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Beyond RNAi: Antiviral defense strategies in *Drosophila* and mosquito

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ABSTRACT

Virus transmission and spread by arthropods is a major economic and public health concern. The ongoing dissemination of arthropod-borne viruses by blood-feeding insects is an important incentive to study antiviral immunity in these animals. RNA interference is a major mechanism for antiviral defense in insects, including the fruit fly *Drosophila melanogaster* and several vector mosquitoes. However, recent data suggest that the evolutionary conserved Toll, Imd and Jak-Stat signaling pathways also contribute to antiviral immunity. Moreover, symbionts, such as the intracellular bacterium *Wolbachia* and the gut microflora, influence the course of virus infection in insects. These results add an additional level of complexity to antiviral immunity, but also provide novel opportunities to control the spread of arboviruses. In this review, we provide an overview of the current knowledge and recent developments in antiviral immunity in Dipteran insects, with a focus on non-RNAi mediated inducible responses.

1. Introduction

Insects are among the most diverse and numerous animals on earth and populate almost every habitat (Chapman, 2009). As agricultural pests, they cause severe economic losses by damaging and killing crops, but insects also pose an important threat to human and animal health. Indeed, insects are vectors for numerous pathogens, including viruses, bacteria, protozoa and nematodes. Over 500 arthropod-borne viruses (arboviruses) have been identified, among which ~100 are harmful to humans (ICTV, 2011). Most arboviruses are RNA viruses from the families of *Bunyaviridae* (e.g. La Crosse virus), *Togaviridae* (e.g. Chikungunya virus) and *Flaviviridae* (e.g. West Nile virus, WNV; Dengue virus, DENV; Yellow fever virus) (Weaver and Reisen, 2010).

Due to increased global travel and urbanization, adaptation of insects to new habitats, and possibly climate change, arboviruses are spreading from tropical areas to new territories (Jones et al., 2008). For example, the incidence of DENV infection increased 30-fold over the last 50 years and the virus now infects an estimated 50 million people annually (WHO, 2009). Recently, DENV infections not associated with travel to endemic countries have been reported in Florida and France (La Ruche et al., 2010; Franco et al., 2010). The rapid spread of WNV throughout Northern America (Kilpatrick, 2011) and the (re-)emergence of Chikungunya in Africa, Asia and Southern Europe (Schuffenecker et al., 2006; Burt et al., 2012) exemplifies how introduction of arboviruses into new territories and adaptation to new vectors can cause epidemics. Insect vector control is instrumental to prevent the spread of arboviruses. The widespread use of insecticides, however, is limited in many ways, for example by the development of resistance of the targeted insect. Other vector control tools are therefore urgently needed (Rivero et al., 2010).

Arbovirus infection of the vector is established upon blood-feeding of a susceptible female mosquito on a viremic vertebrate host. Within the insect vector, arboviruses have a complex life cycle that includes replication in the midgut, followed by systemic dissemination via the hemolymph and replication in the salivary glands (Kuno and Chang, 2005). Transmission of an arbovirus to a naive vertebrate host during blood-feeding requires high viral titers in the saliva. Anatomical and immunological barriers affect the ability of the virus to reach such titers and thus to accomplish successful transmission to a naive host. Despite efficient replication, arboviruses do not cause overt pathology and are only associated with minor fitness costs in the insect vector (Lambrechts and Scott, 2009), suggesting that the insect immune system restricts virus infection to non-pathogenic levels. Understanding the mechanisms of insect antiviral immunity may provide opportunities for restricting the spread of arboviruses.

Innate immunity provides the first line of defense against microbial invaders and is defined by its rapid activation following pathogen recognition by germline-encoded receptors. These receptors recognize small molecular motifs that are conserved among classes of microbes, but are absent from the host, such as bacterial cell wall components and viral double-stranded (ds) RNA. Collectively, these motifs are called pathogen-associated molecular patterns (PAMP). Innate immunity is traditionally distinguished from adaptive immunity, which emerged ~500 million years ago in vertebrates (Hirano et al., 2011). Adaptive immunity is based on pathogen recognition by a large repertoire of highly diverse

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antigen receptors generated by somatic gene rearrangements. In contrast to innate immunity, the adaptive immune system is endowed with long-term memory that allows a more rapid and robust response upon re-exposure to previously encountered pathogens. Whether invertebrates are also capable of generating immunological memory is a long-standing matter of debate. The extreme diverse repertoire of isoforms of the immunoglobulin domain containing receptor DSCAM (Down syndrome cell adhesion molecule) with a role in specific immunity in *Drosophila* and *Anopheles gambiae* (Watson et al., 2005; Dong et al., 2006), as well as the existence of memory traits in the immune systems of several invertebrate species (Kurtz, 2005; Netea et al., 2011) challenged the paradigm that anticipatory immunity and memory were exclusive to the vertebrate immune system.

