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Antiviral immunity and virus-mediated antagonism in disease vector mosquitoes

Glady Hazitha Samuel¹, Zach N. Adelman¹, and Kevin M. Myles^{1,*}

¹Texas A & M University, Department of Entomology, Minnie Belle Heep Center, College Station, TX 77843-2475

Abstract

More than 100 pathogens, spanning multiple virus families, broadly termed “arthropod-borne viruses (arboviruses)” have been associated with human and/or animal diseases. These viruses persist in nature through transmission cycles that involve alternating replication in susceptible vertebrate and invertebrate hosts. Collectively, these viruses are among the greatest burdens to global health, due to their widespread prevalence, and the severe morbidity and mortality they cause in human and animal hosts. Specific examples of mosquito-borne pathogens include Zika virus (ZIKV), West Nile virus (WNV), dengue virus serotypes 1-4 (DENV 1-4), Japanese encephalitis virus (JEV), yellow fever virus (YFV), chikungunya (CHIKV), and Rift Valley fever virus (RVFV). Interactions between arboviruses and the immune pathways of vertebrate hosts have been extensively reviewed. In this review we focus on the antiviral immune pathways present in mosquitoes. We also discuss mechanisms by which mosquito-borne viruses may antagonize antiviral pathways in disease vectors. Finally, we elaborate on the possibility that mosquito – borne viruses may be engaged in an evolutionary arms race with their invertebrate vector hosts, and the possible implications of this for understanding the transmission of mosquito-borne viruses.

Keywords

RNA silencing; RNA interference; RNAi; VSR; suppressor; arbovirus

Importance of antiviral immunity in the disease vector host

Mosquito-borne viruses are responsible for a broad spectrum of human diseases, including arthritis, hemorrhagic fever, encephalitis, and recently microcephaly and Guillain-Barré syndrome [1, 2]. While infections of vertebrate hosts with **arthropod-borne viruses (arboviruses; see Glossary)** are acute and sometimes associated with disease, the natural maintenance cycles of these pathogens generally require the establishment of persistent life-long infections in their mosquito vectors. Importantly, infections of the mosquito hosts also tend to be non-pathogenic. Evidence for this comes from nearly a century of observations,

*Correspondence: mylesk@tamu.edu (K.M. Myles).

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documented in the literature, describing the survival of arbovirus-infected mosquitoes [3]. The longevity of mosquitoes infected with arboviruses is generally similar to those of uninfected mosquitoes [3]. Indeed, with very few notable exceptions, cytological techniques have also generally failed to detect evidence of any significant pathology in mosquitoes infected with arboviruses [3]. In an attempt to explain the apparent resistance of mosquitoes to the potential cytopathic effects of arboviruses, some virologists had as early as the 1970's hypothesized the existence of endogenous mechanisms that quantitatively limit virus yields in mosquito cells [4, 5]. The seemingly tenuous nature of the relationship between vector and virus was also noted, and in fact suggested as a possible point of human intervention for future disease control [4]. However, in the absence of any real evidence of immune pathways targeting these viruses in the insect, such approaches remained purely hypothetical. Multiple studies now implicate RNA interference (RNAi) as the molecular basis for the apparent resistance of mosquito cells to the potential cytopathic effects of arboviruses, vindicating the now decades old hypothesis, and suggesting new genetic targets for disease control strategies. However, a variety of other molecular mechanisms and pathways may contribute to the antiviral immunity of the insect as well, possibly in a pathogen or tissue specific manner, or by functioning as a form of immunological memory. Given a robust RNA silencing-based antiviral defense, and an array of potential secondary responses, it is unclear how pathogens overcome the mosquito's defenses to be transmitted. Evidence increasingly suggests that, similar to a number of plant and insect viruses, mosquito-borne viral pathogens have evolved to encode mechanisms that are antagonistic to RNA silencing pathways.

The siRNA pathway

Multiple small RNA pathways, which fall under the larger umbrella term, RNAi, have now been described in flies. These include the small interfering RNA (siRNA), microRNA (miRNA), and Piwi-interacting RNA (piRNA) pathways. The siRNA pathway is further subdivided into an endogenous pathway, and an exogenous pathway [6]. Exogenous sources of double stranded RNA (dsRNA), such as the **replicative intermediates** produced during infection of a cell with a virus, are processed into siRNAs by the RNase III enzyme, Dicer 2 (Dcr-2; Figure 1) [7–9]. The resulting, 21-nt in length, siRNA duplexes are subsequently loaded into the **RNA induced silencing complex (RISC)** [10]. The siRNA duplexes contain a passenger strand, which will be removed from the activated RISC, and a guide strand that provides sequence specificity to the RISC [11]. The guide strand directs Argonaute 2 (Ago-2), an essential RISC component possessing endonuclease activity, to complementary RNAs in the cell leading to their sequence specific degradation (Figure 1) [12, 13].

Drosophila melanogaster possessing genetic lesions in key components of the siRNA pathway (*dcr-2* and *ago-2*), and exhibiting a loss-of-function phenotype, were essential to demonstrating the siRNA pathway's role in a protective antiviral immune response in fruit flies. Several groups independently demonstrated increased susceptibility of the siRNA pathway null mutant flies to infection with well-characterized insect pathogens that included **flock house virus (FHV)**, **Drosophila C virus (DCV)**, and **Cricket paralysis virus (CrPV)**. When the siRNA pathway null mutants were infected with these viruses they exhibited what was termed an “**enhanced disease phenotype**”, which was characterized by

elevated levels of virus replication and increased mortality, in comparison to wild-type flies, [8, 9, 14].

