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Mosquito immune responses to arbovirus infections

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Abstract

The principal mosquito innate immune response to virus infections, RNA interference (RNAi), differs substantially from the immune response to bacterial and fungal infections. The exo-siRNA pathway constitutes the major anti-arboviral RNAi response and its essential genetic components have been identified. Recent research has also implicated the Piwi-interacting RNA pathway in mosquito anti-arboviral immunity, but Piwi gene-family components involved are not well-defined. Arboviruses must evade or suppress RNAi without causing pathogenesis in the vector to maintain their transmission cycle, but little is known about mechanisms of arbovirus modulation of RNAi. Genetic manipulation of mosquitoes to enhance their RNAi response can limit arbovirus infection and replication and could be used in novel strategies for interruption of arbovirus transmission and greatly reduce disease.

Keywords

RNA interference; RNAi; innate immunity; arbovirus; transgenic mosquito

1. Introduction

Insects, as do all metazoans, mount an innate immune response upon exposure to infectious agents. Innate immunity is the cellular-level first line of defense against infection and is initiated by detection of a pathogen-associated molecular pattern (PAMP) by a host pattern-recognition receptor (PRR). Insect genomes do not encode elements of protein-based adaptive immune responses, and thus must rely totally on innate immunity for protection. The fruit fly *Drosophila melanogaster* is a model organism for the study of anti-arboviral defense in mosquitoes; both are members of the order Diptera and can be infected with arboviruses, although Drosophila are not arbovirus vectors. In addition, experimental infections of Drosophila by intrathoracic injection with high viral doses frequently result in

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pathogenesis. Arbovirus infections of mosquitoes naturally occur by infectious blood-meal ingestion and are generally non-pathogenic and persistent, possibly due to the balance that has evolved between the mosquito innate anti-viral response to control pathogenesis and the arboviral evasion without complete suppression of this response. Availability of the Drosophila genome sequence [1] and use of facile genetic techniques have provided a framework for genetic comparisons with *Anopheles gambiae* [2,3], *Aedes aegypti* [4,5] and *Culex quinquefasciatus* [6,7] as these mosquito genome sequences have been published.

2. Drosophila innate immunity

In the canonical Drosophila innate immune response following bacterial or fungal injection, transcriptional signaling cascades induced by detection of PAMPs by host PRRs result in activation of transcription factors from the NF-κB family (e.g.,Dif, Relish and dorsal) and, ultimately, release of antimicrobial peptides (AMPs) into the insects' hemolymph. Two major signaling pathways with well-defined microbial PAMPs and insect PRRs are Toll, which responds to fungal and Gram-positive bacterial infections and Imd (immune deficiency), which is triggered by Gram-negative bacterial infections [8]. Several studies of the Drosophila transcriptional response to viral infection have implicated elements of Toll and Imd pathways in antiviral immunity, depending upon the virus used [9-13]. Viral PAMPs and identities and functions of host effectors for these pathways have not been characterized for the most part. Injection with Drosophila C virus (DCV) induces the JAK/STAT pathway, which also can be activated by septic injury [14], resulting in transcription of several genes with STAT-binding elements in their promoters [15]. One such STAT-regulated, DCV-responsive gene encodes a small protein called Vago that was shown to control DCV load in the fat body after infection. Intriguingly, induction of *vago* transcription was dependent on the DExD/H-box domain of Dicer 2, the initiator of the anti-viral RNAi response [16].

Recently, Kemp et al. [17] explored the role of the JAK/STAT pathway in Drosophila innate immunity to a diverse set of viruses. Each virus infection resulted in a unique pattern of gene induction; however, flies with a mutation in *hopscotch*, their sole Janus kinase (JAK) gene, were susceptible to significantly increased mortality only after infection with members of the *Dicistroviridae* insect virus family, and not with the arbovirus Sindbis (SINV, *Alphavirus*) or any of the other four viruses tested. In contrast, Drosophila with a mutation in the *dcr2* gene, encoding the PRR for the exo-siRNA pathway of the RNAi response, exhibited increased mortality after infection with insect viruses of all families, including a DNA-containing iridovirus. Their study confirmed previous findings that RNAi, which is the Drosophila innate immune response unique to viral infections, is the most effective and wide-ranging antiviral response in insects [16,18].

