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Small creatures use small RNAs to direct antiviral defenses

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Abstract

Antiviral RNA silencing has been recognized as an important defense mechanism in arthropods against RNA viruses. However, the role of this pathway in DNA virus infection remains largely unexplored. A report in this issue of the *European Journal of Immunology* [Eur J Immunol 2012. XXXX] provides new insight into the role of RNA silencing in antiviral defense against DNA viruses. Huang and Zhang found that the dsDNA virus White Spot Syndrome virus, an agriculturally important pathogen of shrimp, is targeted by the shrimp RNA silencing machinery via the production of virus-derived siRNAs. Furthermore, the authors show that the RNA silencing pathway, and crucially, Dicer-2, is important for restricting viral infection. This study provides novel insights not only into shrimp antiviral defenses but also potentially into antiviral immunity against DNA viruses in a larger spectrum of hosts, as discussed in this Commentary. Furthermore, this study may contribute to the future development of immune-based therapeutics to combat viral pathogens, not only in aquaculture, but also in insect vectors of human diseases.

RNA silencing is an innate antiviral pathway used to target foreign RNA for degradation. This mode of recognition is based on the fact that all RNA viruses produce double-stranded (ds)RNA during their life cycle. Since dsRNAs are not naturally produced in higher organisms, the development of dsRNA-based recognition systems provides a simple strategy for the selective targeting of RNA viruses. Organisms including plants and arthropods use RNA silencing to control both endogenous gene expression and foreign RNAs derived from viruses. In contrast, while mammals have maintained RNA-silencing pathways to control endogenous gene expression, it is thought that they have lost antiviral RNA-silencing activities and instead have evolved the sequence-independent, pan-antiviral interferon response pathway. Like the RNA-silencing pathway, the core function of the interferon pathway lies in the recognition of viral nucleic acids, including dsRNAs, by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), intracellular DExD/H box helicases (RIG-I, MDA5), and kinases (PKR). These receptors discriminate “self” from “nonself” RNA by recognizing several key features of viral RNA, including dsRNA and 5'-triphosphorylated ssRNA, which are not normally present in mammalian cells. Whether arthropods use a combination of sequence-specific and sequence-independent mechanisms to combat viral pathogens has yet to be fully elucidated.

Antiviral RNA interference (RNAi) has been most extensively studied in plants and in the model invertebrate *Drosophila melanogaster* [1]. RNAi is one of several modes of RNA silencing in *Drosophila*, which include the miRNA pathway, which regulates endogenous genes, the piRNA pathway, which represses mobile genetic elements in the germline, and the endogenous siRNA pathway, which responds to transposons in the soma. RNAi is

initiated by the RNaseIII-like enzyme Dicer-2, which generates a 21nt RNA duplex from a larger dsRNA precursor molecule, such as a viral replication intermediate [2]. The resultant small interfering RNA duplex (siRNA) is loaded onto an Argonaute (Ago) protein, Ago2, within the RNA-induced silencing complex (RISC), where one strand of the duplex is preferentially retained, allowing it to guide RISC to cleave the complementary sequence on the mRNA target [3]. Under the prevailing model for the function of the antiviral RNAi pathway, viral RNAs from RNA viruses are targeted by Dicer-2 to produce virus-derived siRNAs, which are incorporated into RISC to guide the slicing of cognate viral RNAs, thereby restricting viral replication (Figure 1B). In support of this, *Drosophila* with mutations in the core siRNA machinery (*Dcr-2* and *AGO2*) display increased sensitivity to infection by an ever-increasing array of RNA viruses [4]. Moreover, additional cellular factors that contribute to antiviral silencing have been identified, including *Ars2*, *Cbp20* and *Cbp80*, which facilitate the dicing activity of Dicer-2 and are required for antiviral defense [5].

Although some of the RNA viruses used in functional studies of the *Drosophila* RNAi pathway are natural *Drosophila* pathogens, such as *Drosophila C* virus, many of the other viruses studied, such as Sindbis virus, do not naturally infect *Drosophila* but rather are classified as arboviruses, which are medically important pathogens transmitted by hematophagous arthropods to vertebrates, including humans. For example, the study of the mosquito antiviral RNAi pathway is an important area of current investigation, since an understanding of the interaction between arboviruses and their natural vector may someday be harnessed to control medically important human pathogens. Studies in mosquitoes have demonstrated that the RNAi pathway restricts the replication of many arboviruses, including O'nyong-nyong virus, Sindbis virus, West Nile virus, and Dengue virus [6].

