



Published in final edited form as:

Future Microbiol. 2011 March ; 6(3): 265–277. doi:10.2217/fmb.11.11.

Mosquito RNAi is the major innate immune pathway controlling arbovirus infection and transmission

Carol D Blair

Arthropod-borne & Infectious Diseases Laboratory, Department of Microbiology, Immunology & Pathology, Colorado State University, 1692 Campus Delivery, Colorado State University, Fort Collins, CO 80523-1692, USA ■ Tel.: +1 970 491 6794, ■ Fax: +1 970 491 8323 ■
cblair@colostate.edu

Abstract

Mosquito-borne arboviruses cause serious diseases in humans that are increasingly becoming public health problems, yet arbovirus infections cause minimal pathology in the mosquito vector, allowing persistent infections and lifelong virus transmission. The principal mosquito innate immune response to virus infections, RNAi, differs substantially from the human immune response and this difference could be the basis for the disparate outcomes of infection in the two hosts. Understanding the mosquito antiviral immune response could lead to strategies for interruption of arbovirus transmission and greatly reduce disease. Research focused on RNAi as the primary mosquito antiviral response has the greatest potential for developing a full understanding of mosquito innate immunity. This article reviews our current knowledge of mosquito antiviral RNAi and charts some of the future directions needed to fill knowledge gaps.

Keywords

Aedes aegypti; alphaviruses; arbovirus; argonaute; dicer; *Drosophila melanogaster*; flaviviruses; innate immunity; RNAi

Arthropod-borne viruses (arboviruses) cause some of the most serious and feared human infectious diseases, such as hemorrhagic fevers and encephalitides, yet their infections of arthropod vectors, which are essential links in their transmission cycles, are almost always nonpathogenic and persistent for the life of the mosquito or tick. Research in the last 10 years has led us to the hypothesis that a major factor in determining the disparate outcomes of vertebrate and mosquito infections by arboviruses is the difference in the immediate or innate immune responses in the two systems. In this article, I will provide support for the postulate that the balance between the RNAi defense response and the viral ability to evade this response in order to cause persistent infection is key to arbovirus transmission.

The vertebrate immune response to arboviruses has been intensely studied, both in attempts to control disease through the development of vaccines or immunotherapeutics, and because it is clear that in some cases immunopathology plays a major role in exacerbation of human diseases. On the other hand, knowledge of the immune response of the vector to arbovirus

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Financial & competing interests disclosure

Research conducted in the author's laboratory was supported by NIH/NIAID grant AI034014. The author has no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

infections is relatively incomplete, but it is gaining attention in order to provide a more comprehensive model for arbovirus–vector interactions, and because enhancement of vector immunity could augment resistance and interrupt arbovirus transmission. A comprehensive review of current knowledge of mosquito innate immune responses has recently been published [1]. My discussion will focus more narrowly on RNAi as the principal virus-specific mosquito innate immune response, with brief reference to the potential roles of both general and virus-specific transcriptionally activated pathways in antiviral defense.

The model organism *Drosophila melanogaster* has provided many insights into the immune systems of both vertebrates and invertebrates; however, one of the most important vertebrate innate antiviral pathways, the interferon (IFN)- α/β , or type I IFN system, was discovered before knowledge of *Drosophila* immunity was developed and is clearly unique to vertebrates. RNAi is the primary and most important antiviral immune response in *Drosophila* but has little or no role in vertebrate innate immunity. Despite this fundamental contrast between the two immune systems, both primary responses appear to be activated by similar pathogen-associated molecular patterns (PAMPs), particularly dsRNA, that are recognized and distinguished from ‘self’ by host cellular pattern-recognition receptors (PRRs).

Thus, I will briefly outline relevant aspects of type I IFN induction and its effects on cells in order to set the stage for the contrasting effects of RNAi and to highlight the background to the search for similar protein-based signaling pathways in mosquitoes. I will then summarize the extensive research on the *Drosophila* innate immune response to virus infection, which is the basis for our current knowledge of mosquito RNAi, and present recent work leading to a more thorough understanding of its important role in transmission of arboviruses by mosquito vectors. Finally, I will outline some areas of mosquito RNAi research that should be explored in the future.

Type I IFN, the vertebrate primary innate immune response to virus infection

Interferons are a group of cytokines secreted by vertebrate cells that have antiviral effects. IFN- α/β , or type I IFN, comprises a family of closely related proteins discovered in 1957 [2]. In the 1960s, it was shown that type I IFN can be induced by extracellular synthetic or virus-specific dsRNA (reviewed in [3]); however, it has since been found that other virus-associated molecules can trigger these pathways. Among the IFN-associated PAMPs and PRRs most relevant to this discussion are the following: first, extracellular or intra-endosomal dsRNA recognized by Toll-like receptor-3 on membranes of endosomes or lysosomes [4]. Second, intraendosomal ssRNA recognized by TLR7/8, localized in endosomes of dendritic cells [5]. Third, intracytoplasmic viral RNA, particularly 5' triphosphorylated RNA, recognized by two widely expressed molecules containing DExD/H-box RNA helicase domains, retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 [6].

