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Review

Advances in dissecting mosquito innate immune responses to arbovirus infection

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Arthropod-borne viruses – arboviruses – are a significant threat to public health. Whilst there is considerable knowledge about arbovirus interactions with vertebrate immunity, relatively little is known about how vectors such as mosquitoes control arbovirus infections. In this review, we discuss novel findings in the field of mosquito antiviral responses to arboviruses, in particular RNA interference, the up-and-coming field of general immune-signalling pathways, and cell death/apoptosis.

Introduction

Arthropod-borne viruses, or arboviruses, are a major public health and veterinary problem in many regions of the world. Billions of people are at risk from the most important arboviral disease, dengue fever (Halstead, 2008; Kyle & Harris, 2008). The spread of West Nile virus through North America, the arrival of bluetongue virus in northern Europe and the UK, and outbreaks of chikungunya virus in the Indian Ocean and in Italy all show the dangers posed by arboviruses that extend beyond their traditional geographical boundaries (Angelini *et al.*, 2007; Gould *et al.*, 2006; Landeg, 2007; Powers & Logue, 2007; Weaver & Barrett, 2004). Most arboviruses are RNA viruses of the families *Bunyaviridae*, *Togaviridae* and *Flaviviridae*, although bluetongue virus of the double-stranded RNA (dsRNA) family *Reoviridae* is a highly important arbovirus of veterinary importance. A list of insect-borne arbovirus families (with relevant examples), as opposed to tick-borne arboviruses, is provided in Table 1. Only one DNA arbovirus, tick-borne African swine fever virus (family *Asfarviridae*), is currently known (Dixon *et al.*, 1995).

In nature, arboviruses are generally maintained in a cycle that requires horizontal transmission by arthropod vectors to vertebrate hosts. Arboviruses replicate in both arthropods and vertebrates, which each exert different pressures on the evolution of these viruses. Haematophagous arthropods such as female mosquitoes become infected by engorging arbovirus-containing blood from a vertebrate (Weaver, 2006; Weaver & Barrett, 2004). Many of the biological factors involved in arbovirus transmission (haematophagy, ecology etc.) have been reviewed elsewhere (Kuno & Chang, 2005) and are beyond the scope of this review. However, one important consequence of such a transmission cycle is the exposure of arboviruses to both vertebrate and invertebrate immune systems.

Mosquito–arbovirus interactions are not always benign to the vector (Lambrechts & Scott, 2009), but infection of arthropod cell cultures usually leads to a persistent infection. It has been assumed that the relatively efficient control of arbovirus infection in mosquitoes is due to innate immune responses. Arthropods do not have the powerful interferon response of vertebrates, although secreted antiviral factors (against alphavirus replication) have been described (Condreay & Brown, 1988; Newton & Dalgarno, 1983). At the present time, knowledge about vertebrate immunity to virus infection (virus nucleic acid sensors, interferons, JAK/STAT signalling etc., and viral interference with these) exceeds by far our knowledge of insect antiviral responses (Randall & Goodbourn, 2008).

The last few years have seen a vast increase in knowledge of mosquito genetics and immunity-related genes, mainly through the *Anopheles gambiae* and *Aedes aegypti* sequencing projects (Christophides *et al.*, 2002; Holt *et al.*, 2002; Nene *et al.*, 2007; Waterhouse *et al.*, 2007). Much of the pioneering work on mosquito immunity stems from work on *Plasmodium* parasites and *Anopheles*, and is reviewed elsewhere (Barillas-Mury & Kumar, 2005; Christophides *et al.*, 2004; Osta *et al.*, 2004; Whitten *et al.*, 2006). Research on mosquito immunity has also been influenced strongly by work on *Drosophila melanogaster*; this has been recently reviewed (Ferrandon *et al.*, 2007; Kemp & Imler, 2009; Lemaitre & Hoffmann, 2007). Arboviruses have a different biology from other (often pathogenic) insect viruses, and this review will focus on recent progress in the field of immune responses to arboviruses in mosquitoes and, in particular, on RNA interference (RNAi), immune-signalling pathways and cell death/apoptosis.

The principal arboviruses discussed below are the alphaviruses Sindbis virus (SINV), Semliki Forest virus (SFV), Venezuelan equine encephalitis virus (VEEV) and o'nyong-nyong virus (ONNV) (family *Togaviridae*); the flaviviruses

Table 1. Arbovirus families and genera transmitted by insect vectors

Important representative viruses are indicated. Arboviruses mentioned in this review and whose interactions with mosquito innate immunity have been studied are indicated in bold. Abbreviations: ss, single-stranded; ds, double-stranded.

Family	Genome	Genus	Important viruses
Rhabdoviridae	ss(−) RNA	*Vesiculovirus*	Vesicular stomatitis virus
		Ephemerovirus	Bovine ephemeral fever virus
Togaviridae	ss(+) RNA	*Alphavirus*	**Sindbis virus**
			Semliki Forest virus
			Chikungunya virus
			O'nyong-nyong virus
			Eastern/Western/Venezuelan equine encephalitis viruses
Flaviviridae	ss(+) RNA	*Flavivirus*	West Nile virus
			Dengue virus
			Japanese encephalitis virus
			Yellow fever virus
Bunyaviridae	ss(−) RNA (three segments)	*Orthobunyavirus*	Oropouche virus
			La Crosse virus
		Phlebovirus	Rift Valley fever virus
Reoviridae	dsRNA (10–12 segments)	*Orbivirus*	Bluetongue virus
			African horse sickness virus

dengue virus (DENV; type is indicated if known), Japanese encephalitis virus (JEV) and West Nile virus (WNV); and the bunyavirus La Crosse virus (LACV). Members of these virus families have (+)-strand (*Togaviridae*, *Flaviviridae*) or (−)-strand (*Bunyaviridae*) RNA genomes, are enveloped and are often transmitted by mosquitoes (also see Table 1).