Virus-derived siRNAs (vsiRNAs) are generated in the host during infection by RNA viruses in both *Drosophila* and mosquitoes. The biogenesis of these vsiRNAs has been the focus of much research to discover the identity of the viral RNA precursor targeted, and to provide insight into how the RNAi pathway mechanistically responds to infection against distinct classes of viruses [1]. Figure 1A diagrams the potential RNA precursors of vsiRNAs generated during RNA virus infection, bearing in mind that these precursors must be in the form of dsRNA. Small RNA sequencing of virus-infected cells or animals has revealed that the dsRNA replication intermediate of RNA viruses is a common target of the antiviral machinery [4, 7-9] (and Sabin and Cherry, unpublished observations). In addition, as RNA viruses have limited coding capacity, they often encode highly structured *cis* elements (structured viral RNA) with double-stranded character that direct transcription, replication and packaging. Therefore, it is perhaps not surprising that the antiviral RNAi machinery is capable of targeting those regions with double-stranded character within the highly structured viral transcripts. Viruses such as Flock House virus, *Drosophila C* virus, and West Nile virus, appear to expose such structures during infection; the majority of the small RNAs generated during their replication derive from only the genomic RNA strand [10]-[12] (and Sabin and Cherry, unpublished observations). This suggests that double-stranded structures within single-stranded RNAs can be processed into siRNAs during infection.

Genetic studies have indicated that robust antiviral RNAi requires not only vsiRNA biogenesis by Dicer-2, but also the action of the core siRNA RISC effector, Ago2; however, only a fraction of vsiRNAs are specifically bound to Ago2 in infected cells [13, 14] with a large proportion of vsiRNAs being stable, but not bound to Ago2. Whether the “free” vsiRNAs are loaded onto another RISC, such as Ago1 RISC, which normally binds miRNAs, or whether the vsiRNAs are stabilized elsewhere remains unknown. Furthermore, while some reporters that bear viral RNA target sequences can be silenced by vsiRNAs produced during infection, this is not always the case [8, 13, 15]. Altogether, these findings

raise questions regarding which vsiRNAs reflect the active pool for viral silencing, and whether viral sequences are indeed generally targeted by Ago2-RISC. Additional studies of the effector step of antiviral RNAi are necessary to resolve these issues.

Since viruses co-evolve with their hosts, one hallmark of an important antiviral pathway is the development of robust countermeasures against the host-encoded antiviral immune factors by viruses. Thus, the discovery that many insect viruses encode suppressors of RNAi underscores the importance of the RNAi pathway in antiviral defense [1]. Furthermore, mechanistic studies have revealed that virally-encoded suppressors can act at different steps in the silencing pathway, including Dicer-2 processing and Ago2 slicing [4], suggesting that indeed, the entire pathway is required for defense.

In contrast to RNA viruses, very little is known about the interactions of DNA viruses with the antiviral RNA silencing machinery, particularly in arthropods. If these viruses were restricted by the RNAi machinery, the DNA genome could not be targeted directly; rather, RNA transcripts from the viral genome would form structures with double-stranded character that would be recognized and processed by Dicer-2 (Figure 1A). In *Drosophila*, a recent study by Bronkhorst et al. [15] found that overlapping bidirectional transcription of the dsDNA virus Invertebrate iridescent virus 6 (IIV-6) likely leads to the formation of dsRNA *in trans*, which is processed by Dicer-2 into small RNAs. Conversely, small RNAs produced in wild-caught mosquitoes infected with a ssDNA densovirus, which has no overlapping convergent transcripts, map predominantly to the viral RNA transcripts, suggesting that local interactions within a single-stranded RNA strand form dsRNA *in cis* that are targeted by antiviral RNAi [16]. However, the mechanism by which the insect RNAi pathway restricts infection of DNA viruses remains poorly understood, and is an important subject of future study.

Shrimp are arthropods of agricultural and ecological importance, and White spot syndrome virus (WSSV) is a highly pathogenic dsDNA virus that impacts aquaculture and is thought to have caused over \$15 billion in losses [17]. It has been demonstrated that sequence-specific long dsRNAs could confer antiviral immunity against WSSV, as well as against the shrimp RNA virus Taura syndrome virus (TSV) [18]. Moreover, injection of a synthetic siRNA against WSSV VP28, a viral envelope protein, conferred sequence-specific antiviral resistance [19]. Therefore, both long dsRNAs and synthetic siRNAs induce sequence-specific antiviral immunity in shrimp. Whether the shrimp RNAi pathway naturally targets RNA or DNA viral pathogens remained unclear. However, in this issue of the European Journal of Immunology, Huang and Zhang examine whether the RNAi pathway directs an antiviral immune response against the dsDNA virus WSSV in shrimp [20]. Since a synthetic siRNA designed to target VP28 (vp28-siRNA) is capable of controlling infection, Huang and Zhang first asked whether vp28-siRNA is produced naturally during infection of the shrimp *Marsupenaeus japonicus* with WSSV. Indeed, vp28-siRNA can be detected by northern blotting and small RNA sequencing of infected tissues. Expression of vp28-siRNA in various shrimp tissues is dependent upon WSSV infection, as the siRNA cannot be detected in tissues where WSSV does not replicate to detectable levels. Thus, vp28-siRNA is a virus-derived small RNA that is generated from WSSV transcripts during infection.