3. RNAi in mosquitoes

3.1 RNAi is an antiviral defense mechanism in invertebrates and plants

Silencing of gene expression by introduction of long double-stranded (ds)RNA with cognate sequence to the silenced gene was described in both the nematode *Caenorhabditis elegans* [19] and Drosophila [20] in 1998. It was soon recognized that a similar gene silencing

phenomenon had been previously observed in plants, and ultimately that RNA-mediated gene silencing was an antiviral defense mechanism in both plants and invertebrates [21-23]. Modeling plant virus studies, we described inhibition of arbovirus replication in mosquitoes and mosquito cells resulting from expression of both virus genome-derived positive-sense and negative-sense RNA from alphavirus transduction vectors (presumably expressed as dsRNA during alphavirus replication) and from expression of virus genome-derived inverted repeat RNA from a plasmid, which formed dsRNA in the mosquito cell nucleus [24-28]. Following the description of dsRNA-mediated gene silencing (RNAi) in *C. elegans* and *D. melanogaster*, we proceeded to characterize the mechanism and machinery of mosquito RNAi in *An. gambiae* [29] and *Ae. aegypti* [30,31].

3.2 Components and mechanisms of mosquito antiviral RNAi

RNAi in mosquitoes is now known to comprise three major pathways, named for the effector RNAs that are their end products: small interfering (si)RNA, micro (mi)RNA, and Piwi-interacting (pi)RNA pathways. Each has a distinct role in either antiviral defense, regulation of development and gene expression, or defense of the genome against transposon mobilization and expression, although in Drosophila some interconnections have been noted [32]. The mosquito genes that encode major participants in each pathway have been identified by homology to Drosophila genes [33]. The exogenous (exo)-siRNA pathway represents the major antiviral innate immune response in mosquitoes [34] and this antiviral response will be the focus of this review. The potential role of piRNAs in antiviral defense is less clear and will be discussed in section 3.2.2.

3.2.1. The exo-siRNA pathway—The exo-siRNA response in arbovirus-infected mosquitoes can be triggered by dsRNA >150 bp in length [35] (Figure 1). In virus-infected cells, the source of dsRNA is thought to be genome replication intermediates, intra-strand RNA secondary structures [30,36-38], or convergent transcription of DNA virus genomes [17]. Other virus-specific RNA structures might also serve as PAMPs, since it has been shown that viruses with negative-sense RNA genomes generate little dsRNA during replication [39], yet infection of insect cells with vesicular stomatitis virus (VSV, *Vesiculovirus*), with a non-segmented negative-sense genome, and La Crosse and Rift Valley bunyaviruses, with three negative sense and/or ambisense genome segments, generates typical virus-specific small RNAs [40-43].

Many of the components and activities of the insect siRNA pathway have been elucidated with *in vitro* reconstitution assays using Drosophila or mosquito cell lysates or purified proteins (Figure 1). The dsRNA PAMP is recognized by the mosquito ortholog of Drosophila Dicer 2 (Dcr2), an RNase III-family dsRNA endonuclease that serves as a PRR to initiate the exo-siRNA pathway [33,44]. Dcr2 is a large protein (1658 amino acids in *Ae. aegypti*) with a DExD/H helicase domain at the N terminus, followed by a second helicase domain, a dsRNA-binding domain, a PAZ domain required for recognition and alignment of dsRNA ends, and two RNase III domains near the C-terminus [33]. Dcr2 binds and cleaves dsRNA into 19-22 bp siRNA duplexes with 2-nt overhangs at the 3′-OH-ends. In arbovirus-infected mosquitoes, the siRNAs are derived from both genome-sense and antisense viral RNA from sites distributed throughout their lengths [37,38,45]. The dsRNA-binding protein