Type I IFNs are synthesized as a result of PAMP recognition by a PRR, resulting in activation of one of several signaling pathways by transcriptional upregulation. IFNs are released from infected cells and bind to specific receptors on the same and surrounding cells, triggering additional signaling cascades that ultimately result in expression of several hundred genes that specify the anti viral state. Key players activated in response to type I IFN binding to its receptor are the Janus/just another kinase (JAK) family and the signal transducers and activators of transcription (STATs; reviewed in [7]). Expression of antiviral gene products can bring about dramatic changes in cellular function, such as mRNA

degradation and translational arrest, in many cases leading to apoptosis and death for the infected cell. Thus the IFN response must be tightly controlled [8].

RNAi & other small RNA pathways in *Drosophila*

The insect antiviral innate immune response has been described more recently and consists primarily of exogenous RNAi (exo-RNAi). In insects, the innate immune response is the immediate and only defense; unlike the vertebrate IFN pathway, the insect innate immune response does not lead to an acquired response. RNAi was first observed in plants as a defense mechanism against aberrant transcription (termed post-transcriptional gene-silencing) and virus infection (termed virus-induced gene silencing) in the early 1990s [9,10]. That dsRNA is the molecular trigger for post-transcriptional gene-silencing/virus-induced gene silencing/RNAi was revealed in 1998 by studies in the nematode *Caenorhabditis elegans* [11] and *D. melanogaster* [12]. Much of what we know about RNAi in insects has been elucidated in *Drosophila* flies and cultured cells. RNAi is one of the molecular mechanisms for regulation of gene expression generally known as RNA silencing. It has a central role in insect antiviral immunity [13-16]. It appears to require minimal transcriptional induction [17-19], although its activation might induce upregulation of other antiviral genes (see below) [20]. RNAi is initiated by intracellular detection of exogenous long dsRNA. Dicer-2 (Dcr2) is an RNase III that recognizes cytoplasmic dsRNA as a PAMP and cleaves it into small interfering RNAs (siRNAs) to initiate the RNAi pathway. The siRNAs are usually 21 bp in length with 5' phosphates and two-nucleotide (nt) overhangs on the 3' hydroxyl ends [21-24]. The siRNAs, in association with Dcr2 and the dsRNA-binding protein R2D2, are loaded into a multiprotein RNA-induced silencing complex (RISC), which contains Argonaute-2 (Ago2) [25-27]. The RISC unwinds and degrades one of the siRNA strands and retains the other strand as a guide for recognition and annealing to long ssRNA, such as mRNA, that is complementary to the siRNA. In the effector stage of the pathway, sequence-specific cleavage of viral mRNA is mediated by the 'slicer' endonuclease activity of Ago2 at the point of complementarity, leading to its further destruction [28-30]. Notably, the RNAi response inhibits virus replication without causing death of the infected cell.

One common feature of vertebrate and invertebrate innate immune responses is the involvement of DExD/H-box helicases, which play a crucial role in the cytosolic detection of viral RNAs in both flies and mammals. Dcr2 belongs to the same DExD/H-box helicase family as the RIG-I-like receptors that sense cytoplasmic viral PAMPs and mediate interferon induction in mammalian cells. Deddouche *et al.* showed that recognition of *Drosophila C* virus (DCV; *Dicistroviridae*) dsRNA by *Drosophila* Dcr2 also induced expression of the *vago* gene, which has antiviral activity [20]. They proposed that members of the DExD/H-box helicase gene family represent an evolutionarily conserved set of sensors (PRRs) that detect viral nucleic acids and direct antiviral responses [20,31].

Several lines of evidence suggest the importance of RNAi in *Drosophila* antiviral immunity: first, flies with mutations in known RNAi pathway components are hypersensitive to RNA virus infections and develop a dramatically increased viral load; second, many insect-pathogenic viruses, as well as plant viruses, encode suppressors of RNAi that counteract the immune defense of the fly; and third, siRNAs derived from the infecting virus genome (viRNAs) have been discovered and characterized in infected cells/flies [31]. The rapid evolution of RNAi pathway genes compared with miRNA pathway genes in *Drosophila* also suggests an ongoing arms race between insect viruses and hosts, and points to the importance of RNA silencing in antiviral defenses [32]. Increasing evidence that cross-talk or redundancy between RNAi and other RNA silencing pathways in *Drosophila* bolsters the antiviral response is also accumulating.

Galiana-Arnoux *et al.* pointed out [13] that an important difference in RNAi in plants compared with *Drosophila* might be that RNAi appears to be cell autonomous in *Drosophila* [33], whereas in plants the RNAi response spreads systemically within the plant to induce protective RNAi at distant sites [34,35]. They suggested that plant cell-to-cell transfer of the silencing signal is essential for the host to protect uninfected cells from viral infection and propose that in *Drosophila*, RNAi might need to be coupled to other defense mechanisms, such as induction of antiviral genes triggered by cytokine signaling in uninfected cells. However, Saleh *et al.* showed that eliciting antiviral RNAi immunity in adult flies by inoculation of long dsRNA required an intact dsRNA uptake pathway [36,37], suggesting that spread of dsRNA from infected to uninfected cells in *Drosophila* is also an essential part of effective antiviral immunity.