Next, Huang and Zhang [20] tested whether known miRNA and siRNA pathway components are important for generating the virus-derived vp28-siRNA. To this end, the authors depleted the siRNA pathway Dicer protein, Dicer-2, as well as the miRNA biogenesis factors Drosha and Dicer-1 from shrimp, and then challenged the shrimp with WSSV. While the levels of vp28-siRNA were unaffected in Drosha- and Dicer-1-depleted animals, knockdown of Dicer-2 abolished vp28-siRNA accumulation. The authors also detected vp28-siRNA in the cytoplasm of wild type infected cells using RNA-FISH, but not

in Dicer-2-depleted animals. Therefore, the siRNA pathway component Dicer-2, but not the miRNA pathway components Drosha or Dicer-1, is required for vp28-siRNA biogenesis in WSSV-infected shrimp.

To investigate whether the vsiRNA functions in the context of RISC, Huang and Zhang [20] used an electrophoretic mobility shift assay to demonstrate that synthetic vp28-siRNA interacts with Ago2, but not Ago1, while a control siRNA specifically interacts with Ago1 rather than Ago2. These results suggest that vp28-siRNAs produced during infection are incorporated into an Ago2-containing RISC. However, additional studies, such as immunoprecipitation and sequencing of Ago2-bound small RNAs from infected shrimp, are necessary to verify this conclusion. It will be essential to determine whether depletion of Ago2 renders shrimp more susceptible to virus infection, since this would demonstrate a role for both the biogenesis and effector steps of the RNAi pathway in antiviral defense.

Arguably the most important discovery of Huang and Zhang [20] is their finding that Dicer-2 is required for antiviral defense against WSSV. Depletion of either Dicer-2 or its product, vp28-siRNA, rendered the shrimp more susceptible to WSSV infection, as evidenced by the replication of WSSV being enhanced more than tenfold at 24 and 48 hours post-infection in these animals. These results clearly implicate the biogenesis step of the shrimp RNAi pathway in suppressing DNA viral infection *in vivo*.

The work of Huang and Zhang [20] raises several important questions that will likely guide future efforts to characterize antiviral responses against DNA viruses. Regarding the biogenesis of vsiRNAs, it is clear that one particular vsiRNA, vp28-siRNA, is generated during WSSV infection, and that it is potently antiviral. How can one particular vsiRNA provide so much protection? Are other vsiRNAs produced during infection? What are the viral precursors that give rise to these small RNAs? Moreover, how do dsDNA viruses differ from RNA viruses in their recognition and processing by the cell? As mentioned previously, in insects, DNA virus-derived siRNAs can be produced from bidirectional transcription [15] or from structured single-stranded RNAs [16] (Figure 1A). Work from our laboratory has found that infection of *Drosophila* cells with Vaccinia virus, a large dsDNA poxvirus, generates abundant vsiRNAs that appear to be produced through both mechanisms, i.e. convergent transcription and local stem-loop structures within longer single-stranded transcripts (Sabin and Cherry, unpublished observations). Therefore, future work in shrimp and other arthropods is needed to clarify the identity of the viral transcripts targeted by the antiviral RNAi pathway. In the case of WSSV and vp28-siRNA, strand-specific RT-PCR of the region of VP28 from which the siRNA derives may aid in determining whether its dsRNA precursor is produced *in trans* or *in cis*.

Another important question raised by the study of Huang and Zhang [20] is how, mechanistically, the RNAi pathway restricts DNA virus infection. Since RNaseIII enzymes such as the Dicer proteins specifically cleave RNA, it is probable that the shrimp Dicers act on the viral RNA transcripts rather than the DNA genome, which likely reduces the levels of these transcripts and hence their encoded proteins. Moreover, there are two straightforward mechanisms by which the vsiRNAs could interfere with viral replication: by suppressing gene expression at either the transcriptional or post-transcriptional level. We favor a post-transcriptional silencing mechanism, whereby an antiviral RISC targets viral mRNAs for degradation, which inhibits the expression of essential viral genes, leading to the suppression of viral replication. Quantification of the stability of viral transcripts in the presence or absence of an intact RNAi response may provide further evidence supporting post-transcriptional gene silencing as the mechanism of suppression of DNA virus infection.