The first evidence of an antiviral role for RNAi in mosquitoes came from studies conducted in an *Anopheles gambiae* cell line, in which replication of a viral RNA was shown to be dependent on the depletion of Ago-2 [15]. Multiple groups subsequently reported elevated levels of virus replication in both adult mosquitoes and cultured cell lines infected with various arboviral pathogens, after knocking down components of the siRNA pathway [15–21]. Several studies also demonstrated that infection of adult mosquitoes and cell lines with various arboviruses results in the production of **virus-derived siRNAs (vsiRNAs)** [19, 22–27]. Further, these siRNAs were shown to direct an antiviral response important to the pathogenic outcome of **alphavirus** infections in the mosquito [24]. Infection of *Aedes aegypti* and *An. gambiae* mosquitoes with recombinant alphaviruses expressing a dsRNA binding protein and **viral suppressor of RNA silencing (VSR)**, significantly decreased accumulation of vsiRNAs, resulting in dramatic increases in both virus replication and mortality [24]. The pathogenesis exhibited by these mosquitoes after infection with the recombinant alphaviruses expressing the VSR protein is similar to the enhanced disease phenotype exhibited by RNAi null mutant flies when infected with viruses [5,6,11]. A similar disease phenotype was recently observed in *Ae. aegypti* infected with Sindbis virus (SINV), in which Dcr-2 had been genetically ablated, suggesting that Dcr-2 is the enzyme responsible for initiating the anti-viral immune response that limits the pathogenicity of the virus in the vector host [28, 29]. In the same study, infection of *dcr-2* null mutant *Ae. aegypti* with YFV significantly increased replication of the virus in comparison to levels observed in wild type mosquitoes, suggesting that RNAi is also an important antiviral mechanism targeting **flaviviruses** in this host.

Viral suppressors of RNA silencing

In a classic example of an evolutionary arms race between host and pathogen a diverse range of plant and insect viruses have evolved to encode VSRs (Figure 1). For example, the P19 protein of Cymbidium ringspot virus (CymRSV) dimerizes to bind siRNA duplexes that are 21-nt in length, specifically interfering with their incorporation into the RISC [30]. In contrast, the FHV B2 protein mediates suppression more generally, by indiscriminately binding dsRNA, interfering with the processing of long dsRNAs by Dcr-2 as well as incorporation of siRNA duplexes into the RISC [31–33]. The DCV 1A suppressor protein binds long dsRNAs, but not short dsRNAs [14]. Other modes of action have also been described. For example, the CrPV 1A protein does not bind dsRNA or siRNA duplexes, but rather inhibits RNA silencing through direct interaction with Ago-2 [34]. The Turnip crinkle virus (TCV) P38 capsid protein (CP) also has been shown to interact directly with the *Arabidopsis* Argonaute 1 (Ago-1) protein [35]. However, the TCV P38 also mediates VSR activity through binding long dsRNAs and siRNA duplexes [36, 37]. Interestingly, the TCV CP appears to have evolved a suppressor function that is separate and distinct from the protein's structural role in the encapsidation of virions [38]. Although VSRs have independently evolved in diverse virus genera, dsRNA binding appears to be emerging as an increasingly common theme [39]. The compact genomes of RNA viruses, necessitated by the high error rates associated with RNA dependent RNA polymerases (RDRPs), typically

encode multifunctional proteins; some with functions that necessitate RNA binding. Some of these proteins may also have acquired the ability to sequester dsRNA, a common cellular recognition pattern associated with virus replication, under strong selective pressure from antiviral immune responses. The influenza NS1 protein possesses multiple immune-modulatory functions stemming from an ability to bind several different types of RNA [40]. Indeed, the protein's ability to sequester dsRNA has also been shown to suppress RNA silencing [15]. Thus, a particularly intriguing hypothesis is that virulence factors in arboviruses may have evolved to modulate both vertebrate and invertebrate immune responses.

Suppressors of RNA silencing in arboviruses

With numerous examples of proteins in plant and insect viruses that function as antagonists of host immune responses, there has been speculation that arboviruses may have evolved similar adaptations to counter antiviral immune pathways in vector mosquitoes. The alternative argument being that the transmission of arboviruses depends upon non-pathogenic infections of mosquito vectors, obviating the evolution of viral proteins that are strongly antagonistic to vector immune pathways. Several studies have attempted to address this question by identifying VSRs encoded in the genomes of arboviruses.

The non-structural protein encoded on the S-segment (NSs) of Bunyamwera virus (BUNV), and the closely related La Crosse virus (LACV), have been proposed to act as VSRs [41–44]. Deleting a portion of the BUNV NSs proved to be less detrimental to virus replication in a *dcr-2* deficient mosquito cell line, than it was in a non-isogenic mosquito cell line possessing a fully functional Dcr-2 [43]. The BUNV NSs deletion mutant also proved to be less infectious to adult *Ae. aegypti* mosquitoes than wild type virus. However, interpretation of the results obtained in cell culture is complicated by the use of non-isogenic cell lines. For example, the BUNV NSs deletion mutant replicated differently in another cell line also containing a null mutation in *dcr-2* [43]. A specific mechanism for the proposed VSR activity was not demonstrated by this study. Using an *in vitro* assay another group demonstrated that the dengue virus (DENV) NS4B protein interferes with processing of siRNAs by Dicer [45]. Gene silencing reporter assays in mammalian cells indicate that transmembrane domains 3 and 5 are somehow involved in the purported VSR activity [45]. While the specific mechanism of suppression remains unclear, it does not appear to involve binding of dsRNA.

Non-coding sequences present in the genomes of flaviviruses have been postulated to act as molecular sponges, or decoys, that antagonize antiviral immunity in invertebrate cells by acting as a competitive substrate for Dicer [46]. **Subgenomic flavivirus RNAs (sfRNAs)** were shown to inhibit a gene silencing reporter assays in both invertebrate and mammalian cell lines, as well as processing of dsRNA by Dicer in an *in vitro* assay [46, 47]. Another study demonstrated an approximately 3-fold decrease in the silencing efficiency of an endogenous gene in *Culex quinquefasciatus* mosquitoes injected with both dsRNA targeting the gene and Kunjin virus (KUNV) [48]. In contrast, silencing of the endogenous gene in *Cx. quinquefasciatus* injected with dsRNA and an sfRNA-deficient KUNV was comparable to mock-infected mosquitoes. In the same study, the 3' un-translated region (UTR) of the