R2D2 associates with the siRNA-Dcr2 complex to facilitate loading into the multi-protein RNA-induced silencing complex (RISC), which is the effector for antiviral RNAi [46]. The essential component of the RISC is Argonaute 2 (Ago2), which contains an endonuclease V-homologous Piwi domain with "slicer" activity [47] and a PAZ domain, which binds and unwinds the siRNA, allowing endonucleolytic degradation of the "passenger" strand [48]. The RISC-bound siRNA "guide" strand forms a perfectly base-paired duplex with the complementary sequence on target viral mRNA [48] and the target is cleaved at the center of the duplex by Ago2 endonuclease activity [47,49,50]. The non-capped, 5′-phosphorylated product of Ago2-cleaved viral mRNA [51,52] can serve as a substrate for further degradation by the cellular 5′-3′ exonuclease XRN1 [53,54]. The essential roles of Dcr2, Ago2 and the exo-siRNA pathway in mosquito antiviral defense were shown by injection of dsRNAs derived from their respective *ago2* and *dcr2* gene sequences into *An. gambiae* and *Ae. aegypti*, followed by arbovirus infection. This RNAi-mediated knock-down of gene expression resulted in ~10-fold higher infectious o'nyong-nyong virus (ONNV, *Alphavirus*) production in *An. gambiae* and dengue virus type 2 (DENV2, *Flavivirus*) in *Ae. aegypti* [29,31]. In addition, we discovered that the C6/36 cell culture line (*Ae. albopictus*) has a single nucleotide deletion in the *dcr2* gene, resulting in a non-functional Dcr2 protein and 10-to-100-fold enhanced arbovirus replication compared to other mosquito cell lines such as Aag2 (*Ae. aegypti*) [38,42]

3.2.2. The piRNA pathway—In Drosophila, Piwi-interacting RNAs (piRNAs) are 24-30 nt small RNAs that regulate transposable element (TE) transcription and transposition and thus protect the genome. piRNA biogenesis in Drosophila involves three proteins from the Piwi clade of Ago family proteins, Piwi, Aubergine (Aub) and Ago3, but is Dicer-independent. Drosophila piRNAs are generated by "ping-pong" amplification, resulting in biases for 5′ U (U_1) on antisense strands and position 10 A (A_{10}) on sense strands [55,56]. Several recent studies have implicated piRNAs in mosquito antiviral defense [38,42,43,45,57]. We found that in DENV2 (*Flavivirus*)-infected, Dcr2-defective C6/36 cells (see section 3.2.1), the predominant virus RNA-derived small RNAs were 27 nt long, whereas viral small RNAs in DENV2-infected, Dcr2-competent Aag2 cells and *Ae. aegypti* mosquitoes were predominantly 21 nt siRNAs. The vast majority (96%) of 27-nt viral small RNAs in C6/36 cells had sense polarity and a strong preference for A_{10}, unlike piRNAs generated by ping-pong amplification [38]. Morazzani et al. [45] found that chikungunya virus (CHIKV, *Alphavirus*)-infected *Ae. albopictus* mosquitoes generated a large proportion of piRNA-like virus-specific small RNAs in addition to siRNAs, and although the 27-nt RNAs largely had positive polarity, they displayed the A_{10}-positive strand, U_1-negative strand signature suggesting ping pong amplification. Similarly, Vodovar et al. [58] showed that exo-siRNA-competent cultured *Ae albopictus* and *Ae aegypti* cells produced both siRNA and piRNA-like virus-specific small RNAs with a ping-pong signature after SINV infection. Léger et al. [43] also observed Rift Valley fever virus (RVFV, *Phlebovirus*)-specific small RNAs with both Dcr2 and Piwi signatures from infected Dcr2-competent Aag2 and U4.4 (*Ae. albopictus*) cell cultures. Interestingly, the 21-nt siRNAs were predominant early in infection whereas 24-27-nt piRNAs were more prominent after persistent infection was established. Overall, the piRNA pathway appears to play a role in mosquito antiviral defense that varies depending upon the mosquito species and possibly