Transcriptional gene silencing is a mechanism by which many organisms, including *Drosophila*, silence mobile genetic elements in germline and somatic tissues [21, 22]. In plants, virus-derived siRNAs can direct epigenetic silencing of DNA viruses such as ssDNA geminiviruses; Dicer-like 3 (DCL3)-derived small RNAs direct DNA methylation and repressive H3K9 methylation of viral genomes [23]. While DNA methylation has been lost in several evolutionary lineages, including invertebrates such as *Drosophila*, these organisms utilize histone modifications to modulate gene expression at the chromatin level. Indeed, recent work has demonstrated that transposon-derived piwi-interacting RNAs (piRNAs) direct the deposition of repressive histone modifications at the promoters of active transposons in *Drosophila* [22]. Therefore, it is possible that virus-derived siRNAs direct repressive modifications onto chromatinized viral genomes to silence gene expression in shrimp. Chromatin immunoprecipitation studies in the presence and absence of a functional RNA silencing pathway will be essential to investigate this possibility. Of course, these mechanisms are not mutually exclusive, and both transcriptional and post-transcriptional mechanisms may be directed by the antiviral silencing pathway.

Consistent with the importance of RNA silencing in the control of RNA viruses, many arthropod-borne RNA viruses encode suppressors of RNA silencing to evade these host-encoded defenses. If DNA viruses are also restricted by the RNA silencing machinery, one would predict that DNA viruses would also encode such suppressors. Indeed, WSSV is capable of inhibiting RNAi-mediated gene silencing of endogenous mRNAs in shrimp [24]. Furthermore, we recently found that the dsDNA poxvirus *Vaccinia* virus also carries a suppressor of silencing [25]. In this case, the *Vaccinia* virus-encoded poly(A)polymerase, VP55, catalyzes 3' polyadenylation of host miRNAs, resulting in their degradation by the host machinery. Although several different poxviruses are able to induce the degradation of miRNAs in both insect and mammalian hosts, siRNAs, which are 2'-O-methylated in insects, are protected from this activity. This suggests that 2'-O-methylation may have evolved in hosts to protect vsiRNAs from degradation by virally-encoded suppressors of silencing. Whether small RNA degradation is a common mechanism of host suppression utilized by other virus families is unknown.

While these data suggest that the RNAi pathway suppresses WSSV infection by targeting and processing viral RNA in shrimp, how this response contributes to the more complex antiviral response triggered by infection is not yet clear. An emerging literature suggests that, in addition to sequence-specific antiviral RNAi, long dsRNA of any sequence can induce an antiviral response in shrimp. Injection of non-specific dsRNA into the shrimp *Litopenaeus vannamei* induced a protective response against two unrelated viruses, WSSV and TSV [26]. More recent studies have expanded upon this work and, although it is now clear that injection of long dsRNA induces an antiviral state in the shrimp, reports are conflicting as to whether siRNAs are also capable of inducing a sequence-independent antiviral response [18, 27, 28]. Moreover, the mechanism by which cells are able to detect foreign dsRNA has not yet been uncovered. Plasma membrane-associated dsRNA transporters may play a role in this response (Figure 1B) and Labreuche et al. [28] have identified a shrimp ortholog (Lv-Sid1) of the *Caenorhabditis elegans* cell-surface Sid-1 protein that transports dsRNA into cells [29]. *Drosophila*, however, encode a scavenger receptor rather than a Sid-1 ortholog to internalize dsRNA [30, 31].

Considering the fact that both sequence-specific and sequence-independent antiviral responses are triggered by dsRNA in shrimp, how these two pathways synergize at an organismal level to defend against viral infection is unknown. We propose a model that combines both mechanisms of dsRNA-based immunity where dsRNA serves as both a functional, sequence-specific substrate of the antiviral RNAi pathway, as well as a sequence-independent danger signal, or pathogen-associated molecular pattern (PAMP),

which induces additional antiviral responses (Figure 1B). This sequence-independent antiviral response to dsRNA is analogous to responses in mammals, where dsRNA is an important PAMP recognized by different classes of PRRs including RIG-I and MDA5, to induce antiviral responses such as the interferon pathway. Intriguingly, the closest insect ortholog of the intracellular sensors RIG-I and MDA5 is Dicer-2 and virus infection in *Drosophila* initiates a specific transcriptional response, including the induction of the antiviral effector Vago, whose expression is dependent upon Dicer-2 [32]. This suggests that Dicer-2-driven signaling contributes to the induction of a specific set of antiviral effectors during infection. The spectrum of Dicer-2-dependent downstream signaling events, and whether this function of Dicer-2 is conserved in shrimp and other invertebrates, has yet to be elucidated.