virus, which includes the sfRNA, was specifically enriched after immunoprecipitation of human Dicer and Ago-2, suggesting a possible mechanism of action [48]. Curiously, the replication of WT KUNV and the sfRNA-deficient KUNV did not appear to differ significantly in the infected *Cx. quinquefasciatus*. Similarly, it was reported that the replication of two different West Nile virus (WNV) isolates and an sfRNA1-deficient mutant WNV exhibited similar replication kinetics in both *dcr-2⁺ U4.4* and *dcr-2* null C6/36 *Aedes albopictus* cell lines [49]. Although sequencing small RNAs from WNV infected *Culex pipiens* and mapping to the viral genome did not reveal a disproportionate number of 21-nt reads derived from the region encoding the sfRNA, specific hotspots mapping to the sfRNA were noted [49]. However, overall both the production and distribution of vsiRNA generating loci in these mosquitoes was similar to that observed in *Cx. pipiens* infected with the sfRNA-deficient mutant virus [49]. Additional studies with *dcr-2* null mutants would be helpful in determining if the small RNAs mapping to the sfRNA hotspots are indeed products of the RNAi machinery. While this particular study produced little direct evidence for the WNV sfRNA acting as a VSR, the sequence was found to increase both dissemination of WNV from the midgut of the insect, as well as transmission of WNV by the vector mosquito. Interestingly, another group recently reported that two epidemiologically relevant substitutions in the 3' UTR of DENV-2 specifically increased virus production in the salivary glands of *Ae. aegypti*, resulting in more infectious saliva[50]. These results were correlated with increased production of the DENV-2 sfRNA and inhibition of the Toll immune pathway, specifically in the salivary glands[50]. Thus, this study suggests a tissue specific role for the sfRNA that may be independent of any VSR activity.

Expression of heterologous VSR proteins, employing diverse mechanisms of RNA silencing, from recombinant SIN viruses has been shown to correlate with a disease phenotype in *Ae. aegypti* [29]. In contrast with these results, infection of *Ae. aegypti* with recombinant SIN viruses expressing the WNV sfRNA, DENV NS4B or BUNV NSs does not appear to significantly increase virus replication or mortality, i.e., hallmarks of a disease phenotype, suggesting that these sequences provide little to no protection from the RNA silencing response induced by the replicating SINV[29]. In the case of the WNV sfRNA, these results are consistent with previous findings demonstrating that the replication of KUNV and WNV mutants, deficient for the production of sfRNAs, were comparable to that of WT viruses in both mosquitoes and cells [48, 49]. However, SINV expressing the capsid (YFC) protein of YFV was recently shown to produce a disease phenotype in *Ae. aegypti*, similar to that observed when well-characterized VSR proteins are expressed from the virus [29]. Genetic rescue experiments demonstrating that the replication and virulence of SINV expressing the YFC was equivalent to that of SINV containing a non-translatable version of the YFC in a *dcr-2* null mutant background, specifically implicates the protein as an antagonist of the siRNA pathway[29]. These experiments were similar to those performed in studies that have previously implicated the TCV P38, Cucumber mosaic virus 2b, FHV B2, Nora virus VP1, CrPV 1A, and DCV 1A proteins as VSRs [8, 34, 51–53]. Genetic rescue experiments involving the Nora virus VP1, CrPV 1A and DCV 1A proteins also involved expression from a recombinant SINV, as infectious clones were not available for these viruses [34, 54]. Antagonism of RNA silencing by YFC appears to be mediated by non-specific binding of

long dsRNAs, interfering with the production of vsiRNAs by Dicer [29]. Although VSR proteins are incredibly diverse, non-specific RNA binding is a common theme [39], with some of the most well characterized suppressor proteins, for example the FHV B2 protein, having this mechanism of action [32, 53]. A VSR function has previously been identified in the P38 CP of the plant virus TCV [36]. TCV is also a positive-strand virus, and high affinity coat protein binding sites have been identified on the ssRNA viral genome [55]. The TCV CP has also been found to bind dsRNA non-specifically [39]. Thus, there is precedent for both ssRNA and dsRNA-binding in a viral protein with functions similar to those of the YFC. The same study found that dsRNA binding is a general strategy for the suppression of RNA silencing that has evolved independently many different times in the VSRs of a large number of unrelated plant viruses[39]. Further, the authors hypothesized that the most ancient VSRs may have been non-specific dsRNA-binding proteins. Identification of a VSR function in a flavivirus protein (YFC) with many similarities to the previously identified TCV CP, may suggest independent evolution of VSR functions in functionally similar capsid proteins. While the YFC is clearly an antagonist of RNA silencing, genetic rescue experiments in the context of the YFV genome will be required to confirm the suppressor activity of YFC during YFV infection.

PIWI-interacting RNAs

The primary role of piRNAs is believed to be in silencing **transposons**, and maintaining the integrity of the animal's germline [56–58]. These small RNAs originate from distinct genomic loci, termed piRNA clusters [58–60]. These piRNA clusters give rise to long, single-stranded RNA transcripts that are then processed into piRNAs. These piRNA clusters appear to be a biological equivalent of malware definition files, distinguishing transposons from host genome sequences, facilitating selective silencing of the selfish genetic elements by the piRNA pathway. Dicer doesn't appear to be involved in processing piRNAs. However, piRNAs can be further classified based upon distinct mechanisms of biogenesis [60, 61]. Zucchini (Zuc) is the ribonuclease that processes the 5' end of piRNAs in follicular cells (Zuc-mediated processing) that are then loaded into the slicer protein, Piwi [62, 63]. In nurse cells, an alternative "ping-pong" mechanism has been described for the biogenesis of piRNAs that will be loaded into the slicer proteins, Aubergine (Aub) and Ago-3 (slicer-mediated processing) [60, 61]. In this mechanism, piRNAs direct the production of additional piRNAs from opposite strands. For example, piRNAs bound by Aub will direct the cleavage of precursors that will be bound by Ago-3, in a process that determines the 5' ends of the piRNAs. The cleavage that generates the 5' end of the product occurs exactly 10 nucleotides (nt) from the 5' end of the guide piRNA sequence. This results in enrichment for an adenine at the 10th position of ping-pong-derived piRNAs, which correlates with the characteristic U-bias found at the 5' end of piRNAs. The 3' ends of piRNAs can be formed by Zuc cleavage, slicer processing, or exonucleolytic trimming[60, 64].

