persistent infection necessary for efficient arbovirus transmission. Further studies defining the nature of the transmitted signal and the mechanisms by which it is shared will allow a much deeper understanding of this aspect of insect antiviral immunity.

Finally, it is important to note that much of the current picture of the insect immune response has been elucidated in *Drosophila*. While *Drosophila* has proven an invaluable tool for understanding the virus-insect host interaction, it is important to keep in mind when looking at the vector-arbovirus interaction that the virus and mosquito immune pathways have co-evolved. Hence, as mosquito species become genetically more accessible it will be important to confirm findings from *Drosophila* studies in the natural vector species.

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Highlights

- Innate immunity plays a critical role in the outcome of virus infection of insects
- RNAi is a major mechanism of antiviral defense and is a systemic response
- Multiple pathways are activated in response to virus infection
- Connections between these pathways have been uncovered
- Pathway interactions lead to a tailored immune response to viruses

Fig. 1. siRNA pathway

Cytoplasmic, virus-derived dsRNA is recognized by Dicer-2 in complex with R2D2. Dicer-2 cleaves the dsRNA into 21nt siRNAs. The siRNA is loaded into the pre-RISC complex where duplex unwinding and selection of a guide strand occurs. The guide strand functions to direct RISC to the viral RNA target through base-pairing. The Ago2 protein in the RISC cleaves the viral RNA target inhibiting virus replication. Points in the pathway at which viral suppressors of RNAi function are shown.

Fig. 2. Insect Jak-STAT pathway

The pathway is initiated by the binding of a ligand (upd3 or vago) to their receptor (dome or unknown receptor, respectively.) After dimerization of the receptor, the associated Janus kinases (hop) transphosphorylate each other, recruit and phosphorylate STATs, resulting in translocation to the nucleus and regulation of transcription. SOCS and dPIAS are known to act as inhibitors of hop or STAT, respectively. It was recently found that recognition of viral dsRNA by Dicer2 induces the expression of *vago* in an RNAi-independent mechanism. Vago is secreted and can activate the Jak-STAT pathway through an as-yet uncharacterized receptor, inducing the transcription of *vir-1*, *attC*, and potentially other antiviral molecules.

Fig. 3. Insect $\text{Nf-}\kappa\text{B}$ -related immune pathways

A) Imd signaling pathway. Transmembrane receptors PGRP-LCs and intracellular receptor PGRP-LE sense the diaminopimelic-containing peptidoglycan of Gram-positive bacteria and *Bacillus*. The receptor molecules multimerize upon activation and transmit the signal to the adaptor molecule IMD. IMD activation recruits dFADD which recruits a caspase, DREDD. Activation of DREDD leads to polyubiquitination of IMD. TAK1 binds to the polyubiquitin chain and is responsible for the assembly and activation of the IKK complex (IKK β and IKK γ). The IKK complex mediates the phosphorylation of Relish. Phosphorylated Relish is subsequently cleaved by DREDD, leaving the C-terminal ankyrin repeats in the cytosol. The N-terminal DNA binding domain of Relish translocates to the nucleus and regulates transcription of effector molecules such as *diptericin* and *diptericinB*. **B) Toll signaling pathway.** Toll pathway responds to Gram-positive bacteria and fungi infections. Lys-type peptidoglycan on bacteria cell wall is detected by PGRP-SA, PGRP-SD and GNBP-1. GNBP-3 detects β -glucan on fungal cell wall. Protease Persephone can also sense foreign proteolytic activities result from fungal infection. Both signals from bacteria and fungi are integrated into the same proteolytic cascade and thus lead to the activation of the Toll ligand, Spätzle. Spätzle binds to the Toll receptors and transduce the signals to Cactus-Dif through Toll-induced-signaling complex which contains MyD88, Tube and Pelle. Cactus is phosphorylated and cleaved from Dif, allowing Dif to translocate into nucleus and regulate the transcription of effector genes. The point at which viruses interact with each of these pathways is currently unresolved.