Immune-signalling pathways and antiviral immunity

Antibacterial and antifungal responses in *D. melanogaster* rely mainly on signalling via Toll for fungi and most Gram-positive bacteria and Imd for Gram-negative bacteria (Ferrandon *et al.*, 2007; Lemaitre & Hoffmann, 2007). Pathogen-recognition receptors activate these Toll or Imd signalling cascades, resulting in nuclear translocation of the NF-κB/Rel family transcription factors Dif (adults)/Dorsal (larvae/adults) and Relish, respectively, which initiate transcription of effectors such as antimicrobial peptides. In addition, Imd signalling can also activate the JNK pathway.

The *Drosophila* Toll pathway shares similarities with vertebrate interleukin-1 and Toll-like receptor signalling, whilst the *imd* gene encodes a protein similar to receptor-interacting protein (RIP) of the vertebrate tumour necrosis factor receptor pathway (Lemaitre & Hoffmann, 2007).

Sequencing and annotation of the *A. gambiae* and *Ae. aegypti* genomes have been major steps towards understanding the immune system of disease-carrying insects (Christophides *et al.*, 2002; Holt *et al.*, 2002; Nene *et al.*, 2007; Waterhouse *et al.*, 2007). Mosquitoes lack Dif, but rely on the Dorsal orthologue Rel1 and the Relish orthologue Rel2 to induce the expression of antimicrobial

molecules. At least for *Ae. aegypti*, Rel1 exists in two isoforms, Rel1-A and Rel1-B, which act cooperatively to enhance gene expression (Shin *et al.*, 2005). Rel2 exists in three isoforms (long, short and IκB-type). Rel2-long, the predominant isoform, is similar to *D. melanogaster* Relish and contains histidine/glutamine-rich and serine-rich regions, REL-homology domains, inhibitor IκB-like ankyrin and Death domains; Rel2-short lacks ankyrin and Death domains, whilst the IκB-type consists mainly of an IκB domain (Shin *et al.*, 2002). Only two Rel2 isoforms exist in *A. gambiae* (Meister *et al.*, 2005). In *D. melanogaster*, antiviral immunity can also be mediated in part by the JAK/STAT signalling pathway, which has counterparts in vertebrates (Dostert *et al.*, 2005). In addition, bacterial infection can also activate STAT signalling in *A. gambiae* (Barillas-Mury *et al.*, 1999) and STAT proteins have been described in *Aedes albopictus* (Lin *et al.*, 2004).

Immune signalling in response to arbovirus infections

Analysis of immune pathways involved in arbovirus–mosquito interactions has largely relied on genomic studies to identify differentially regulated genes (Sanders *et al.*, 2005; Sim *et al.*, 2005; Xi *et al.*, 2008); recent key research in this field is summarized (by mosquito species) in Table 2. At least one molecule with increased levels post-infection, the heat-shock protein cognate 70B, has antiviral activity; silencing of this gene reduced the lifespan of *A. gambiae* mosquitoes infected with ONNV (Kang *et al.*, 2008; Sim *et al.*, 2007). In midguts of *Ae. aegypti* infected with SINV, upregulation of the Toll pathway (seen as early as 1 day post-infection) is followed by activation of JNK signalling and is probably preceded by Imd activation (both pathways

Table 2. Summary of recent key research on antiviral responses against arboviruses, as described in anopheline (*Anopheles gambiae*) and aedine (*Aedes aegypti* and *Aedes albopictus*) mosquito species

Mosquito species	Anti-arboviral pathway or activity	Key references
A. gambiae	RNAi	Keene *et al.* (2004); Myles *et al.* (2008, 2009)
	Heat-shock protein cognate 70B	Sim *et al.* (2007)
Ae. aegypti	RNAi	Myles *et al.* (2008); Cirimotich *et al.* (2009); Sanchez-Vargas *et al.* (2009); Campbell *et al.* (2008a, b)
	Immune signalling	Sanders *et al.* (2005); Xi *et al.* (2008)
Ae. albopictus	RNAi (including systemic)	Attarzadeh-Yazdi *et al.* (2009)
	Immune signalling	Fragkoudis *et al.* (2008)

are linked in *D. melanogaster*; see above) (Sanders *et al.*, 2005). In addition, other immune molecules such as serine proteases are upregulated; these enzymes play many roles in innate immunity (Lemaitre & Hoffmann, 2007), but their role in response to virus infections remains unclear. In the case of another alphavirus, SFV, if activated before infection, not Toll- but Gram-negative-mediated signalling (JAK/STAT or Imd/Jnk) can inhibit virus replication in mosquito cell cultures (Fragkoudis *et al.*, 2008). Bacterial infection also induces resistance to various RNA viruses in *D. melanogaster*, whilst the JAK/STAT pathway is a mediator of antiviral activities (Dostert *et al.*, 2005; Hedges *et al.*, 2008; Teixeira *et al.*, 2008). The bacterial endosymbiont *Wolbachia* is reduced in *Ae. aegypti* infected with the alphavirus chikungunya virus, although it remains to be determined whether this is related to virus activation of immune signalling (Tortosa *et al.*, 2008).

A recent detailed study has provided important insights into *Ae. aegypti* immune responses to DENV-2 infection (Xi *et al.*, 2008). Oxidative-defence enzymes were mainly repressed, but strong upregulation of the Toll and JAK/STAT pathways was observed, 10 days post-infection. RNAi-mediated knockdown studies demonstrated that the Toll pathway plays a role in the control of DENV-2, although the contribution of JAK/STAT was not analysed. Differential regulation of serine proteases, serine protease inhibitors and thioester-containing proteins was also observed. The Toll pathway was previously implicated in the control of *Drosophila* X virus infection of *D. melanogaster* (Zambon *et al.*, 2005). No involvement of the Imd pathway in the control of DENV-2 was found, but as this is a more acute response relative to later Toll activation, at least in *D. melanogaster* (Lemaitre & Hoffmann, 2007), it might not have been observed in this analysis. However, comparison of alphavirus- and flavivirus-infected mosquitoes through genomic studies does suggest different dynamics of immune responses, which might be due to the characteristics of the viruses (Sanders *et al.*, 2005; Xi *et al.*, 2008). Interestingly, infection of thrips by tomato spotted wilt bunyavirus also activates classical immune-signalling pathways (Medeiros *et al.*, 2004), indicating that these observations are not only valid in mosquitoes. It should, however, be noted that previous work on *D. melanogaster* infection with *Drosophila* C virus

has shown that activation of host gene expression at the transcriptional level does not always translate into production of the corresponding proteins (Dostert *et al.*, 2005; Sabatier *et al.*, 2003).