While it is often presumed that small RNA pathways are conserved among **dipterans**, it is becoming increasingly apparent that there are major differences in the small RNA pathways of the fruit fly and those of the mosquito. The *Drosophila* Piwi-clade is comprised of the Ago slicer proteins: Piwi, Aub and Ago-3. Mutations in **Piwi-clade Ago proteins** have

previously been shown to increase expression of transposable elements (TEs) in the fly ovaries [56, 57]. However, no clear role for this pathway has been established outside the germline. Ago-3 and Aub do not appear to be expressed in the *Drosophila* soma [60, 61, 65–67]. While Piwi and Zuc are expressed in follicular cells, with the production of mature piRNAs in these cells occurring exclusively through Zuc-mediated processing [56, 68], the piRNA pathway does not appear to operate more broadly in soma of the fruit fly. *Aedes* and *Culex* mosquitoes have an expanded repertoire of Piwi-clade Ago proteins [69, 70]. The Piwi-clade Ago proteins of *Ae. aegypti* include Piwi 1-7 and Ago-3, which in contrast to *D. melanogaster* are broadly expressed in the soma. Further, several groups have reported on the production of a class of ping-pong dependent **virus-derived piRNAs (vpiRNAs)** in mosquitoes and mosquito cell lines, infected with various arboviruses [22, 27, 71–76](Figure 2). Evidence indicates that production of these vpiRNAs occurs in the mosquito soma, unlike ping-pong-dependent piRNAs that have been described previously from repetitive elements or piRNA clusters. Northern blotting of small RNAs after immunoprecipitation of Piwi-clade Ago proteins from an *Ae. aegypti* cell line infected with SINV indicated specific enrichment of virus derived piRNAs with Ago-3 and Piwi-5 [77]. Sequencing these small RNAs indicated that antisense vpiRNAs were preferentially bound by Piwi-5, while sense strand vpiRNAs were preferentially bound by Ago-3, suggesting a possible role for these two proteins in a ping-pong mechanism [77]. Although the production of viral piRNAs in response to virus infection has been well documented, an antiviral role for the piRNA pathway has yet to be directly demonstrated. Interestingly, knockdown of Piwi-4 in **Aag2 cells** results in enhanced replication of Semliki Forest virus (SFV), but Piwi-4 does not appear to be involved in the production of viral piRNAs, rather an antiviral role has been proposed for the Piwi-4 protein independent of the piRNA pathway [18, 77, 78].

Interestingly, infection of *dcr-2* null mutant cells with a recombinant CHIKV expressing B2 significantly decreased accumulation of viral piRNAs, while expression of a B2 with an R54Q mutation, previously shown to abolish the protein's ability to bind long dsRNA, exhibited no such inhibitory effect on the production of viral piRNAs [71]. Decreases in viral piRNAs were accompanied by cytopathological changes in the infected cells, as well as an increased number of viral (+) strands per piRNA. This has led to a hierarchical model of antiviral immunity in aedine mosquitoes [71]. In this model, the siRNA pathway serves as the primary antiviral defense, with the somatic piRNA pathway providing a subordinate antiviral activity that provides robustness to the vector's antiviral immune response. While it remains unclear how the B2 protein's ability to bind dsRNA inhibited the production of viral piRNAs in these cells, the results raise the intriguing possibility that VSRs capable of sequestering dsRNA may interfere with both the siRNA and piRNA pathway. The involvement of viral dsRNA replicative intermediates in the production of vpiRNAs is also attractive as a hypothesis to explain the selective targeting of viral RNAs by the pathway. The positive sense CHIKV genomic RNA is capable of initiating virogenesis even when deproteinized [79]. An essential requirement for this to occur is that the cellular machinery for protein production efficiently translates the viral mRNAs. The genomic and mRNAs of alphaviruses are generally indistinguishable from cellular mRNAs, possessing a 5'-terminal cap and 3'-terminal poly (A) tract [79]. The piRNA pathway does not appear to target endogenous cytoplasmic mRNAs possessing similar properties. It also seems unlikely that

the RNA of alphaviruses possess sequence specific triggers of an antiviral piRNA response, as the pathway also targets other RNA viruses (flaviviruses, **phleboviruses** and **orthobunyaviruses**), with diverse genome sequences and configurations (segmented, negative polarity, etc.) [22, 72, 75, 76]. However, dsRNA is a well-established molecular pattern commonly recognized by both vertebrate and invertebrate antiviral immune pathways.

Regulation of arbovirus replication by miRNAs

MicroRNAs are processed from endogenous hairpin transcripts and regulate host gene expression by binding mRNAs, influencing their translation or relative abundance. These endogenous transcripts are transcribed by RNA polymerase II as primary miRNAs (pri-miRNA) [80]. The pri-miRNAs are then processed by the nuclear protein, Drosha, into pre-miRNAs, which are subsequently exported from the nucleus into the cytoplasm [81]. In the cytoplasm, pre-miRNAs are further processed into ~22-nt duplex miRNAs by Dcr-1 [82]. The strand of the duplex that is less thermodynamically stable at its 5'-end will be loaded into a RISC containing Ago-1, serving as the guide that mediates sequence specific translational repression or mRNA degradation [83, 84].