Several small RNA-mediated pathways that regulate cellular gene expression have been recognized in *Drosophila*. miRNAs are 22–23-nt RNA guides that regulate cellular functions, such as differentiation and development and metabolic homeostasis. Unlike exogenous siRNAs, miRNAs also occur in and have similar functions in vertebrate cells. They are transcribed from the cellular genome as primary miRNAs by RNA polymerase II and processed sequentially by two distinct endonucleases in the RNase III family, nuclear Drosha and cytoplasmic Dicer 1 (Dcr1; the only ortholog of the *dcr* gene family in humans). Dcr1 acts with dsRNA-binding protein Loquacious (Loqs), or R3D1 (R3D1/Loqs) to process pre-miRNA and load the miRNA duplex into an Ago1-containing RISC [38]. Originally it was shown that miRNAs typically recognize targets in the 3' noncoding region of cellular mRNAs by imperfect complementarity and inhibit their translation [39]. More recently, it has been found in *Drosophila* that miRNAs produced by Dcr1 may be loaded into Ago2-containing RISCs by Dcr 2 plus R2D2 to inhibit mRNA expression by sequence-specific endonucleolytic action [40,41], illustrating cross-talk between RNA silencing pathways in insects.

Obbard *et al.* compared the rates of evolution between the RNAi genes *dcr2*, *ago2* and *r2d2* and the paralogous miRNA genes *dcr1*, *ago1* and *r3d1* in several species of *Drosophila* and found that the RNAi genes, which are required for effective antiviral defense, are among the top 3% of the most rapidly evolving in the entire genome [32]. They acquire nonsynonymous amino acid substitutions at a rate significantly higher than other components of innate immunity and 24-fold greater than their miRNA paralogs. They speculated that this was due to equally rapid evolution of insect-pathogenic viral suppressors of RNAi, constituting an evolutionary arms race between hosts and parasites [42].

A recently discovered *Drosophila* pathway for production of small silencing RNAs from cellular transcripts is termed endogenous siRNA (endo-siRNA) [43-47]. The endo-siRNA pathway was first found in germ-line cells and was shown to defend against transcription of transposable elements (TEs); it has subsequently been demonstrated in somatic cells and can regulate protein-coding genes. Production of endo-siRNA can result from transcription of overlapping 3' untranslated regions on opposite strands of the genome and genomic loci that form long hairpin transcripts. The pathway produces 21-nt small RNAs and uses Dcr2 and Ago2-RISC, but Dcr2 associates with the miRNA pathway dsRNA-binding protein R3D1/Loqs for RISC-loading, providing another example of blurred distinctions between small RNA pathways, their components and their functions [43,44].

Another recently discovered RNA-silencing pathway in *Drosophila*, which has also been identified in mammalian cells, uses Piwi-interacting RNA (piRNA) [48,49]. piRNAs associate with members of the Piwi clade of the Argonaute proteins, which includes Piwi, Aubergine (Aub) and Argonaute 3 (Ago3) in *Drosophila*. piRNAs are approximately 24–30 nt in length and are modified by DmHEN1 (also known as Pimet) 2'-O-methylation on their

3' termini [50,51], as are siRNAs but not miRNAs. The piRNA pathway trigger appears to be ssRNA, since the small RNAs are almost always of a single polarity, and their biogenesis is Dcr1- and Dcr2-independent, possibly using the endonuclease activity of the Piwi proteins. The piRNA pathway is limited to germ-line cells and is believed to have important roles in controlling the activity of TEs in the genome and in development of reproductive tissues. Recently, RNA virus genome-derived piRNAs were discovered in cultured *Drosophila* ovary somatic sheet cells [52].

Mosquito RNAi & arboviruses

Arbovirus infection in mosquito cells & mosquitoes

Aedes aegypti is a major vector for yellow fever, dengue (DEN; *Flaviviridae*, *Flavivirus*), and chikungunya (*Togaviridae*, *Alphavirus*) viruses. Because DEN fever and DEN hemorrhagic fever are major and growing public health burdens throughout the tropics and subtropical regions of the world [53], much of our research has focused on dengue virus (DENV)–*A. aegypti* interactions. No licensed vaccines or specific antivirals are available to prevent or treat DENV infections, and vector control is currently the most effective means to limit these disease threats. Ultimately, our aim is to use knowledge of virus–vector interactions to develop new strategies for vector control, which will be an important component of a coordinated disease control armamentarium that also includes vaccines and therapeutics.

Dengue virus, along with other arboviruses, is capable of causing persistent infections of cultured mosquito cells as well as adult female mosquitoes. Intracellular viral RNA and infectious virus released into the cell culture medium exhibit a burst of replication and exponential expansion to a peak approximately 72 h after infection, then decline to reach plateau levels, which may vary over a tenfold range, for the life of the culture [54,55].

In the natural infection cycle, the first mosquito tissue encountered by DENVs ingested in a blood meal from an acutely infected human is the midgut. To be transmitted, the virus must successfully infect the midgut epithelial cells, amplify there by intracellular replication, and progeny virions must escape the midgut cells to disseminate to the salivary glands and other tissues. After further replication in salivary gland cells, DENVs emerge into the ducts to be transmitted in saliva when the female mosquito takes another blood meal. Thus, initial infection of, amplification in, and dissemination from the midgut is critical; after successful oral infection of competent mosquitoes by DENV2 in an artificial blood meal, viral RNA and proteins are readily detected in midguts beginning 2–3 days post-blood meal and reach peak titer there approximately 10 days post-blood meal, when infectious virus begins to disseminate [56,57]. Approximately 10–14 days are required for the extrinsic incubation period, the time between initial infection and transmission [57], by which time persistent infection is established. Inhibition of early stages of DENV midgut infection (e.g., receptor binding, cell entry, RNA translation or replication) is defined as a midgut infection barrier (MIB). Failure of infectious virions to disseminate from the midgut (or failure to infect secondary target organs), is considered a midgut escape barrier (MEB) [58]. Complex *A. aegypti* genetic determinants influence both DENV infection of and dissemination from the midgut. Both MIB and MEB map to multiple loci on the three *A. aegypti* chromosomes, indicating that these are quantitative traits [59,60]. Although there is evidence that robustness of the RNAi response influences both MIB and MEB [19], none of the genes comprising the quantitative trait loci have been identified directly.