One potential mechanism to explain the non-specific immunity triggered by dsRNA in shrimp is that the detection of dsRNA, either by Dicer-2 or an additional sensor (Figure 1B), triggers a feed-forward loop, whereby the RNAi machinery itself is transcriptionally upregulated, thus setting up a cellular environment that is poised to attack and degrade additional foreign nucleic acids. A recent study found that injection of dsRNA leads to the specific upregulation of Ago2 and Sid-1 mRNA in the shrimp *Litopenaeus vannamei* [28]. Moreover, WSSV infection induced Dicer-2 mRNA in *Litopenaeus vannamei* [33]. Recent work in our laboratory has shown that virus infection of *Drosophila* induces the upregulation of the RNAi pathway components Dicer-2 and Ars2 [34]. However, the viral PAMPs involved in inducing this response are not likely dsRNA, since the transcriptional upregulation of antiviral effectors occurs prior to viral replication. The shrimp Ars2 ortholog was recently identified and cloned [35]; it will therefore be important to investigate whether Ars2 and additional members of the RNA silencing pathways in shrimp are regulated by infection.

Although vsiRNAs are produced in an infected cell, whether these small RNAs or other viral RNA species, such as dsRNA, are released from infected cells remains unknown. It is possible that the release of nucleic acids from infected cells alerts neighboring cells or even distant cells to the presence of infection. Accordingly, a local infection could lead to systemic antiviral defenses. This would also present opportunities for synergies between sequence-specific responses, which act cell-autonomously, and sequence-independent responses, which generate a non-autonomous antiviral state. Studies in *Drosophila* have demonstrated that systemic RNAi can suppress viral replication [36]. Further exploration of these possibilities will likely reveal additional aspects of immunity to viral pathogens, but altogether will reinforce the fact that the initiation of antiviral immunity in response to the detection of viral PAMPs, including dsRNA, is a defense strategy conserved through evolution.

In summary, dsRNA seems to play a dual role in shrimp: it serves as a substrate of the RNAi pathway in order to direct sequence-specific antiviral silencing, while also functioning as a sequence-independent danger signal to induce an antiviral transcriptional response. Therefore, shrimp antiviral immunity combines aspects of the insect antiviral RNAi pathway with aspects of the mammalian dsRNA response. Whether this is also the case for other arthropods or other organisms thought to exclusively rely on antiviral silencing, remains unclear. Of note, while there is no specific therapeutic against WSSV, genetic selection for shrimps that are resistant to infection by WSSV or that do not develop the pathological consequences of infection (white spot disease) has led to the development of three selected lines of *P. vannamei*. While there was still some mortality post WSSV challenge, all infection survivors were qPCR negative for WSSV [37] but whether this is due to an increase in the efficacy of antiviral silencing is unknown. Nevertheless, harnessing this cocktail of antiviral responses may one day be used to protect marine animals and valuable

food sources from viral pathogens. Moreover, an understanding of the antiviral pathways conserved between shrimp and insects, such as mosquitoes, may aid in efforts to develop immune-based therapies against human arboviruses.

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(A) Potential dsRNA precursors of vsiRNAs generated by RNA viruses (left) or DNA viruses (right) during replication. The potential source of each precursor molecule is indicated below the diagram, and the region that contains double-stranded character is boxed in red. Viral RNA-dependent RNA polymerase, vRdRp.

(B) Model for sequence-dependent and -independent antiviral immunity triggered by dsRNA in shrimp. During the sequence-specific antiviral response, dsRNA is processed by Dicer-2 to yield virus-derived siRNAs (vsiRNAs), which are bound by an Ago2-containing RISC and target cognate viral RNAs for degradation. During the sequence-independent induction of shrimp antiviral immunity, dsRNA is detected by an as yet-unidentified sensor (diagrammed in blue) or combination of sensors, including Dicer-2 itself, as indicated by the dashed arrow. Recognition of cytoplasmic dsRNA initiates a signaling cascade, which results in the transcriptional activation of antiviral effectors and the upregulation of the RNAi machinery. Signaling in response to dsRNA could also potentially activate additional antiviral factors, shown in green, in a post-transcriptional manner. In addition, systemic antiviral defenses may be triggered by the release of dsRNA from virally-infected cells but this requires further investigation.