A number of RNA viruses, including multiple arboviruses, have been found to exploit host miRNAs to evade vertebrate antiviral immune responses, resulting in increased replication and pathogenicity [85]. Similar phenomena have not yet been widely described in invertebrates, despite abundant regulation of protein-coding genes in these animals. However, a miRNA-like small RNA has been found to be encoded in the 3' UTR of WNV [86]. The mature sequence (termed KUN-miR-1) was detected by northern blot in WNV-infected *Ae. aegypti* and *Ae. albopictus* cell lines. Production of the small RNA in mosquito cells was shown to be dependent on Dicer 1, but not Dicer 2. KUN-miR-1 appeared to be required for efficient replication of the virus in mosquito cells by up-regulating the expression of GATA4, a transcription factor [86]. It would be interesting to know if the GATA4 transcription factor is similarly up-regulated in *Culex* mosquitoes, the natural vector species of WNV, by expression of KUN-mir-1. Six virus derived miRNA-like small RNAs, termed vsRNA 1-6, have also been identified in *Ae. aegypti* mosquitoes infected with DENV-2 (New Guinea C strain) by next generation sequencing [87]. While DENV-2-vsRNA5 was associated with increased replication of the virus, the candidate miRNA sequence remains somewhat controversial due to its relatively low abundance in adult mosquitoes and cells [88]. Mosquitoes possessing loss-of-function mutations in key components of the miRNA pathway would be helpful in confirming the identity, and determining the mechanism of biogenesis for the KUN-mir-1 and DENV-2-vsRNA 1-6 sequences. Certainly, infection of mosquitoes with arboviruses also results in the differential expression of a number of miRNAs [89–93]. Whether any of these changes are directly attributable to a specific viral mechanism that has evolved to the benefit of the pathogen requires further investigation.

Other evolutionarily conserved antiviral pathways

While the siRNA pathway appears to be a robust pan-antiviral response present in mosquitoes, there is some evidence to suggest a role for other evolutionarily conserved pathways, such as the **Jak-STAT**, **Immune deficiency (Imd)** and **Toll pathways** in vector responses to viral infection [94, 95] (Figure 2). While these pathways have primarily been characterized in the genetic model organism, *Drosophila melanogaster*, key components of the transcriptional responses are conserved in the major vector species, *Ae. aegypti* [96].

Initial evidence for the involvement of Jak-STAT in the antiviral responses of insects came from studies conducted in fruit flies infected with DCV [97]. Infection of flies with DCV resulted in the up-regulation of a distinct set of genes, including some with promoters possessing consensus **STAT (signal transducers and activators of transcription)** binding motifs. Notably, the majority of up-regulated genes were distinct from previously well-characterized genes encoding **antimicrobial peptides (AMPs)**. Mutating the STAT-binding site in one of the differentially expressed genes, *vir-1*, reduced its activity in the *D. melanogaster* S2 cell line [97]. In *Drosophila*, a single STAT factor (STAT92E) is regulated by a single Jak (Janus) kinase, Hopscotch (Hop). Similarly, transcription of *vir-1* was substantially lower in flies possessing loss-of-function mutations in *hop* when infected with DCV. In both this study, and a subsequent study, *hop*-deficient flies were found to exhibit increased viral titers and decreased survival when infected with DCV or CrPV, also suggesting an antiviral role for this pathway [97, 98]. However, later studies with the *hop* mutant flies and a panel of different viruses found that the contribution of the JAK-STAT pathway to antiviral immunity may be virus-specific, as the *hop*-deficient flies did not exhibit an “enhanced disease” phenotype with all of the viruses tested [98]. While this study found that flies possessing loss-of-function mutations in key components of the siRNA pathway were more susceptible to infection with the large panel of viruses tested, including the DNA virus, IIV-6, transcriptional responses induced by Jak-STAT were virus-specific, and only critical during infection with members of the *Dicistoviridae* (DCV, CrPV, etc.). Infection of *D. melanogaster* with DCV has also been shown to induce the expression of the vago gene, the product of which limits replication of the virus in a tissue specific manner (i.e., fat body) [99]. Interestingly, the expression of Vago appears to be activated by Dcr-2, in a manner independent of its role in RNA silencing, suggesting a possible connection between tissue specific inducible antiviral factors and the more general RNA silencing pathway (Figure 2). A *Culex* orthologue of Vago (*CxVago*) was found to be up-regulated in a *Cx. quinquefasciatus* cell line (Hsu) infected with WNV [100]. The *CxVago* protein was also found to have antiviral activity. The antiviral activity of the protein was diminished in Hsu cells treated with dsRNA targeting Dcr-2, suggesting Dcr-2-dependent activation of Vago. Interestingly, knockdown of *CxSTAT2* or *CxJak*, a Hop orthologue, diminished antiviral activity in Hsu cells treated with secreted Vago protein, suggesting the protein’s antiviral properties may involve activation of the Jak-STAT pathway [100].

Unfortunately, the Jak-STAT pathway’s role in the mosquito’s immune response to viruses is not as well characterized as it is in the model organism *D. melanogaster*. Similar to studies in *Drosophila*, infection of *Ae. aegypti* mosquitoes with arboviruses has been shown to up-regulate the expression of various Jak-STAT pathway genes [101, 102]. *Ae. aegypti*, in

which components of the Jak-STAT pathway were knocked down, have also been shown to exhibit increased susceptibility to DENV infection; although, significant increases in virus replication and decreased survival were not reported [103]. In contrast, infection of an *Ae. albopictus* cell line (U4.4) with the alphavirus, Semliki Forest virus (SFV) did not activate the STAT pathway, suggesting that similar to *Drosophila* the antiviral role of the pathway may be pathogen or tissue specific [104].

In vertebrates, arboviruses, including Japanese encephalitis virus (JEV), WNV, and DENV have been shown to interfere with the Jak-STAT pathway by inhibiting Jak phosphorylation, thereby preventing translocation of STAT1 and STAT2 to the nucleus, or altering the expression of ISG genes [105–107]. Although there is no direct evidence for arboviral interference with the Jak-STAT pathway in the mosquito, inhibition of STAT phosphorylation and DNA binding activity has been reported in C6/36 mosquito cells infected with JEV [108]. Similarly, activation of the Jak-STAT pathway in U4.4 cells exposed to heat inactivated bacteria was more limited in the presence of SFV-infections [104]. Thus, additional studies may be warranted to determine if arboviruses have evolved to encode specific antagonists of Jak-STAT pathways in disease vectors.