RNAi in mosquitoes

Publication of the complete genome sequences of *Anopheles gambiae* [61] and *A. aegypti* [62] and release of the genome sequence of *Culex quinquefasciatus* [63] enabled

identification and comparison of orthologs of *D. melanogaster* innate immunity genes, including *dcr2*, *r2d2* and *ago2* in vector mosquitoes [64,65]. In the absence of facile systems to introduce mutations into mosquito genomes, further identification of immunity-related genes, their functions, and their expression has largely relied on microarray gene-expression analyses and RNAi knock-downs.

We have shown that profound inhibition of alphavirus and flavivirus replication in cultured *A. albopictus* and *A. aegypti* cells and *A. gambiae* and *A. aegypti* mosquitoes can be triggered by transient expression or introduction into the cytoplasm of a long dsRNA derived from the virus genome sequence [54,66-71]. The role of RNAi in mediating dsRNA-induced virus resistance in mosquitoes was confirmed by transient RNA silencing of *dcr2*, *ago2* and *r2d2*. Injection of long dsRNA cognate to these genes demonstrated that functional Dcr2, R2D2 and Ago2 are crucial for limiting viral RNA accumulation and infectious virus production and dissemination in mosquitoes [18,54,69,72]. Knock-down of *dcr2* resulted in a greater than fourfold increase in the amount of viral genome mRNA and a greater than tenfold increase in viral load at 14 days post-infection (PI). In *dcr2*-knockdown mosquitoes the extrinsic incubation period was reduced from the normal 10–12 days to as little as 7 days [54].

We have also shown that transformation of cultured mosquito cells or *A. aegypti* embryos with a DNA construct capable of expressing a long dsRNA transcript cognate to the DENV2 genome results in cells or organisms with heritable resistance to the cognate virus [72,73]. Importantly, we have shown that DENV2 infection of cultured *A. aegypti* cells and oral infection of adult female mosquitoes generates cytoplasmic dsRNA, the trigger for RNAi, and that viRNAs can be detected in infected cells [54]. We have recently analyzed the DENV2 RNA-derived small RNA in infected *A. aegypti* Aag2 cells and mosquitoes by deep sequencing and shown that it has the characteristics of exo-siRNA; it is 21 nt long, has both positive- and negative-polarity with regard to the viral genome and has sequences distributed along the entire genome [55]. Surprisingly, despite the presence of abundant dsRNA, the viRNA in infected cells and mosquitoes constituted a very small proportion of total small RNA (0.01–0.05% of 12×10^6 reads from deep sequencing at 5 days PI in cell cultures and 9 days PI in mosquitoes) [55]. Similar results were obtained in deep-sequencing analysis of viRNA in West Nile flavivirus-infected *C. quinquefasciatus* mosquitoes (0.05% of small RNA reads at 7 days PI) [74]. Long-term persistent infection of *A. aegypti* Aag2 cell cultures by the insect-only cell fusing agent flavivirus generated a tenfold higher but still very minor proportion of viRNAs (0.6–0.7%) [55]. Alphavirus replication in mosquito cells and mosquitoes appears to generate a higher proportion of virus-specific small RNAs. Approximately 10% of the 18–24-nt RNAs sequenced from Sindbis virus (SINV; *Togaviridae*, *Alphavirus*)-infected *A. aegypti* mosquitoes were matches to the SINV genome [75], and in *O'nyong-nyong* virus-infected *A. gambiae* mosquitoes the proportion of virus-specific small RNAs was 1.2% of the total small RNA reads [76]. Siu *et al.* determined that 2.1% of small RNAs in Semliki Forest virus (SFV; *Togaviridae*, *Alphavirus*)-infected U4.4 (*A. albopictus*) cells mapped to the viral genome [77]. The higher proportions of alphavirus small RNAs compared with flavivirus small RNAs may be due to differences in accessibility of the RNAi machinery to viral dsRNA during viral replication, or because more Dcr2 substrate is available due to rapid viral replication to higher titers during the initial acute phase of infection of alphavirus-infected mosquito cells [70,77], or due to different RNAi evasion capabilities of the two arbovirus genera.