The fruit fly *Drosophila melanogaster* is a powerful model to study innate immunity. Over the years, detailed insight in antibacterial and antifungal immunity has been obtained, greatly facilitated by the extensive genetic toolkit for this model organism (Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007). However, *Drosophila* is also a host to numerous viruses (Huszar and Imler, 2008). Natural populations of *D. melanogaster* can be infected with RNA viruses, such as Sigma virus, (DmelSV) (Fleuriet, 1981a,b), *Drosophila* C Virus (DCV) (Plus et al., 1975), and Nora virus (Habayeb et al., 2006). Several other RNA viruses efficiently replicate in *Drosophila* after experimental inoculation, including Cricket paralysis virus (CrPV) (Reinganum and Scotti, 1976), *Drosophila* X virus (DXV) (Teninges et al., 1979), Flock House virus (FHV) (Galiana-Arnoux et al., 2006; Wang et al., 2006), Sindbis virus (SINV) (Herreng, 1967), and Vesicular stomatitis virus (VSV) (Peries et al., 1966). Recently, a DNA virus has been identified in *Drosophila innubila* (Unckless, 2011), but no DNA virus has yet been recovered from natural *D. melanogaster* populations. The infection models that are discussed in this review are presented in Table 1.

Antiviral defense mechanisms of insects remain poorly understood. Many studies on antiviral immunity have been performed in *Drosophila*, but the functional dissection of vector competence in mosquitoes is gaining momentum. The completion of the genome sequence of important vector species (*A. gambiae*, *Aedes aegypti* and *Culex quinquefasciatus*) and the development of genome-wide transcriptomics and RNA interference (RNAi) technology has been instrumental in studies of mosquito immunity (Holt et al., 2002; Nene et al., 2007; Bartholomay et al., 2010). Both in *Drosophila* and mosquitoes, RNAi plays a crucial role in the defense against RNA viruses (reviewed by Ding and Voinnet, 2007; van Rij and Berezikov, 2009; Kemp and Imler, 2009; Blair, 2011). More recently, the piwi-interacting (pi) RNA pathway, a related but distinct small RNA silencing pathway, has also been implicated in antiviral defense in these insects (van Mierlo et al., 2010; Wu et al., 2010; Morazzani et al., 2012; Vodovar et al., 2012). In this review, we will go beyond antiviral RNAi responses and explore the role of evolutionary conserved immune signaling cascades, including the Toll, Imd and Jak-Stat pathways, in antiviral defense. We will also discuss recent work that demonstrates a crucial role of bacterial symbionts in the control of pathogen burden and the outcome of viral infections.

2. Immune pathways in insects

The Toll and Imd (Immune deficiency) pathways mediate systemic immunity against bacteria and fungi in *Drosophila*. We will give a brief overview here (Fig. 1); more detailed reviews are available elsewhere (Ferrandon et al., 2007; Lemaitre and Hoffmann, 2007). Upon infection by Gram-positive bacteria or fungal pathogens, the Toll pathway is activated by two pathogen-sensing systems. First, the PAMPs of entomopathogenic fungi (beta-glucans) and Gram-positive bacteria (Lysine-type peptidoglycan) are detected by pattern-recognition receptors, such as Gram-negative binding protein (GNBP)-1 and -3, and peptidoglycan recognition proteins (PGRP)-SA and -SD, respectively. Second, the proteolytic activity that accompanies microbe invasion of the hemolymph, which can be considered a danger signal, is sensed by the protease Persephone (Gottar et al., 2006; El Chamy et al., 2008). Both branches initiate an extracellular signaling cascade that leads to the cleavage of pro-Spätzle into the active cytokine Spätzle, which then binds the transmembrane receptor Toll. Subsequently, an intracellular signaling pathway leads to the translocation of the NF-κB like transcription factors Dif (Dorsal-related immunity factor, in adults) and Dorsal (in larvae and adults) to the nucleus. Here, Dif and Dorsal bind to NF-κB responsive elements, thereby inducing the expression of many genes, including a specific set of antimicrobial peptides (AMPs), such as *Drosomycin* and *Defensin*.

Gram-negative bacteria activate the Imd pathway via the detection of monomeric or polymeric diaminopimelic (DAP) type peptidoglycans by PGRP-LC and -LE. These receptors then recruit Imd and activate the intracellular signaling cascade that leads to the activation of another NF-κB like factor, Relish, after cleavage of its inhibitory IκB domain. Upon activation, the Rel domain of Relish translocates to the nucleus and induces transcription of another set of immune genes and AMPs, such as *Diptericin* and *Cecropin*.