tissue, the arbovirus family and the magnitude of the exo-siRNA response. By comparison to Drosophila, the Piwi-subfamily genes have expanded greatly in culicine mosquitoes. *Ae. aegypti* have *ago3* and *piwi1-7* genes from the Piwi subfamily and *Cx. quinquefasciatus* have *ago3* and *piwi1-6* [33]. The potential role of each in mosquito antiviral pathways has not been clearly defined, although *ago3* was implicated in ONNV-infected *An. gambiae* [29] and *piwi4* in Semliki Forest virus (*Alphavirus*)-infected *Aedes* cell cultures [59].

4. Other potential antiviral immune mechanisms in mosquitoes

4.1. Transcriptional induction

A number of studies have examined transcriptional activation of known immunity genes after arbovirus infection by phylogenomic comparisons with Drosophila and/or between mosquitoes [7,60-62]. In a meta-analysis, Bartholomay et al. [7] determined the response of *Cx. quinquefasciatus* to infection by West Nile virus (WNV) and compared previous studies of *Ae. aegypti, Ae. albopictus* and *An. gambiae* mosquitoes and cultured cells infected by SINV, WNV, DENVs and ONNV. Although large numbers of infection-response genes were induced in both midgut and carcass of *Cx. quinquefasciatus* by 14 days after a WNV-infectious blood-meal, reflecting spread of infection throughout the midgut and dissemination to fat body, salivary glands and other tissues, relatively few of these were canonical immunity genes. Activated genes included orthologs of a few Toll, Imd and JAK/STAT pathway components; activation of Toll and JAK/STAT genes had previously been observed to play a role in control of DENV infection by *Ae. aegypti* [61-63]. Arboviral PAMPs and effector molecules were not identified in most studies; however, Souza-Neto et al. [62] identified two DENV restriction factors that contain putative STAT-binding elements in their promoter regions and Luplertlop et al. [64] demonstrated that the anti-bacterial peptide cecropin, which was induced in DENV-infected *Ae aegypti* salivary glands, had anti-DENV and anti-CHIKV activity as well as anti-bacterial activity. In addition, Paradkar et al. [65,66] showed that the *Culex pipiens molestus* ortholog of Drosophila *vago* (See Section 2) was induced in a Dcr2-dependent manner following WNV infection and restricted viral infection in cultured mosquito cells by activating the JAK/STAT pathway.

It is important to note that although *Cx. quinquefasciatus*, like *Ae. aegypti*, encodes an expanded repertoire of RNAi pathway orthologs, none of these had significantly modulated expression levels during infection by WNV, SINV, or DENV2 [7,30,57]. This might be related to the necessity for a balance between the potentially potent RNAi response of the host and arbovirus evasion of this immune response without pathogenesis in order for virus persistence and transmission to occur [34].

4.2. Apoptosis

Apoptosis is a highly-regulated process of programmed cell death required for development and homeostasis in metazoans through removal of unneeded or damaged cells, and may also serve in innate antiviral immunity in insects [67]. Apoptosis correlated with WNV infection in midguts of a refractory strain of *Cx. pipiens pipiens* and was proposed as the basis for limited midgut infection and inhibition of disseminated infection [68]. Large accumulations of apoptotic cells were also observed in *Cx. p. quinquefasciatus* salivary glands at 28 days

after WNV infection and appearance of this pathology was associated with a lower proportion of virus-transmitting mosquitoes [69]. To answer the question whether apoptosis is an early innate immune response that can prevent/limit viral infection or simply one of the cellular outcomes associated with viral infection, Liu et al. [70] provided DENV-infectious blood-meals to both refractory and susceptible strains of *Ae aegypti*. They observed that the pro-apoptotic gene *michelob_x* (an ortholog of Drosophila *reaper*) was rapidly induced only within the refractory mosquito strain. On the other hand, some arboviruses may exploit the apoptotic pathway to enhance disseminated infection in mosquitoes. Induction of apoptosis in *Ae. aegypti* by injection of dsRNA to silence the inhibitor of apoptosis gene (*Aeiap*) followed by oral infection with SINV resulted in increased midgut infection and dissemination to other tissues, and inhibition of apoptosis by silencing the initiator caspase *Aedronc* had the opposite effect [71]. Viral PAMPs, potential virus-encoded regulators of apoptosis, and detailed mechanisms for apoptotic effects on arboviral replication have not been determined.