Surprisingly, we found that a tenfold higher amount (0.2%) of small RNAs in DENV-infected *A. albopictus* C6/36 cell cultures than in *A. aegypti* cultures were virus-specific [55]; however, additional analysis showed that they did not have characteristics of siRNA generated by Dcr2, but resembled piRNAs – that is, they were 27 nt in length and virtually

all positive-polarity. Further analysis of these cells suggested that their Dcr2 activity is defective. Brackney *et al.* demonstrated that C6/36 cells also exhibit a defective RNAi response after infection with arboviruses from two other families (*Togaviridae* and *Bunyaviridae*) [78]. The Dcr2 mutation that results in deficiency in their RNAi response might account for the ability of C6/36 cells to propagate arboviruses to much higher titers than other cultured mosquito cells [79] and emphasizes the central role of the exo-RNAi response initiated by Dcr2 in limiting arbovirus infections in vectors. Production of virus-specific pi-like-RNAs in response to DENV infection in Dcr2-defective C6/36 cells also suggests that cross-talk between small RNA pathways can occur in mosquito cells to allow other RNA silencing pathways to provide a less-effective 'back-up' in the absence of a robust exo-RNAi response. Most importantly, these results demonstrate the necessity of an RNA silencing response, which cannot be supplanted by other innate immune responses, for control of arbovirus infections in mosquitoes.

In further support of the idea that crosstalk occurs between RNA silencing pathways, Bernhardt compared rates of molecular evolution in the antiviral siRNA genes *dcr2*, *r2d2* and *ago2* with their miRNA paralogs *dcr1*, *r3d1* and *ago1* within and among geographically dispersed natural populations of *A. aegypti* by examining the ratio of replacement to silent amino acid substitutions (K_A/K_S) [80]. In *A. aegypti*, unlike *Drosophila* [32], both siRNA and miRNA pathway genes appeared to be subject to diversifying selection, suggesting possible overlap and redundancy in the innate immune functions of the small RNA pathways.

Corresponding to the demonstration of systemic spread of RNAi in *Drosophila* [36], Attarzadeh-Yazdi *et al.* presented results indicating that the spread of an RNAi signal from cell to cell in SFV-infected *A. albopictus*-derived mosquito cell cultures contributes to limiting virus dissemination [81]. They showed that SFV infection could not prevent either the establishment of a cell-autonomous antiviral RNAi response or the spread of protective signal, suggesting that SFV does not encode a canonical suppressor of RNAi. However, expression of a plant virus siRNA-binding protein (tombusvirus p19) by SFV strongly enhanced virus spread between cultured cells, suggesting that the spreading signal was siRNA. Thus mosquitoes, like plants and flies, appear to have a mechanism for RNAi-based protection of uninfected cells from disseminating virus, suggesting that RNAi alone may be sufficient to restrict the infection and protect the organism from pathology due to arbovirus infections.

Viral counter-defenses to RNAi

The ability of arboviruses such as DENV to persistently infect both mosquito cells and mosquitoes despite the RNAi response has led to speculation about viral mechanisms of immune suppression or evasion in insect cells. Although few mechanistic studies have been reported, several viral anti-RNAi strategies can be envisaged.

A number of plant viruses and insect pathogenic viruses express a virus-encoded protein suppressor of RNAi (VSR) during replication [82]. Expression of VSRs in insect virus-infected cells results in enhanced virus production, but in most cases these are virulence factors that greatly increase the pathogenicity of the viral infection. To date, a VSR that functions during mosquito infection has not been identified for any arbovirus [81,83,84]. One of the most widely studied and potent insect virus VSRs is the B2 protein encoded by Flock House virus (FHV; *Nodaviridae*) [85,86]. Expression of FHV B2 protein by recombinant SINV resulted in increased cytopathology in infected mosquito cell cultures and mortality in infected mosquitoes [71,75]. Such pathogenic, rather than persistent, infections of mosquitoes by arboviruses would be detrimental to virus transmission and maintenance in nature. In contrast to laboratory studies, many insect viruses naturally cause

persistent infections and not all VSRs cause equally devastating effects in their host cells. In nature, infection of *Drosophila* by the insect dicistrovirus DCV causes low mortality and persistent infection, whereas the closely related cricket paralysis virus (CrPV) causes a lethal infection. Each virus encodes a structurally and functionally dissimilar VSR; the DCV suppressor binds long dsRNA, protecting it from Dcr2, whereas the CrPV suppressor directly antagonizes the RISC endonuclease Ago2 [87]. A recent study contrasted their mechanisms of RNAi suppression and consequences for the host by infecting *Drosophila* with recombinant SINVs expressing either the DCV or CrPV VSR [87]. Flies infected with SINV expressing the CrPV VSR exhibited a marked increase in production of infectious virus and mortality, whereas expression of the DCV VSR caused only modest increases in viral load and pathogenicity. It is possible that arboviruses are able to persist in their mosquito hosts by expressing a subtle, more DCV-like suppressor of RNAi that has not been detected in standard assays.

On the other hand, flaviviruses might evade RNAi by sequestering the viral dsRNA PAMP that is recognized by Dcr2. A number of microscopic and biochemical studies have shown that flavivirus dsRNA replicative complexes are found in the cytoplasm of infected mammalian cells enclosed in double-membrane structures derived from the endoplasmic reticulum [88,89]. Geiss *et al.* found that West Nile virus infection and replication in human cells were greatly reduced when siRNAs were introduced by cytoplasmic-targeted transfection prior to but not after the establishment of viral replication [90], suggesting that replicating flavivirus RNA was specifically protected from RISC cleavage. We have used cellular organelle fractionation and analysis combined with immunofluorescent staining for dsRNA to show that double-membrane vesicles that arise from the endoplasmic reticulum are associated with both positive-sense and negative-sense viral RNA in DENV2-infected mosquito cell cultures and *A. aegypti*, suggesting membrane enclosure of dsRNA in mosquito as well as mammalian cells [91].