In addition to roles in defense against bacterial and fungal pathogens, the Toll and Imd pathways have also been implicated in the antiviral responses of insects. These pathways rely on the activation of pattern recognition receptors to mediate downstream antiviral responses via antimicrobial peptides [109–113]. In *Drosophila*, mutations in the Imd pathway resulted in increased viral replication upon infection with SINV [109]. Similar findings were obtained with o'nyong nyong virus (ONNV) in *An. Gambiae*, in which components of the Imd pathway had been depleted [114]. The Toll pathway has also been suggested to play a role in the antiviral immunity of other disease vector mosquitoes. Infection of *Ae. aegypti* with DENV-2 results in the up regulation of genes involved in the Toll pathway [111]. Activation of the Toll pathway genes in *Ae. aegypti* was correlated with decreased susceptibility to DENV-2, while inhibition of the pathway was associated with increased viral load [111, 115]. However, in contrast, studies with alphaviruses have failed to find any role for the Toll pathway with regard to *Drosophila* or mosquito antiviral responses [104, 109, 116]. Thus, similar to the JAK-STAT pathway the importance of the Toll pathway to antiviral immunity may also be virus specific (Figure 2).

Adaptive Immunity

Recently, it has been proposed that viral-derived DNA plays a role in the establishment of persistent arboviral infections in mosquitoes. This is thought to occur through the activity of endogenous retrotranscriptases that transcribe DNA intermediates from viral sequences [75, 117] (Figure 2). Inhibiting cellular reverse transcriptase activity with the drug **azidothymidine (AZT)**, in *Ae. albopictus* infected with CHIKV, and *Ae. aegypti* infected with DENV, decreased production of viral siRNAs and piRNAs, and increased mortality [75]. Interestingly, concomitant increases in the replication of these viruses were not observed, leaving the underlying reason(s) for the decreased survival of the virus infected, and AZT-treated mosquitoes unexplained [75]. Nevertheless, the notion that DNA intermediates may be involved in the antiviral response, suggests the possibility of

potentially long-lasting immunity. Indeed, another study recently described a non-canonical population of virus-derived siRNAs present in *Drosophila* **hemocytes** [118]. The authors of this study have proposed that the non-canonical siRNAs are secondary products amplified from reverse transcribed viral DNAs, which were also found to be present in hemocyte cells; although, the specific mechanism for the production of the “secondary viral siRNAs” was not demonstrated (Figure 2). It is questionable whether these products should be termed “secondary” vsRNAs as they exhibit biochemical properties that are distinct from those exhibited by the canonical siRNAs processed by D-2. Indeed, the authors speculated that they might be processed from single stranded RNAs, in a mechanism with similarities to piRNA biogenesis. Interestingly, the authors of this study showed that exosome-like vesicles purified from fruit flies infected with SINV, and containing the non-canonical viral siRNAs, were able to passively immunize naïve flies against subsequent viral infection. Thus, it is proposed that hemocytes release exosome-like vesicles containing the non-canonical viral siRNAs, which disseminate to uninfected naïve tissues and cells distal to the sites of active infection, conferring immunity in a manner that, in some respects, parallels the long-lasting adaptive immune responses of vertebrate mammalian organisms [118]. It is presently unknown if mosquitoes possess an analogous mechanism for amplifying and spreading the antiviral immune response.

Concluding Remarks

The majority of human viral pathogens transmitted by mosquitoes appear to have co-evolved with their insect hosts so as to minimize adverse effects on survival. Until relatively recently there was little evidence explaining the apparent resistance of mosquito cells to the potential cytopathic effects of these viruses. However, in a little more than a decade, the mosquito has been found to possess multiple immune pathways, many of which appear to contribute, at least in some capacity, to the antiviral defenses of the insect (Figure 2). In spite of this, mosquito-borne viruses persist in nature causing widespread morbidity and mortality in human and animal hosts. Evidence is beginning to mount that arboviruses have evolved adaptations to actively block RNA silencing, the primary innate immune response in mosquitoes to infection with viruses. The presence of VSRs in the genomes of these viruses is potentially transformative to our understanding of multiple aspects of pathogen transmission by mosquito vectors, but most notably with regard to the emergence and re-emergence of diseases. Viruses and their hosts often have adversarial relationships in which a beneficial adaptation in one organism, the virus’s ability to infect and replicate in a host for example, imposes a cost to the other organism, for example, the manifestation of disease. Over time, the evolutionary pressures associated with this relationship may result in reciprocal adaptive genetic changes to both species, particularly host proteins involved in antiviral immunity, and viral proteins involved in blocking immunity, which has been termed an evolutionary “arms race” [119]. Although evidence of co-evolution between hosts and pathogens can in practice be difficult to demonstrate, the evolution of VSR proteins is hypothesized to result from “arms races”. This is supported by the fact that previously characterized VSR proteins exhibit evidence of rapid evolution. Comparing VSRs across virus families reveals little conservation of structure, even in cases where function is conserved [38, 120]. Even within some viral species, VSR proteins have been shown to

exhibit evidence of rapid evolution[121]. Consistent with this, components of the *Drosophila* siRNA pathway, which are directly antagonized by VSR proteins, have been demonstrated to be among the most rapidly evolving genes in the fruit fly[122, 123].