A recent study showed that the *D. melanogaster* DExD/H-box RNA helicase Dicer-2 (Dcr-2), which has a crucial role in RNAi, also mediates the induction of antiviral genes (Deddouche *et al.*, 2008), a role similar to vertebrate RIG-I-like receptors (RNA helicases RIG-I, Mda-5, LPG2), a family of cytoplasmic sensors involved in detecting virus nucleic acids and mediating antiviral signalling (Randall & Goodbourn, 2008; Yoneyama & Fujita, 2009), to which Dcr-2 belongs. dsRNA is known to activate antiviral signalling in shrimp (Robalino *et al.*, 2004, 2005, 2007) and possibly Lepidoptera (Hirai *et al.*, 2004); however, global activation of immune responses by dsRNA has not yet been described or observed in mosquitoes. We have carried out several studies with dsRNA and immune-signalling reporters in mosquito cells and observed no activation of immune responses; however, these studies were performed by liposome-mediated transfection of the dsRNA mimic poly(I:C) and the possibility that nucleic acid sensors failed to detect this molecule [e.g. through localization of sensors in a different cellular compartment from the introduced poly(I:C)] cannot be excluded. Similar findings have been reported for *D. melanogaster* (Hedges & Johnson, 2008). Nevertheless, the induction of individual gene products such as Vago through an RNA helicase-dependent pathway shows that, in Diptera, such antiviral signalling pathways do exist (Deddouche *et al.*, 2008). Therefore, it would not be surprising to identify a similar non-RNAi antiviral function for mosquito Dcr-2.

Taken together, the studies detailed above suggest that there is induction of antimicrobial immune pathways, including Toll, JAK/STAT and Imd/Jnk, in arbovirus-infected mosquitoes; the activators of these systems remain unknown (Fig. 1). In the absence of mosquito genetic mutants, silencing immune-pathway components or other genes with possible immune functions by RNAi technology might be the best way forward to really understand the contribution of individual pathways. The contribution of each pathway might be more or less important according to the arbovirus–mosquito combination, but their role is
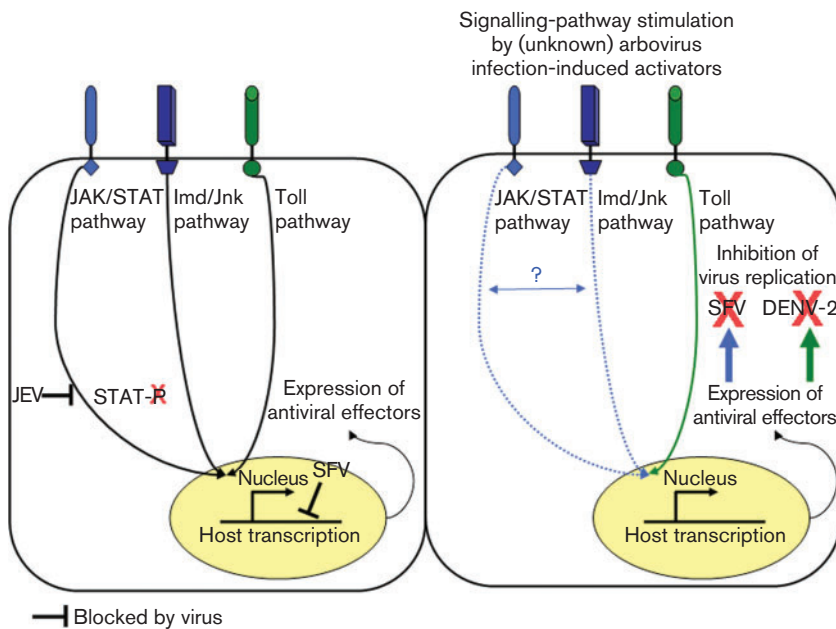
Fig. 1. Antiviral signalling pathways and viral suppression of cellular signalling in mosquito cells. Activation of immune-signalling pathways (Imd/Jnk, JAK/STAT, Toll) leads to host gene transcription and expression of effector molecules; JEV blocks STAT phosphorylation (a key event when JAK/STAT signalling is activated), whilst SFV infection reduces host gene transcription (and thus possibly production of antiviral effectors). When these immune-signalling pathways are activated (it is not yet known how arbovirus infection triggers activation), Toll signalling (green arrow) is important in controlling DENV-2, whilst (in all likelihood) stimulation of Imd/Jnk or JAK/STAT signalling inhibits SFV replication (dotted blue arrows; it is not clear yet which pathway mediates this antiviral activity).

now recognized and the challenge will be to identify the antiviral effectors.

Virus suppression of immune signalling in mosquito cells

Most, if not all, known viruses of animals and plants interfere with host responses in order to replicate and propagate. Viruses encode protein(s) interfering with host antiviral mechanisms in a variety of ways; the subject is too extensive to be discussed here and we recommend recent reviews for further reading (Bowie & Unterholzner, 2008; Randall & Goodbourn, 2008). As mentioned above, immune-signalling pathways in *D. melanogaster* display similarities to vertebrate immunity, e.g. Toll receptors, NF-κB-type transcription factors and JAK/STAT signalling. Many viruses target immune-signalling pathways in vertebrate cells and it would not be surprising if arboviruses were to do so in arthropod cells.