Inefficient RISC-mediated degradation of FHV RNA as a mechanism of viral persistence was suggested by the findings of Flynt and colleagues, that bulk 21-nt viRNAs in FHV-persistently infected S2 *Drosophila* cells are poorly loaded into RISC [92]. Inefficient loading of flavivirus viRNAs into mosquito cell RISC could render the exo-RNAi pathway downstream of Dcr2 ineffective, allowing the virus to maintain an infection in mosquitoes; however, competent Dcr2 activity, acting upstream of RISC, could limit the number of viral dsRNA replicative intermediates, thus preventing the virus from compromising vector fitness.

A large portion of the viRNA population in infected mosquito cells could be derived from 'hotspots' on the viral genome that, possibly due to intrastrand secondary structures, are inaccessible or poor targets for Ago2 cleavage. We have shown that there are Dcr2 target sites on both DENV2 and CFAV genomes that are over-represented in the viRNA populations [55]. Similar hotspot-derived viRNAs in SFV-infected *A. albopictus* cells were demonstrated to be significantly less efficient at mediating antiviral RNAi than cold spot viRNAs [77], suggesting a viral 'decoy' mechanism of RNAi evasion. Alternatively, or in addition, over-expression of viRNAs from hotspots in the viral genome could select for enhanced proliferation of 'escape mutants' among the viral genome population that fail to be targeted by the vast majority of viRNAs [74]. Brackney *et al.* characterized WNV-specific viRNAs in midguts of orally exposed *Culex pipiens quinquefasciatus* by deep sequencing and demonstrated that regions of the WNV genome that were more intensely targeted by RNAi were more likely to contain point mutations compared with weakly targeted regions [74], suggesting that, under natural conditions, positive selection of WNV RNA within mosquitoes is stronger in regions highly targeted by the host RNAi response. Whether or not

the viral genome structure actively promotes either of these counterdefenses that result in ineffective RISC-mediated cleavage has not been investigated.

It is possible that the mosquito vector's exo-RNAi pathway and arboviruses have evolved a balance that involves more than one of these mechanisms.

Transcriptionally activated insect innate immune pathways

The *Drosophila* model has been important in understanding the innate immune response in insects despite obvious limitations in comparisons with disease vectors such as mosquitoes. Studies of *Drosophila* responses to infection are conducted by intrathoracic injection of infectious agents, followed by transcriptional analysis as well as use of gene knockout mutants. Interpretation of results is tempered by the knowledge that injection of pathogens does not mimic the normal dose or route of infection and may trigger nonspecific responses to trauma and injury. The first descriptions of *Drosophila* responses to microbial infection were of three evolutionarily conserved signaling pathways, Toll, Imd and JAK–STAT, each of which is transcriptionally induced by specific host receptor recognition of particular PAMPs of bacteria and fungi, such as lipopolysaccharides, peptidoglycans and β -1,3-glucans, and results in expression of antimicrobial peptides and activation of cellular immune responses via specific transcription factor-induction pathways [93,94]. Orthologs of most of the genes in these pathways have been identified in mosquito genomes [64].

Although it has been reported that many viral infections trigger a transcriptional response in *Drosophila*, whether viruses induce, are controlled by, or suppress similar immune signaling pathways to extracellular pathogens appears to vary with the virus and the arthropod host. An investigation of transcriptionally activated antiviral immunity conducted with *Drosophila* that had mutations in genes with known or suggested immune activity to bacteria and fungi showed that the Toll pathway modulated infections with *Drosophila X* virus (dsRNA genome; *Birnaviridae*) [95]. A genome-wide microarray analysis of DCV (ssRNA)-infected flies revealed that viral infection triggered increased expression of approximately 150 genes, all of which were distinct from those regulated by the Toll and Imd pathways. This transcriptional analysis suggested that DCV infection of *Drosophila* triggers induction of an unidentified cytokine that activates the Domeless receptor and the JAK kinase Hopscotch, leading to activation of a virus infection-dedicated, STAT-mediated gene expression pathway [17]. Some of the genes induced by DCV infection and triggered by dedicated JAK–STAT activity, notably the viral infection marker *vir-1*, were shown to have STAT-binding sites in their promoters. Infection of flies with mutations in the *hopscotch* gene demonstrated that activation of this JAK–STAT pathway was involved in control of viral load; however, the Domeless-activating cytokine, PRR(s) and PAMP(s) that triggered induction were not identified [17]. Interestingly, both Toll-like receptors and the JAK–STAT pathway are also part of the mammalian antiviral interferon innate immune response.

The JAK–STAT-induced pathway triggered by DCV infection was not sufficient to establish the antiviral state, and additional, JAK–STAT-independent antiviral gene expression was noted in DCV-infected flies [20]. One of the genes induced was *vago*, which encodes a unique cysteine-rich polypeptide that controlled viral load in the fat body after DCV infection, although the effector mechanism was not determined. Notably, *Vago* expression was also induced by SINV but not FHV infections of *Drosophila*. DCV infections of mutant flies showed that induction of *Vago* was dependent on Dcr2, but not R2D2 or Ago2, suggesting that, in addition to its role in RNAi, Dcr2 might act as a PRR that triggers a signaling pathway for specific antiviral gene expression [20].