While the presence of VSRs in arboviruses suggests an “arms race” with the antiviral immune pathways of vectors, it will be interesting to see if these co-evolutionary relationships are characterized by positive (diversifying) selection, as would be expected of a conventional arms race. We have previously hypothesized that persistent infections of mosquito vectors, necessary for the maintenance of arboviruses in nature, would require the potency of any potential VSRs to be balanced with the replication kinetics of the virus and the antiviral responses of the host [124] (Figure 3; Key figure). Viruses exhibit diverse strategies for mRNA production, replication and protein translation, and these strategies have co-evolved with the cellular targets and defenses of natural hosts, contributing to the overall replication kinetics of the virus observed in that host. Expressing a heterologous VSR from SINV likely disturbs the equilibrium that has co-evolved to balance the replication kinetics of the virus with the antiviral response of the host. However, these results do not necessarily suggest that same VSR should render the virus whence it was originally derived lethal to its’ insect host, as mentioned above many factors contribute to the co-evolution of a virus with its natural host. For example, DCV is associated with low mortality and establishes persistent infections in its native host. Nevertheless, when the viruses VSR protein, DCV-1A, is expressed from a recombinant SINV it increases the pathogenicity of the alphavirus infection in both a fruit fly and mosquito host [34] [29]. Thus, in the case of mosquito-borne pathogens, we can hypothesize that overly robust antagonism of RNA silencing in the vector host should be selected against, as this would result in disease and possibly death, both of which would be highly detrimental to transmission of the virus (Figure 3). Similarly, we also hypothesize that VSRs too weakly antagonistic to result in productive infections of the vector host should also be selected against, as this would very obviously also be detrimental to transmission of the virus (Figure 3). Thus, the requirement for arboviruses to establish persistent, non-pathogenic infections in the vector host might limit rapid diversifying evolution of host proteins involved in antiviral immunity, and viral proteins involved in blocking immunity. The opposing forces of immunity and virus-mediated antagonism, regardless of mechanism, likely have a large role in determining the optimal levels of virus replication necessary for transmission to a vertebrate host (Figure 3). However, as other viral proteins directly influence the spatial, temporal, and level of expression of the antagonistic mechanism in the insect host cell, the necessity of maintaining this equilibrium might act as a general constraint to the evolution of the proteins encoded in the pathogen’s genome, rather than just those involved in antagonizing immunity. Alternating replication of arboviruses in vertebrate and invertebrate hosts has previously been hypothesized to limit the rate of reciprocal adaptations to either host, constraining the evolution of these viruses[125, 126]. In support of this, vector-borne RNA viruses have been shown to exhibit less evidence of positive (diversifying) selection than viruses with other routes of transmission, this being most apparent in proteins present on the surface of the virion [127]. However, this does not exclude the possibility that other aspects of the biology of these viruses also necessitate constraints on evolution. Interestingly, analysis of VSRs from twelve different plant viruses showed that only a subset exhibited evidence of

persistent positive selection, when compared to the other viral genes [128]. While the study was unable to find any mechanistic basis to explain why the VSRs as a group did not demonstrate evidence of positive selection, the VSRs were not analyzed on the basis of whether or not they were associated with viruses producing persistent or acute infections. A similar analysis with the VSRs of insect viruses associated with pathogenic or non-pathogenic persistent infections in their respective hosts would be interesting to perform in the future (see Outstanding Questions).

As discussed, evolutionary trade offs may be required to maintain the transmission cycles of arboviruses in nature. This may serve to constrain not only adaptation to new vertebrate hosts but also new invertebrate hosts, i.e., vector switching. Exploiting a vector species is a co-evolutionary process that involves, among other things, adapting the mRNA production, replication and protein translation of the virus to the immune responses of the insect. We are only just beginning to understand the immune defenses of insects and the antagonistic mechanisms encoded by viruses to counteract them. However, the molecular genetics of these host-pathogen interactions are, in theory, major determinants of the emergence and re-emergence of vector-borne diseases. In the future, it may be possible to exploit knowledge of virus-vector interactions to inform the design of powerful new strategies of disease control.

GLOSSARY

Aag2 cells

Ae. aegypti cell line of embryonic origin that is used as a model to study mosquito immunity

Aedes aegypti

Widely known as the yellow fever mosquito for its role in the transmission of yellow fever virus, but also a primary vector for pathogens such as Zika virus, dengue virus (serotypes 1-4), and chikungunya virus

Aedes albopictus

An invasive mosquito species and known vector of chikungunya virus, dengue virus (serotypes 1-4) and potentially Zika virus

Alphavirus

A genus of single-stranded positive-sense RNA viruses that includes mosquito-borne pathogens such as chikungunya virus, Sindbis virus, Semliki forest virus, and o'nyong-nyong virus

Anopheles gambiae

A major mosquito vector of malaria, but also of o'nyong-nyong virus, one of only a few viruses vectored by anopheline species mosquitoes

Antimicrobial peptide

Inducible antimicrobial factors released through activation of innate immune responses to bacterial, fungal and viral pathogens

Arthropod borne viruses

A non-taxonomic grouping of viruses biologically transmitted by hematophagous arthropods. While these viruses span multiple taxonomic groups, many examples can be found in four genera: *Flavivirus*, *Alphavirus*, *Orthobunyavirus* and *Phlebovirus*, including some of the most important mosquito-borne pathogens causing severe morbidity and mortality in humans and animals

Azidothymidine (AZT)

Inhibitor of reverse transcriptases

Cricket paralysis virus

A positive strand RNA virus, belonging to the family *Dicistroviridae*, and causing pathogenic infections of fruit flies and field crickets

Culex pipiens

A complex of closely related mosquito species including *Cx.pipiens.pipiens* and *Cx.pipiens.quinquefascitus* that are vectors of West Nile virus

Culex quinquefascitus

A mosquito belonging to the *Cx.pipiens* complex and vector of West Nile virus

Dipterans

Members of the order *diptera* meaning “two wings” having one pair of wings and sucking mouthparts. Mosquitoes and flies are members of this order

Drosophila C virus

A positive strand RNA virus, belonging to the family *Dicistroviridae*, and establishing persistent, non-lethal infections, in strains of *Drosophila*

Enhanced disease phenotype

Phenotype exhibited by immuno-compromised fruit flies when infected with viruses, characterized by elevated levels of viral replication and increased mortality

Flavivirus

A genus of single-strand positive-sense RNA viruses that includes mosquito-borne pathogens such as yellow fever virus, Zika virus, West Nile virus, Kunjin virus, dengue virus (serotypes 1-4) and Japanese encephalitis virus

Flock house virus

A non-enveloped, positive strand RNA virus with a bipartite genome, belonging to the family *Nodaviridae*, and causing pathogenic infections of beetles

Hemocytes

Macrophage-like immune cells, found circulating in the hemolymph of insects

Imd pathway

An evolutionarily conserved signaling pathway controlling the release of antimicrobial peptides

JAK-STAT pathway

An evolutionarily conserved inducible signaling pathway that mediates its activity through the activation of transcription factors and expression of genes involved in a variety of cellular processes, including immunity

Orthobunyavirus

A genus of tri-segmented negative strand viruses that includes the mosquito-borne pathogens bunyamwera virus and LaCrosse virus