4.3. Autophagy

Autophagy is also a normal cellular process in which damaged or unwanted proteins and organelles are sequestered in double-membrane structures for degradation in response to nutrient deprivation or stress such as virus infection [72]. A potential protective role for autophagy was shown in VSV infection of Drosophila [73]; however, little is known about autophagy in arbovirus-mosquito interactions. Studies in mammalian cell cultures have shown that arboviruses such as DENV subvert the autophagic mechanism for use in formation of the cytoplasmic double-membrane vesicles required to sequester viral RNA replication [74] and to regulate lipid metabolism [75], suggesting that the autophagy machinery might also facilitate DENV replication in mosquito cells. Thus, autophagy, as well as several other cellular processes normally assumed to participate in innate antiviral immunity, might play diverse anti-or pro-viral roles depending on the arbovirus-vector combination.

5. Genetic manipulation of mosquitoes to enhance innate immunity

The powerful anti-viral RNAi response of mosquitoes suggests novel ways of controlling arbovirus replication and transmission. As early as 2000, plant geneticists developed transgenic *Arabidopsis thaliana* that transcribed inverted repeat (IR) RNA that mediated silencing of expression of targeted plant genes [76]. The RNA transcript contained tandem gene-specific sequences in inverted sense and antisense orientations that formed dsRNAs to trigger an RNAi response. In 2003, Drosophila geneticists developed IR transgenes that expressed as heritable phenotypes with RNAi-targeted gene silencing. The IRs were separated by a functional intron such that the transgene transcript formed a loopless hairpin RNA following splicing [77]. Inclusion of a functional intron was necessary because IR gene sequences are difficult to clone in bacterial plasmids and the intron spacer greatly enhanced their stability, facilitating cloning. Using a similar strategy to target DENV2 instead of a host gene, we generated a transposable element (*mariner*, *Mos1*)-transformed *Ae. aegypti* line, Carb77 [78] that was DENV2-resistant. The transgenic mosquitoes expressed 587 nt IR RNA from the DENV2 prM gene in sense and antisense orientations

separated by an active *Ae. aegypti* sialokinin intron sequence. Expression of the transgene was placed under control of the *carboxypeptidase A* promoter so that IR RNA transcription was induced soon after the mosquito acquired a blood-meal. In a more recent study, we developed new transgenic lines using the identical transgene as Carb77. One of these lines, Carb109M, has been genetically stable and refractory to DENV2 infection for >33 generations [79]. Genetic analyses and physical chromosome mapping identified two closely linked transgene integration sites in Carb109M mosquitoes associated with chromosome 3. Northern blot analysis detected abundant, transient expression of the IR-RNA 24h after a blood-meal. NexGen sequencing of midgut small RNAs from blood-fed, but uninfected, Carb109M revealed that the IR-RNA was rapidly processed into 21 nt small RNAs with sequences corresponding to the 587-base target region of the DENV2 RNA genome. Carb109M mosquitoes were refractory to infection with different DENV2 genotypes, but not to other DENV serotypes due to the sequence specificity of RNAi. Expression of a DENV2 sequence-derived IR-RNA in the mosquito midgut initiated the antiviral intracellular exo-siRNA response early in the initial site of infection, efficiently blocked DENV2 infection and profoundly impaired vector competence for DENV2. The two transgene integration sites were stable after multiple generations and following introgression into a genetically-diverse laboratory strain (GDLS) *Ae. aegypti* population from Mexico. Introgression of the transgene into GDLS, with a different genetic background from Carb109M, changed the GDLS population from a highly DENV2-permissive phenotype to a DENV2-refractory phenotype. Significantly, the DENV2-refractory homozygous line, Carb109M/GDLS.BC5.HZ, exhibited (relative to GDLS) minimal fitness loss associated with the transgene [79].