In development of a novel *Drosophila*-arbovirus infection model, Hardy and colleagues noted that replication of SINV replicon RNA expressed from the fly genome, as well as exogenous SINV genome RNA, was enhanced in flies with mutations in components of the Imd pathway but not the Toll pathway [96]. They concluded that viral RNA was the intracellular PAMP that activated the Imd pathway, but that components of the RNAi pathway, including Dcr2, were not PRRs.

Among the first descriptions of transcriptional regulation following arbovirus infection of mosquitoes was that of *A. aegypti* orally infected with SINV, using a midgut-derived microarray [97]. Early (1 day PI) Toll pathway activation was followed by return to background expression levels at later time-points, as virus replication and spread within the mosquito tissues increased, suggesting active viral downregulation, possibly due to decreased expression of ubiquitin kinase levels.

An analysis of SFV infection of cultured U4.4 (*A. albopictus*) cells using STAT-, Imd- or Toll-inducible reporter genes showed that none of these signaling pathways was activated by virus infection; however, activation of STAT/Imd but not Toll pathways before infection resulted in reduced subsequent viral gene expression and RNA levels. In addition, activation of these pathways by heat-killed bacteria was strongly reduced in virus-infected cells, possibly due to moderate reduction in cellular gene expression by virus infection [98]. This general reduction of host transcription might also occur in other alphavirus (SINV)- and/or flavivirus (DENV)-infected mosquito cells [99], possibly accounting for nonspecific suppression of immune pathways.

To characterize expression of immune-related genes in DENV-infected *A. aegypti* immunity, Dimopoulos and colleagues carried out genome-wide microarray-based transcriptional analysis at 10 days post-oral DENV infection, after establishment of viral persistence, and verified their findings by RNAi-based knockdown analyses of selected genes. They observed transcriptional activation of genes putatively linked with regulation of the Toll immune signaling pathway as well as induction of four genes with JAK-STAT pathway-related orthologs [100]. Mosquitoes' susceptibility to DENV infection increased, resulting in relatively modest two- to three-fold increases in virus load, when Toll pathway gene expression was suppressed through RNAi silencing. In analogous studies to those cited previously in *Drosophila*, the Dimopoulos group used RNAi knockdown of *domeless* and *hopscotch* to show that the susceptibility of *A. aegypti* to DENV infection increased when the JAK-STAT pathway was suppressed and identified two genes encoding DENV restriction factors with putative STAT-binding sites in their promoters [101]. Sim and Dimopoulos used microarray analysis to show that DENV infection of cultured *A. aegypti* (Aag2) cells resulted in changes of expression of a wide array of putative immune pathway genes, including Toll pathway components and bacterial PAMP receptors [99]. They suggested that their observation of down- as well as up-regulation of selected genes indicated that DENV selectively evades or suppresses certain aspects of the mosquito immune response.

In the studies cited above, with the exception of the virus-specific, Dcr2-activated pathway leading to Vago induction, neither the viral molecular pattern, the arthropod recognition receptor, nor the antiviral effector mechanism in any of these transcriptionally activated pathways was identified. In contrast to RNAi, where impairment of the pathway by RNA silencing or mutation of key components can result in dramatic increases in viral load (>tenfold), silencing or mutation of most transcriptionally activated genes resulted in modest increases in virus titers (two- to four-fold). Therefore, while it seems clear that arbovirus infection of vectors frequently induces changes in the expression levels of genes in

canonical immune pathways, this effect may be a nonspecific, general response to stress or other perturbations of homeostasis rather than specifically to virus infection.

What additional knowledge of the mosquito innate immune response & its evasion by arboviruses is needed for applications to control disease?

Despite the evidence presented here for the central role of RNAi in mosquito antiviral defense, during natural, persistent infections of DENV2-competent mosquitoes, viral RNA is readily detected in midguts beginning 2–3 days PI and in salivary glands from approximately 10–12 days PI, indicating that RNAi is not totally effective in preventing replication. The following future directions for mosquito RNAi research are suggested to help resolve this paradox and bring us closer to applications for control of arbovirus diseases.

As detailed above, there have been few documented studies of the mechanism of exo-RNAi evasion by arboviruses; however, a number of possibilities have been suggested and additional hypothesis-driven research should be directed at this question.

The research described above also suggests the possibility that enhancement of the RNAi response before or early in infection could be used to render mosquitoes genetically resistant to arbovirus infection and thus the transmission cycle could be interrupted. Indeed, we demonstrated that transformation of cultured mosquito (C6/36) cells with a plasmid that constitutively expressed an approximately 500-bp inverted repeat sequence derived from the DENV2 genome resulted in virtually complete, specific, heritable resistance to DENV2 infection [73]. These cells retained virus resistance for more than 50 passages. We followed this by constructing a transgenic line of *A. aegypti* mosquitoes, Carb 77, that expressed a similar inverted repeat DENV2-RNA from the *A. aegypti carboxypeptidase A* promoter in midgut epithelial cells after ingestion of a blood meal. These mosquitoes exhibited greatly reduced DENV2 replication and transmission after oral exposure to virus in an artificial blood meal [72]. The presence of DENV2-derived siRNAs in RNA extracts from midguts of Carb77 mosquitoes and the loss of the resistance phenotype when the RNAi pathway was interrupted are evidence that DENV2 resistance was caused by an RNAi response [72]. Resistance was maintained in the line for 13 generations. These findings hold promise for eventual implementation of a population-replacement strategy in which DENV-susceptible mosquito populations would be replaced by genetically modified, refractory mosquitoes. However, in both cases, the mosquito cells and mosquitoes lost their DENV2-resistant phenotype over time [POOLE-SMITH K, UNPUBLISHED OBSERVATIONS; 102]. In the transgenic mosquitoes, loss of resistance appeared to be due to loss of expression of the inverted repeat effector transcript. The reason for this alteration in gene expression has not been determined [102]. The causes of the lack of permanence of genetically manipulated RNAi in mosquito cells is another direction that needs to be researched thoroughly to give a more complete understanding of vector immune responses mediated by RNA silencing.