Phlebovirus

A genus of tri-segmented negative strand viruses that includes the mosquito-borne pathogen Rift Valley fever virus

Piwi-clade Ago proteins

Drosophila slicer proteins Argonaute 3, Piwi and Aubergine belonging to the Piwi clade of the Argonaute superfamily

Replicative intermediates

Intermediate forms of viral nucleic acids produced during replication. Although nearly all viruses produce at least some dsRNA, this molecule is a replicative intermediate of RNA viruses

RNAi induced silencing complex (RISC)

A multi-protein complex that in *Drosophila* includes the slicer protein Argonaute 2 and a siRNA, which acts as a guide to target cognate mRNAs

Signal transducer and activator of transcription (STAT)

Transcription factors, activated by Janus Kinase (Jak), involved in multiple aspects of cellular immunity

Subgenomic flavivirus RNA (sfRNA)

Non-coding RNA produced from the 3' UTR of flaviviruses during infection of vertebrate and invertebrate cells. The sfRNA is a highly resistant structure that is not amenable to degradation by the cellular endonuclease XRN1

Toll Pathway

An evolutionarily conserved inducible signaling pathway activating cellular immunity and antimicrobial peptides in response to gram-positive bacteria and fungi

Transposons

Transposable elements (TE) or “jumping genes” are potentially mobile selfish genetic elements that invaded the genome. The endo-siRNA and piRNA pathways are involved in regulating TEs, maintaining the integrity of the animal germline

Viral suppressor of RNA silencing (VSRs)

Proteins encoded in viral genomes that inhibit the RNAi pathway

Virus-derived siRNAs (vsiRNAs)

siRNAs that are derived from the double stranded replicative intermediates of RNA viruses

Virus-derived piRNAs (vpiRNAs)

piRNAs derived from the nucleic acids of RNA viruses

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Highlights

- Multiple studies implicate the small interfering RNA (siRNA) pathway as the major antiviral response of vector mosquitoes.
- Mounting evidence suggests that the genomes of arthropod-borne viruses encode antagonists of antiviral immune pathways.
- Infection of vector mosquitoes with arboviruses results in virus-derived Piwi-interacting RNAs (piRNAs), but these small RNA products have not yet been ascribed an antiviral function.
- Some evidence suggests the Jak-STAT, Imd, and Toll pathways may contribute to the antiviral immunity of the vector, possibly in a pathogen or tissue specific manner.
- DNA intermediates transcribed from viral sequences by endogenous retrotranscriptases may participate in antiviral immunity through virus-derived piRNAs or a non-canonical class of siRNAs trafficked by hemocytes, suggesting that insects may possess an adaptive form of immunological memory.

OUTSTANDING QUESTIONS BOX

1. What role do evolutionary conserved immune pathways play in the antiviral responses of disease vector mosquitoes, and do arboviruses encode antagonists of these pathways? What are the molecular and mechanistic details of these interactions?
2. Have evolutionary conserved immune pathways evolved functions unique to disease vectors, and not present in genetic model organisms.
3. Do disease vector mosquitoes have both innate and adaptive immune responses to viral infections, including systemic immunological memory with parallels to the immune systems of mammalian vertebrate animals?
4. Do arboviruses exploit viral- or host- encoded miRNAs to evade invertebrate antiviral immune responses?
5. Does a co-evolutionary virus-vector relationship necessitating persistent infections for maintenance of natural transmission cycles require evolutionary trade offs that serve to limit rapid genetic changes to host proteins involved in antiviral immunity, and viral proteins involved in antagonism of immunity?

Figure 1. Viruses have evolved diverse proteins (VSRs) that interfere with various steps of the RNA silencing pathway

Viral replication triggers Dcr-2 processing of dsRNA intermediates into siRNA duplexes. Virus-derived siRNAs are then loaded into the RISC, a guide strand acting as a specificity determinant for the targeting and degradation of cognate viral mRNAs. VSRs bind long dsRNAs (YFV C, FHV B2, DCV 1A, H1N1 NS1 or TCV P38) preventing processing by Dicer, and/or the smaller siRNA duplexes (FHV B2, H1N1 NS1, TCV P38 or CymRSV) preventing loading into the RISC. VSRs may in some cases also interact directly with Ago2 (CrPV 1A or TCV P38) to inhibit slicing activity.

Figure 2. Overview of molecular pathways with antiviral or potential antiviral roles in mosquitoes

A) The RNAi pathway has emerged as the major antiviral response in mosquitoes; although, most of the mechanistic details of the pathway are derived from work in *D. melanogaster* or other model organisms. Recognition of viral dsRNA triggers Dicer-2 to process dsRNA into viral siRNAs which are then loaded into the RISC complex to target cognate viral RNAs for sequence specific degradation. B) Virus-derived piRNAs exhibit hallmarks of a ping-pong-mediated mechanism for biogenesis, but these products have not yet been ascribed any specific role in antiviral immunity. C) There is some evidence to suggest that the evolutionarily conserved Imd, Toll and Jak-STAT pathways contribute to the antiviral response in mosquitoes, possibly in a virus or tissue specific role. A connection between the siRNA and Jak-STAT pathways mediated by Vago, a secreted protein, has been postulated. D) The mosquito has recently been postulated to have adaptive antiviral responses mediated by reverse transcribed viral DNA (vDNA), which has been proposed to enter the piRNA pathway. E) In flies, a mechanism for the production of a non-canonical class of viral siRNAs released from exosome-like vesicles in circulating hemocytes has been described. As yet, it remains unclear if similar mechanisms exist in mosquito vectors.

Figure 3. Model for the co-evolutionary relationship between mosquito-borne viral pathogens and their disease vectors

If viral mechanisms antagonizing antiviral immunity are too weak, viral replication will decrease to unsustainable levels leading to non-productive infections. However, viral mechanisms antagonizing antiviral immunity too robustly will result in levels of virus replication consistent with disease and possibly death, which would also be disruptive to the transmission cycles maintaining these viruses in nature. Thus, levels of viral replication conducive for transmission to a susceptible vertebrate host must be defined by upper and lower limits, which may serve as a constraint on the evolution of pathogens with this type of biology.