In *Ae. albopictus* C6/36 cells, lipopolysaccharide stimulation leads to tyrosine phosphorylation of STAT. This process can be blocked by JEV infection (Lin *et al.*, 2004). Presumably this results from the activity of the JEV NS5 protein, which in vertebrate cells prevents STAT1 and TYK-2 phosphorylation, thus blocking interferon signalling (Lin *et al.*, 2006).

In vertebrate cells, mosquito-borne alphaviruses reduce host-cell gene transcription, and this affects at least some host defence responses. This is mediated by the alphavirus nsP2 protein in the case of Old World alphaviruses (SINV, SFV) and the capsid protein in the case of New World alphaviruses (VEEV) (Aguilar *et al.*, 2007; Breakwell *et al.*, 2007; Garmashova *et al.*, 2006, 2007a, b). Given our current understanding of insect innate immunity, the long-standing observation that host-cell RNA levels are reduced

in SINV-infected *Ae. albopictus* cells has a new importance (Sarver & Stollar, 1977). We have recently reported that mild reduction of host gene expression also occurs in SFV-infected *Ae. albopictus* U4.4 cells (Fragkoudis *et al.*, 2008). In this case, it also appears that host RNA synthesis is reduced early in infection (our unpublished observations). JAK/STAT, Toll and Imd signalling pathways were not activated by SFV, and SFV effectively suppressed these signalling pathways after activation (possibly due to suppression of cellular gene expression) (Fragkoudis *et al.*, 2008). These studies suggest that inhibition of gene expression in mosquito cells has effects similar to those in vertebrate cells. A similar mechanism might also explain the suggested suppression of Toll signalling by SINV, where Toll-pathway activation in *Ae. aegypti* midguts is down-regulated as virus titres increase post-infection; however, a more targeted effect on the Toll pathway cannot be ruled out (Sanders *et al.*, 2005). A comparable phenomenon has yet to be demonstrated for New World alphaviruses in arthropods. In the case of VEEV, the capsid protein was found to block nuclear import efficiently in vertebrate cells, but not in a mosquito cell line (Atasheva *et al.*, 2008). It is unclear at the present time how inhibition of nuclear import relates to transcriptional shut-off or viral interference with immunity. New World alphavirus interactions with mosquito immunity therefore remain largely unknown. Inhibition of host gene expression might indeed be a more widespread phenomenon in arbovirus-infected mosquito cells, and has also been described in *Ae. albopictus* cells and cell extracts infected with the rhabdovirus vesicular stomatitis virus (VSV) (Gillies & Stollar, 1982a, b).

As in vertebrates, suppression of signalling in individual infected cells and activation of signalling pathways in

tissues are not mutually exclusive; therefore, genomic studies in mosquitoes and signalling experiments in cell culture complement each other to reveal different facets of virus–host interactions within the complexity of a living organism. In mosquitoes, apoptotic cells, inactive virus particles and released dsRNA might be important initiators of antiviral responses. It has recently been shown that alphaviruses can infect mosquito haemocytes, and there is little knowledge about immune-signalling patterns in these putative effector cells (Parikh *et al.*, 2009). Mosquito antiviral defences appear strong enough to control arbovirus infection, despite arbovirus interference with arthropod signalling pathways (see Fig. 1). Whether arboviruses have evolved actively to suppress antiviral signalling in arthropod cells, or whether this is an unavoidable consequence of the evolution of suppression of vertebrate cell defences that happens, due to similarities of fundamental pathways, to also have an effect in arthropod cells, is unclear. Arboviruses need to replicate to sufficiently high titres in vertebrate hosts to reinfect arthropod vectors through a blood meal; selective pressure to this generates viral mechanisms that counteract immune signalling in vertebrates. This may also affect arthropod cells, as arthropod immune-signalling pathways have antiviral effects. If virus inhibition of immune signalling in arthropod cells was sufficient to increase virus replication and to compromise vector survival and virus transmission, it could be speculated that only the existence of another powerful antiviral defence system in arthropods, antiviral RNAi (see below), might allow the arbovirus transmission cycle.

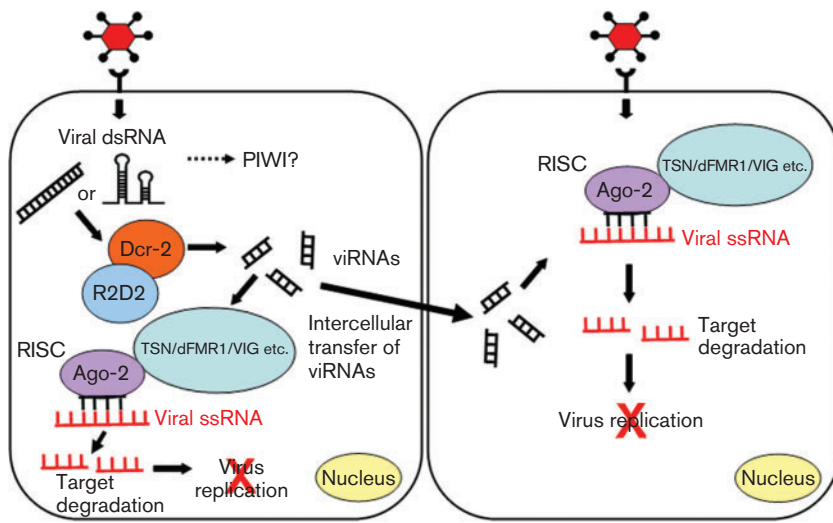## Antiviral RNAi as a defence mechanism in mosquitoes

Small interfering RNA (siRNA)-mediated RNAi is an important antiviral mechanism in mosquitoes (Keene *et al.*, 2004; Li *et al.*, 2004). Many of the experiments leading to the discovery of RNAi in mosquitoes have been reviewed before (Sanchez-Vargas *et al.*, 2004) and will not be discussed here.