The molecular nature of the optimal trigger for antiarboviral RNAi needs to be more clearly defined. Are the dsRNA replicative intermediate or secondary structures, such as stem-loops on viral ssRNA, more important? Are there other viral PAMPs that engage the RNA silencing response? A related puzzle is the nature of viral PAMPs in mosquito infection by arboviruses with negative-sense RNA genomes such as bunyaviruses. Weber *et al.* have demonstrated that dsRNA is not detectable in negative-strand RNA virus-infected mammalian cells [103], and we have found the same to be true of La Crosse virus (*Bunyaviridae*)-infected mosquito cells [91], yet La Crosse virus RNA-derived si-like RNA is readily found in infected mosquito cells [84] and *Drosophila* cells [78]. A recent study also demonstrated a potent RNAi response and the presence of typical viRNAs in

Drosophila S2 cells infected with the negative-strand RNA virus vesicular stomatitis virus (*Rhabdoviridae*) [104]. Is Dcr2 capable of recognizing other features, such as 5'-phosphorylated viral RNAs, as the phylogenetically related RIG-I-like proteins of mammalian cells are [20]?

The findings that piRNAs are elicited in response to viral infection in cultured *Drosophila* germ-line cells [52], that miRNA pathway genes, such as *dcr1* appear to evolve at a rate similar to RNAi pathway genes in *A. aegypti* [80], and that transient knockdown of *ago3* in *A. gambiae* results in enhanced replication of the alphavirus o'nyong-nyong [69] suggest that components outside the canonical RNAi pathway may have roles in antiviral RNA silencing in insects. Cross-talk between pathways and RNA silencing by alternative or induced pathways in various mosquito tissues such as the fat body [20] are other areas for investigation to aid our understanding and applications of the mosquito innate immune response to virus infection.

Imler and colleagues have described dedicated, transcriptionally activated gene expression pathways that are unique to virus infection in *Drosophila* and appear to have roles in control of virus infections [13,17,20]. Induction of at least one antiviral gene, *Vago*, was dependent on Dcr2, suggesting a link between the PRR that initiates RNAi and other antiviral defenses. Although orthologs of *Vago* have not been reported in mosquitoes, these intriguing findings should be further pursued in disease vector research.

Conclusion & future perspective

Exo-RNAi is the most important mosquito antiviral innate immune response; it is initiated early in infection and limits but does not eradicate arbovirus replication. Absent RNAi or other RNA silencing responses, arboviruses may replicate to levels that begin to place their vectors' survival in jeopardy. Future work should aim to identify and comprehensively characterize components of the RNAi pathway, their functions and their regulation and to explore genetic determinants of effectiveness of the exo-RNAi response in both mosquitoes and viruses. Understanding and exploiting natural variations in mosquito and virus populations could lead to new strategies for vector control and arboviral disease reduction.

Executive summary

Introduction

- Arboviruses cause serious diseases in vertebrates, yet they cause nonpathogenic, persistent infections in arthropod vectors.
- We hypothesize that the different outcomes of infection have bases in the host innate immune response.

Type I interferon, the vertebrate primary innate immune response to virus infection

- The type I interferon response is triggered by dsRNA, is transcriptionally activated, and ultimately results in death of the infected cell.

RNAi and other small RNA pathways in *Drosophila*

- RNAi is the principal insect innate immune response; *Drosophila* is the model organism for characterization.
- RNAi is triggered by dsRNA and results in degradation of cognate viral mRNA without harm to the infected cell.
- Other small RNA silencing pathways might have roles in antiviral defense.

Mosquito RNAi & arboviruses

- The components and mechanisms of RNAi in mosquitoes are similar to those in *Drosophila*.
- RNAi controls but does not abolish arbovirus infections of mosquitoes.
- Mechanism(s) of arbovirus evasion of mosquito RNAi have not been well characterized.

Transcriptionally activated insect innate immune pathways

- Antimicrobial transcriptionally activated immune pathways modestly control virus infections in *Drosophila* and mosquitoes.
- *Drosophila* have unique antiviral transcriptionally activated innate immune pathways.

What additional knowledge of the mosquito innate immune response is needed for applications to control arbovirus disease?

- Mechanisms of arbovirus evasion of RNAi.
- Strategies for enhancement of mosquito RNAi.
- Complete molecular characteristics, functions and regulation of mosquito RNAi.
- Interactions of exo-siRNA and other small RNA silencing pathways.
- Discovery of possible unique, transcriptionally activated antiviral pathways in mosquitoes.

Conclusion

- Mosquito RNAi is the major innate immune pathway controlling arbovirus infection and transmission.

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