6. Viral counterdefenses to mosquito RNA-mediated innate immunity

Many insect-pathogenic viruses are known to encode proteins that are potent suppressors of RNAi (VSRs) and thus virulence factors; however, none has been identified in arboviruses. Complete suppression of the major mosquito antiviral defense mechanism and resultant viral pathogenesis might be detrimental to establishment of persistent infections [36,80]. Nevertheless, viral evasion of the mosquito immune response may be necessary in order to infect, disseminate and be transmitted. Viruses with RNA genomes (including almost all arboviruses) acquire mutations at every round of replication due to an error-prone RNA-dependent RNA polymerase (RdRP) [81]. Evidence has been presented for selection of genomes with mutations in regions highly targeted by RNAi as a mechanism of evasion [37,82].

A candidate for limited suppression of RNAi is the subgenomic flavivirus RNA (sfRNA) [53]. One proposed suppressive mechanism is inhibition of Dcr proteins; reduced activity of recombinant human Dcr was observed when WNV-sfRNA was added to an *in vitro* assay. Direct inhibition of mosquito cell Dcr2 was not demonstrated [83]. The sfRNA also was shown to sequester mosquito XRN1, which might be a required downstream step following Ago2 cleavage of viral mRNA to complete degradation [54,84].

7. Future directions

Although evidence has been presented for modulation of arbovirus infections of mosquitoes by canonical innate immune pathways, knowledge that the exo-siRNA pathway is the unique and most potent mechanism of antiviral innate immunity in mosquitoes provides opportunities to develop novel strategies for controlling arbovirus transmission and incentives to more completely understand the mechanism. A few areas in which future research might be pursued are the following: What viral RNA structures in addition to long dsRNA can serve as PAMPs for Dcr2 recognition? What activities are required downstream from Ago2 cleavage of viral RNA to complete the RNAi pathway? What is the role of piRNAs in anti-viral immunity? Which Piwi-clade proteins are involved? What are arboviral strategies for RNAi evasion?

As described in section 5, both genetic manipulation studies and modeling research have suggested the feasibility of transgenic, arbovirus-resistant mosquitoes. Nevertheless, a number of challenges remain to be solved before using RNAi-based genetically modified mosquitoes as an effective strategy for significantly reducing arbovirus transmission and impacting human arboviral disease. In the case of DENVs, IR transgenes need to be developed that target for cleavage RNA genomes of all four DENV serotypes. We are currently designing a tetravalent IR effector gene incorporating the conserved NS5 (RdRP) coding regions of DENV 1-4. In transgenic plants, several examples have been described in which RNAi-based approaches were used to develop resistance to multiple tospoviruses [85,86]. Second, in principle the anti-pathogen gene conferring a DENV-refractory phenotype would require introgression into existing DENV susceptible mosquito populations, in essence replacing DENV-competent mosquito populations with DENV-refractory populations. To accomplish this, the anti-DENV IR effector gene may require linkage with an *Ae. aegypti*-specific selfish genetic-element or gene drive system to enable fixation of the transgene in the target vector population [87]. Killer-rescue based gene drive systems such as *Medea* are currently under development in *Ae. aegypti* [88]; however, gene drive approaches are not likely to be implemented as large-scale public health measures in the near future. Recently, Okamoto et al. [89] used a stochastic, spatially explicit model of *Ae. aegypti* populations from Iquitos, Peru, to evaluate whether population replacement is feasible absent gene-drive. The modeling indicated that releasing mosquitoes carrying only an anti-pathogen construct can negatively impact vector competence of a natural population at ratios well below those considered necessary for transgenic technologies involving population reduction [89-91]. Moreover, Okamoto and colleagues found that introgression of the effector gene could occur locally in a reasonable timeframe and releasing mosquitoes carrying only an anti-pathogen gene is considerably more robust for immigration into wild-type mosquito populations than other strategies modeled. A third challenge is to determine whether an RNAi-based approach will select for DENV quasispecies populations that escape the heritable RNAi-based strategy. A final challenge is that any genetically modified vector approach will need extensive field testing, encounter regulatory hurdles, and require local and regional consent prior to release of the modified vector [92,93].