Much of the understanding of siRNA-mediated antiviral RNAi in arthropods derived from studies on *D. melanogaster*, where RNAi is crucial in controlling various *Drosophila* viruses of several RNA virus families and also arboviruses such as SINV and WNV. This *Drosophila* research identified the key proteins and events of antiviral RNAi (Chotkowski *et al.*, 2008; Galiana-Arnoux *et al.*, 2006; van Rij *et al.*, 2006; Wang *et al.*, 2006; Zambon *et al.*, 2006). Virus-derived long dsRNA is cleaved by the RNase III enzyme Dcr-2 into siRNA of 21–25 bp, often called viRNAs. Dcr-2 and the dsRNA-binding protein R2D2 then interact and integrate one unwound strand (the guide strand) of a viRNA into a multiprotein RNA-induced silencing complex (RISC); the other – passenger – viRNA strand is degraded. This activated RISC complex then mediates target recognition and sequence-specific cleavage

of viral single-stranded RNA through the 'slicer' Argonaute-2 (Ago-2) protein (Ding & Voinnet, 2007; Kemp & Imler, 2009). Other key proteins in the RISC are TSN (Tudor staphylococcal endonuclease), dFMR1 (*Drosophila* homologue of fragile X mental retardation protein) and VIG (Caudy *et al.*, 2002, 2003); homologues of TSN, dFRM1 and VIG proteins have been identified in some mosquito species (Campbell *et al.*, 2008a). The rapid evolution of RNAi genes suggests an ongoing arms race between insect viruses and hosts, and points to their importance in antiviral defences (Obbard *et al.*, 2006).

Orthologues of Dcr-2, R2D2 and Ago-2 exist in vector mosquitoes such as *A. gambiae*, *Culex pipiens* and *Ae. aegypti*, and the *Ago-2* gene was also found to evolve rapidly (Campbell *et al.*, 2008a). Genetic mutants for these key proteins are not yet available for mosquitoes, but it is possible to silence the silencing machinery itself by injection of long dsRNA for a given target (Dcr-2 etc.). Although by its nature this is always self-limiting, this approach has shown that Dcr-2, R2D2 and Ago-2 are important in RNAi responses against flaviviruses and alphaviruses and limit virus production and/or dissemination (Campbell *et al.*, 2008b; Keene *et al.*, 2004; Sanchez-Vargas *et al.*, 2009). In mosquito responses against SINV, the RISC protein TSN is involved in limiting virus dissemination and is also upregulated during infection (Campbell *et al.*, 2008b; Sanders *et al.*, 2005). At least in *D. melanogaster*, Ago-2 (but curiously not Dcr-2) is involved in controlling WNV (Chotkowski *et al.*, 2008). It also seems possible that another RNAi pathway, the PIWI-associated RNAi pathway, which involves three other Ago proteins and is involved in the control of mobile genetic elements (Kemp & Imler, 2009), is involved in controlling antiviral defences. *D. melanogaster spindle-E* and *piwi* mutants are more susceptible to WNV, and silencing of another PIWI protein, Ago-3, affects responses to ONNV in *A. gambiae* (Chotkowski *et al.*, 2008; Keene *et al.*, 2004). Recent key research in this field is summarized (by mosquito species) in Table 2.

Other interesting aspects of antiviral RNAi responses in insects have been revealed recently. In plants, systemic or non-cell-autonomous RNAi, which refers to the spread of RNAi from cell to cell or through the entire plant, is crucially important in limiting virus infections and spread (Voinnet, 2005; Xie & Guo, 2006). A similar phenomenon has recently been shown to exist in insects. A systemic aspect to antiviral RNAi responses, which relies on dsRNA uptake from the cellular environment, has been demonstrated in *D. melanogaster* (Saleh *et al.*, 2009) and we demonstrated a systemic component to antiviral RNAi in SFV-infected mosquito cells (Attarzadeh-Yazdi *et al.*, 2009). The latter relies on direct cell-to-cell spread of viRNAs (and possibly also longer dsRNAs) and inhibits the replication of incoming virus in cells neighbouring infected cells; the exact mechanism is not yet known. Current understanding of the processes involved in the induction/spread of antiviral RNAi in mosquito cells is summarized in Fig. 2.

Fig. 2. siRNA-mediated antiviral RNAi, a mosquito cell-defence mechanism against arboviruses. Arbovirus-induced dsRNAs (replication intermediates or genome structures) are cleaved into virus-induced siRNAs (viRNAs) by the RNase III enzyme Dcr-2, which also interacts with the dsRNA-binding protein R2D2. One strand of the viRNA (guide strand) is then integrated into the RISC. The RISC contains several proteins, including TSN, Ago-2, dFMR1 and VIG. Viral target single-strangled RNA (ssRNA) represents viral mRNA or genome/antigenome and is cleaved by the RISC 'slicer' enzyme Ago-2, resulting in inhibition of arbovirus replication. Spread of viRNAs into neighbouring cells inhibits incoming arbovirus replication. The involvement of the PIWI pathway in this antiviral-defence mechanism is not yet well understood (see main text).

The origin of arbovirus-derived siRNAs

Arboviruses generally induce RNAi responses in mosquito and other arthropod cells, and viRNAs are detected (Blakqori *et al.*, 2007; Campbell *et al.*, 2008b; Chotkowski *et al.*, 2008; Cirimotich *et al.*, 2009; Garcia *et al.*, 2005; Myles *et al.*, 2008; Sanchez-Vargas *et al.*, 2004, 2009). Much effort has been put into characterizing viRNAs in more detail. In the case of alphaviruses, viRNAs from SINV- or ONNV-infected mosquitoes were cloned, sequenced and found to be predominantly 21 nt in length (Myles *et al.*, 2008, 2009), confirming Northern blotting studies (Cirimotich *et al.*, 2009; Sanchez-Vargas *et al.*, 2004). Spread of SFV in U4.4 cells is enhanced by expression of the tombusvirus p19 protein, which binds 21 nt siRNAs with high affinity (Attarzadeh-Yazdi *et al.*, 2009; Scholthof, 2006); initial observations confirm the presence of almost exclusively 21 nt viRNAs in SFV-infected mosquito cells (R. W. Siu & J. K. Fazakerley, personal communication). In contrast, infection of tick cells with SFV produces a more heterogeneous population of viRNAs, indicating that viRNA production differs between arthropods (Garcia *et al.*, 2005). Little is known about bunyavirus viRNAs, but LACV viRNAs appear similar in size to other viRNAs in mosquito cells (Blakqori *et al.*, 2007). In the case of flaviviruses, viRNA populations are more heterogeneous. viRNAs of 18–22 nt were detected in DENV-2-infected *Ae. aegypti* cells; however, infection of *Drosophila* cells with WNV generated viRNAs of 25 nt (Chotkowski *et al.*, 2008; Sanchez-Vargas *et al.*, 2009). Overall, these findings suggest that induction of antiviral RNAi in mosquitoes resembles that of other arthropods, although the details may vary.

The viral dsRNA substrate that serves as the substrate for Dcr-2 (or related) activities to produce viRNAs is currently unknown; however, several observations are of particular interest. Flavivirus (DENV-2) (Sanchez-Vargas *et al.*, 2009) and alphavirus – SINV (Stollar *et al.*, 1972) or SFV (our unpublished observations) – infections of mosquito cells lead to production of long dsRNAs; these dsRNAs could be secondary structures within viral RNA genomes/antigenomes and/or replication intermediates. In plants, secondary structures in (+)-strand RNA virus genomes and replication intermediates have been described as Dicer substrates from plant viruses and viroids (Ho *et al.*, 2006; Itaya *et al.*, 2007; Molnar *et al.*, 2005; Yoo *et al.*, 2004). Much information about viRNAs in insect cells stems from *D. melanogaster* or *Drosophila* cells infected with a (+)-strand RNA insect virus, flock house virus (family *Nodaviridae*) (Aliyari *et al.*, 2008; Flynt *et al.*, 2009; van Rij & Berezikov, 2009). Roughly equal amounts of viRNAs of (+) and (−) polarity were detected, suggesting viRNAs generated from dsRNA replication intermediates as the main substrate for *Drosophila* Dcr-2 activity; dsRNA of the genome or antigenome RNA structures would be expected to produce an excess of viRNAs of (+) polarity, as infected cells contain much more (+)-strand RNA genome than (−)-strand RNA antigenome. viRNAs were derived from the entire viral genome, but with 'hot spots', particularly at the 5′ ends of the bipartite flock house virus genome.

Characterization of viRNA pools from SINV-infected *Ae. aegypti* or ONNV-infected *A. gambiae* gives a slightly different picture. Again, viRNAs are derived from the viral genome and antigenome, but with a bias towards genome-derived viRNAs (Myles *et al.*, 2008, 2009). There are noticeable 'hot spots' for viRNA production, which do not always overlap on the genome and antigenome. The presence of mainly genome-derived SINV viRNAs in infected mosquitoes was also described elsewhere (Campbell *et al.*, 2008b). This pattern suggests that many of these viRNAs are derived from the genomic RNA,

presumably areas of dsRNA secondary structure, with an important contribution of dsRNA replication intermediates indicated by overlapping genome- and antigenome-derived viRNAs. The presence of 'hot spots' for viRNA production and a bias towards genome-derived viRNAs has also been described in DENV-2-infected *Ae. aegypti* cells; this also suggests dsRNA secondary structures in DENV-2 genomes as (although probably not only) substrates for Dicer activities (Sanchez-Vargas *et al.*, 2009). The role of dsRNA replication intermediates in viRNA origin is currently debated (Myles *et al.*, 2009); indeed, a thorough bioinformatic analysis of RNA secondary structures in arbovirus genomes/antigenomes and their overlap with viRNA sequences or the use of replication-deficient mutants is required to assess the origins of arbovirus-induced viRNAs further.

Suppression or evasion of antiviral RNAi by arboviruses?

Many plant-infecting viruses or pathogenic viruses of insects express proteins that suppress the RNAi response of the host by blocking key steps in this process, for example binding long dsRNA or siRNA; these viral suppressors of RNAi (VSRs) are crucial to the replication and propagation of these viruses (Ding & Voinnet, 2007; Gordon & Waterhouse, 2006; Kemp & Imler, 2009; Li & Ding, 2006). Given the importance of VSRs, it is not surprising that much effort was dedicated to identifying similar functions in arboviruses, despite their biology being different from insect-only viruses. Early efforts indicated that none of the mature DENV proteins displays VSR activity (Li & Ding, 2005). A role for the LACV NSs protein as a VSR in vertebrate cells has been suggested, but a similar role in mosquito cells was recently dismissed (Blakqori *et al.*, 2007; Soldan *et al.*, 2005). The presence of a VSR was investigated thoroughly by using reporter systems and reporter gene-expressing SFV, but no evidence of VSR function was found (Attarzadeh-Yazdi *et al.*, 2009).

Reverse engineering of VSRs into arboviruses or arbovirus replicons suggests biological reasons for their (probable) absence in arboviruses. VSR expression leads to a modest but significant increase in arbovirus replicon replication in mosquito and tick cells (Blakqori *et al.*, 2007; Garcia *et al.*, 2006). SINV expression of the flock house virus VSR (dsRNA-binding protein B2) dramatically reduces viRNA production and enhances viral RNA synthesis, virus dissemination and growth, but reduces the survival of infected mosquitoes (Cirimotich *et al.*, 2009; Myles *et al.*, 2008). SFV expression of the tombusvirus VSR p19 (which binds siRNAs) inhibits the systemic RNAi response, at least in cultured mosquito cells (Attarzadeh-Yazdi *et al.*, 2009). Taken together, these experiments suggest that arboviruses do not encode VSRs, but that replication of these viruses in mosquito cells can be enhanced by the presence of a VSR. It is probable that VSR evolution as been selected against by reducing vector fitness and arbovirus transmissibility.