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Highlights

- RNA interference (RNAi) is the major mosquito immune response against arboviruses
- The exo-siRNA pathway plays the key role in anti-arboviral immunity
- The piRNA pathway also evidences involvement in anti-arboviral immunity
- Arboviruses must evade mosquito RNAi to maintain transmission cycles
- Genetic manipulation of RNAi can be used to generate arbovirus-resistant mosquitoes

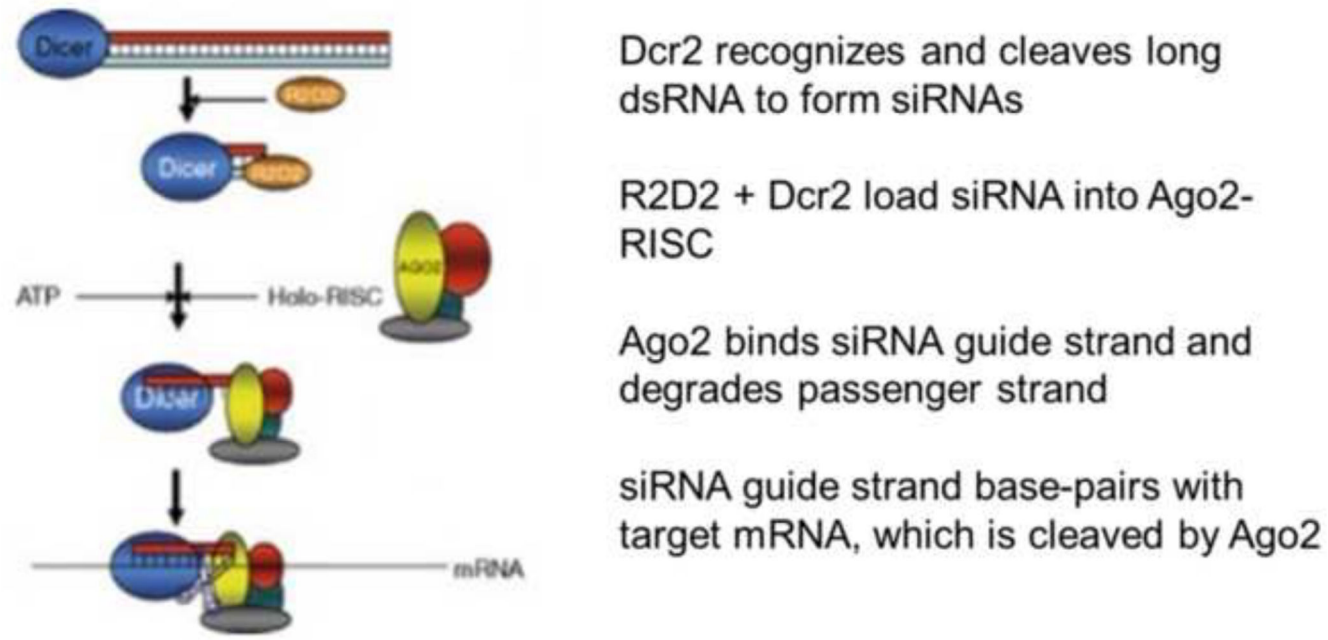

Figure 1.

Diagram showing the essential components of the mosquito exo-siRNA pathway that is the major anti-viral immune response: long virus-specific dsRNA, which serves as the PAMP; Dicer 2 (Dicer), which serves as the PRR; R2D2, the dsRNA-binding protein; Argonaute 2 (Ago2), which acts as effector in cleaving target viral mRNA.