Whilst arboviruses seem unlikely to have evolved to suppress RNAi, they may nevertheless have evolved to evade it. One genotype of SINV was found to be a stronger inducer of viRNAs (and to replicate less well) than another in mosquitoes (Campbell *et al.*, 2008b). WNV infection of C6/36 mosquito cells does not result in viRNA production, and this virus evades, rather than actively inhibits, siRNA-mediated silencing in human cells if siRNAs are added after infection (Chotkowski *et al.*, 2008; Geiss *et al.*, 2005). A recent report on DENV-2-infected *Ae. aegypti* mosquitoes and mosquito cells suggested that this virus also evades antiviral RNAi (Sanchez-Vargas *et al.*, 2009); however, as a reduction in virus genome RNA levels and/or levelling/reduction of virus production coincide with the detection of viRNAs, this remains unclear, especially if viRNAs are derived from DENV-2 genomes, which would have to accumulate before viRNAs can be detected. It has been suggested that sequestration of alphavirus replication complexes into membrane vesicles protects from RNAi (Sanchez-Vargas *et al.*, 2009). There are, however, other possibilities. In the case of flock house virus infection of *D. melanogaster* cells, 'hot spot'-derived viRNAs have poor biological activities (Flynt *et al.*, 2009). This could point to a genome nucleic acid-mediated resistance to RNAi that allows replication without the need for a VSR (although it was also found that the bulk of viRNAs were not loaded into Ago-2, which might account for the lack of silencing activity). In the case of a potato spindle tuber viroid, this was found to be the case (Itaya *et al.*, 2007). Viroid-derived siRNAs mostly stem from secondary structures within the viroid RNA; however, these secondary structures are in turn highly resistant to RISC-mediated cleavage. Similar mechanisms, genome secondary structures inaccessible to viRNAs generated at high-frequency 'hot spots', may also be involved in arbovirus evasion of mosquito RNAi responses.

Arbovirus-induced cell death and apoptosis in mosquito cells

Whilst important in development, at least in vertebrates, apoptosis (programmed cell death) is also an innate response to virus infection that can limit virus replication and spread (Best, 2008). Little is known about apoptotic processes in arbovirus-infected mosquitoes or mosquito cell lines. Several reports describe alphavirus- and flavivirus-induced pathology and sometimes apoptotic cell death in infected mosquitoes (Bowers *et al.*, 2003; Girard *et al.*, 2005, 2007; Mims *et al.*, 1966; Vaidyanathan & Scott, 2006; Weaver *et al.*, 1988, 1992). Whilst there is differential regulation of enzymes involved in apoptosis in DENV-2-infected mosquitoes (Xi *et al.*, 2008), the extent and role of apoptosis, if any, in this infection remains unclear. Roles in resistance and virus transmission potential have been suggested (Girard *et al.*, 2005, 2007; Vaidyanathan & Scott, 2006). Infection of mosquito cell lines by arboviruses usually leads to persistent infection with no cytopathic effects, and survival of the culture. Cytopathic effects have

occasionally been described in mosquito cell lines and this seems to depend on particular arboviruses, cell lines or clones of cell lines (Condreay & Brown, 1988; Sarver & Stollar, 1977; Stalder *et al.*, 1983; Stollar *et al.*, 1979). Cell-cycle perturbations can take place in some virus–cell combinations (Karpf *et al.*, 1997). For LACV, no apoptosis was detected in infected *Ae. albopictus* cells and levels of IAP1 (inhibitor of apoptosis protein 1) were unchanged (Blitvich *et al.*, 2002; Borucki *et al.*, 2002).

It is not clear whether arboviruses actively inhibit apoptosis or whether mosquitoes lack the pathways necessary to activate apoptosis upon infection. A recent study analysed this by infecting mosquito cells with recombinant SINV expressing the apoptosis inducers Michelob_x (Mx) or Reaper (Rpr) of *D. melanogaster* or *Ae. aegypti*, or the anti-apoptotic baculovirus protein p35 (Wang *et al.*, 2008). Expression of pro-apoptotic genes activated apoptotic cell death, whereas control virus had no immediate effect on cell viability, suggesting that SINV did not actively suppress apoptosis, or at least could not suppress apoptosis initiated by these pro-apoptotic proteins. The initial burst of virus production (before the persistent phase with low virus production) was not affected significantly by Mx- or Rpr-induced apoptosis, suggesting that, even when it did occur, apoptosis was not effective at reducing early virus production (although virus production is reduced later, as cells die). Interestingly, expression of the B2 RNAi inhibitor by alphaviruses leads to death of infected mosquitoes and cytopathic effects in cultured mosquito cells (Cirimotich *et al.*, 2009; Myles *et al.*, 2008).

Taken together, these studies suggest that arbovirus infection of mosquito cells most probably triggers cell death when virus replication exceeds a threshold level. Apoptosis affects virus production negatively in persistently infected cells (Karpf *et al.*, 1997) and induction of apoptosis does not confer an advantage to the virus (whenever analysed). For alphaviruses, apoptosis appears unlikely to play a role in the maintenance of persistent infection (Karpf *et al.*, 1997). Avoiding apoptosis and relying on other control mechanisms such as RNAi before initiating apoptotic (or other) cell death *in extremis* might therefore be a useful trade-off for both parties.

From innate immunity towards control of arboviral pathogens

Many early applications of antiviral RNAi in mosquitoes have been reviewed elsewhere (Blair *et al.*, 2000; Olson *et al.*, 2002) and will not be discussed here. However, recent particular highlights to come out of RNAi research, such as the development of DENV-2-resistant *Ae. aygypti* mosquitoes, are recommended for further reading on this subject (Franz *et al.*, 2006; Travanty *et al.*, 2004). Future work in this field will also aim to discover antiviral effector molecules under control of the Toll, JAK/STAT etc. signalling pathways, which might be useful to engineer arbovirus-resistant mosquitoes. Whilst *Wolbachia*-infected

Ae. aegypti mosquitoes have a reduced lifespan (McMeniman *et al.*, 2009), it will also be interesting to verify whether activation of immune pathways by these bacteria could induce resistance to virus infection in mosquitoes, as is the case in *D. melanogaster* (Hedges *et al.*, 2008; Teixeira *et al.*, 2008). There are still many biological obstacles (in addition to public acceptance) before these applications of innate immunity research will impact on public health; these include implications for vector fitness by transgene expression and spread in wild-type populations, and have been reviewed elsewhere (Alphey, 2009).

Obstacles in current research

We still know very little about mosquito innate immune responses to arbovirus infections and the field lacks many tools compared with *Anopheles*/malaria or *Drosophila* research, although this is now improving. In particular, only a few mutant aedine mosquitoes have been described, and not many antibodies or molecular tools for cell-culture work are available. In addition, much cell culture-based work has traditionally been carried out with *Ae. albopictus*-derived cell lines; whilst some have functional immune responses and are excellent tools, *Ae. aegypti* is preferred for work with the live mosquito and its genome sequence is now known. *Ae. albopictus* is also of importance to Europe (i.e. chikungunya virus in Italy and the French overseas department of Réunion) and its genome sequence would be helpful to the arbovirus community. There is also a lack of cell lines from other mosquito species that would allow comparison of arbovirus replication in different host backgrounds and in relatively simple systems. It is certain that research on antiviral immunity in *D. melanogaster* – with its many tools, mutants and reagents – will continue to influence mosquito/arbovirus research, and this model organism has so far proven to be reliable; perhaps *Drosophila* geneticists might benefit in return from using the numerous arbovirus tools and mutant viruses.

Conclusions and perspectives

The last few years have seen considerable progress in understanding how mosquito responses control arbovirus replication. This is mainly due to genomic studies and increased understanding of antiviral RNAi. Whilst the classical pathways that insects use to fight bacteria and fungi have now been shown to also be involved in antiviral responses, it remains to be determined how, when and under what circumstances these pathways are activated by arboviruses and how they mediate antiviral responses (there appear to be considerable differences between arbovirus families). Identifying the inhibitory antiviral activities and effector molecules will be an important challenge. The role of serine proteases or their inhibitors (serpins) in virus infections remains to be investigated (Sanders *et al.*, 2005; Xi *et al.*, 2008); these are known to feed into immune-signalling pathways in *D. melanogaster*

(Lemaitre & Hoffmann, 2007). How these various pathways interplay and regulate each other remains to be seen, although Sanders *et al.* (2005) observed a downregulation of Dcr-2 in SINV-infected *Ae. aegypti* late after infection, so links between antiviral pathways might exist. Equally intriguing is the observation that immune-signalling pathways can be suppressed by arboviruses, possibly in a manner the same as or similar to that in infected vertebrate cells. Several questions arise on how effective and important these pathways are in antiviral responses. Is suppression of one set of antiviral activities sufficient or necessary to assure virus propagation? Is a concerted effort of RNAi and immune pathways required to maintain the delicate balance between vector survival and virus transmission? The survival of arbovirus-infected mosquitoes with immune deficiencies in Imd or Toll signalling pathways should answer many of these questions and complement RNAi-based knockdown studies (Bian *et al.*, 2005; Shin *et al.*, 2003).

Many questions regarding the mechanisms of antiviral RNAi have yet to be solved. Very little is known about how RNAi is induced in the infected cell. Any arbovirus-evasion mechanisms remain to be determined; typical VSRs might not be involved, but other strategies may exist. The question whether low vector competence relates to effective immune responses has been raised in the past (Campbell *et al.*, 2008a). In this context, it is interesting to note that the antiviral RNAi response influences DENV-2 transmission in *Ae. aegypti* directly; this finding has important implications for further understanding vector biology and epidemiology (Sanchez-Vargas *et al.*, 2009). The role of apoptosis in mosquito-cell responses to virus infection remains largely unclear. Virus-induced cell death might yet turn out to be an important factor in arbovirus tropism/transmission or a last attempt (possibly detrimental to the host) to contain arboviruses if mechanisms such as RNAi fail to control replication. Currently, we simply do not know enough to evaluate the contribution of antiviral apoptotic host responses. As suggested by Wang *et al.* (2008), infection of mosquitoes with recombinant arboviruses expressing activators or inhibitors of apoptosis (such as those described by these authors) might answer some fundamental questions regarding apoptosis and possible roles in mosquito antiviral responses.

Much of the published work has focused on (+)-strand RNA viruses, and it remains to be seen how these findings relate to mosquito-borne (−)-strand RNA viruses such as bunyaviruses, for which we still know very little with regards to their interactions with arthropod immunity, or dsRNA reoviruses such as midge-borne bluetongue virus. Research on mosquitoes is also likely to influence research on tick-borne arboviruses. As tick cells can induce antiviral RNAi responses, it is likely that this antiviral defence plays a major role in arachnids (Garcia *et al.*, 2005, 2006). Particularly intriguing in ticks is the presence of RNA-dependent RNA polymerases (Gordon & Waterhouse, 2007) that may have the potential to amplify systemic

RNAi, as occurs in plants (Voinnet, 2005). Moreover, the role of autophagy, described recently in *Drosophila* immunity to VSV (Shelly *et al.*, 2009), in mosquito antiviral immunity remains to be investigated. We are probably only just beginning to comprehend how complex the interactions between arboviruses and their arthropod vectors really are.

Acknowledgements

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