Table 1
Model viruses of *Drosophila* and mosquito.[a]

Host insect	Virus	Abbr.	Family	Genome	Envelope
Drosophila melanogaster	Cricket paralysis virus	CrPV	Dicistroviridae	(+) ssRNA	No
	Drosophila C virus	DCV	Dicistroviridae	(+) ssRNA	No
	Drosophila X virus	DXV	Birnaviridae	bipartite dsRNA	No
	Flock House virus	FHV	Nodaviridae	bipartite (+) ssRNA	No
	Invertebrate Iridescent virus 6	IIV-6	Iridoviridae	dsDNA	–[b]
	Nora virus	–	Unassigned	(+) ssRNA	No
	Sigma virus	DmelSV	Rhabdoviridae	(−) ssRNA	Yes
	Sindbis virus	SINV	Togaviridae	(+) ssRNA	Yes
	Vesicular stomatitis virus	VSV	Rhabdoviridae	(−) ssRNA	Yes
Aedes aegypti	Dengue virus	DENV	Flaviviridae	(+) ssRNA	Yes
	Sindbis virus	SINV	Togaviridae	(+) ssRNA	Yes
	West Nile virus	WNV	Flaviviridae	(+) ssRNA	Yes
	Yellow Fever virus	YFV	Flaviviridae	(+) ssRNA	Yes
Aedes albopictus	Semliki Forest virus	SFV	Togaviridae	(+) ssRNA	Yes
Anopheles gambiae	O'nyong-nyong	ONNV	Togaviridae	(+) ssRNA	Yes

[a] We report here the infection models that are discussed in this review. Note that not all reported infection models represent natural virus-host combinations.
[b] IIV-6 virions can be enveloped, when released by budding, or non-enveloped, when released by cell lysis.

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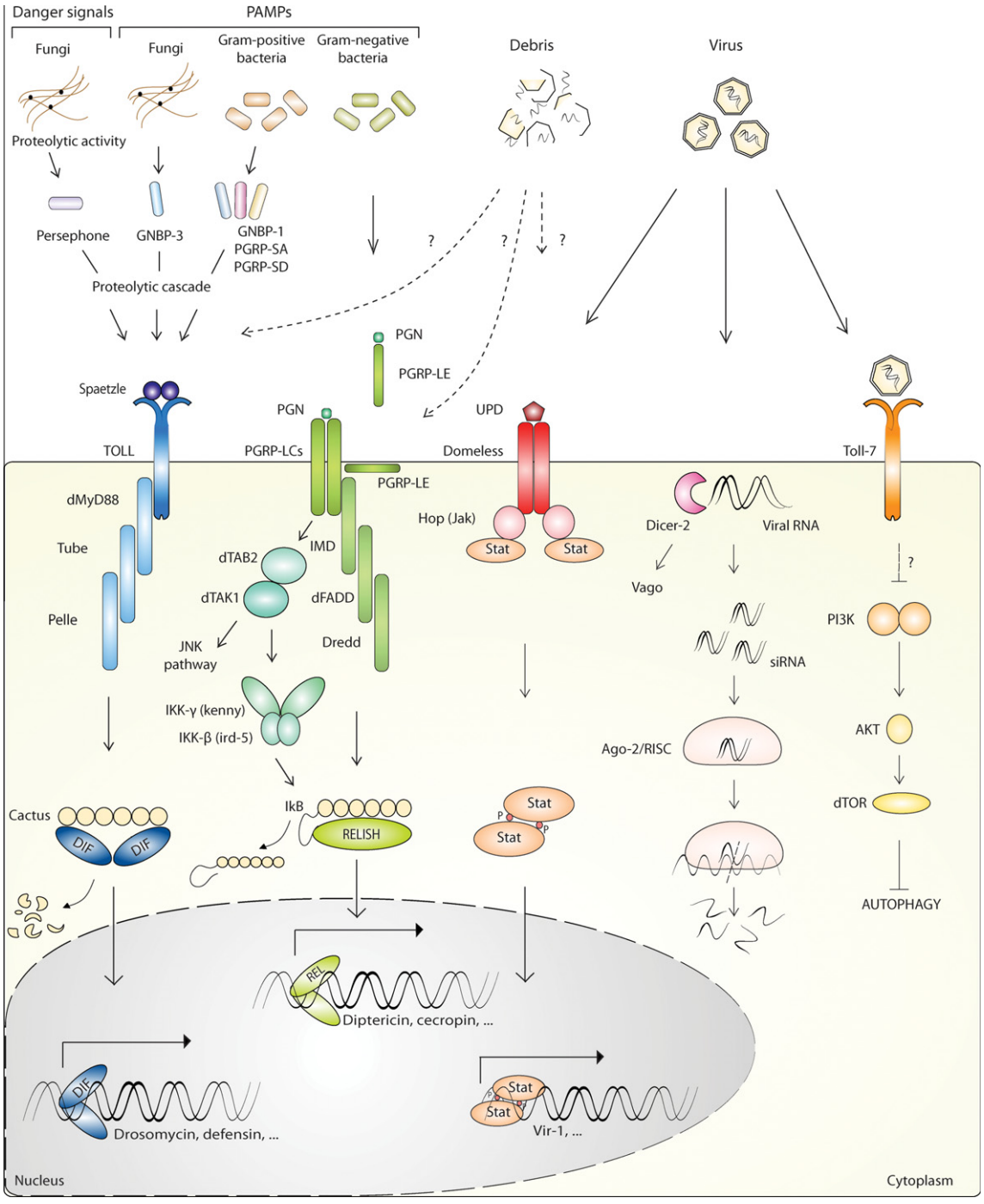

Fig. 1. Inducible immune pathways in *Drosophila*. Toll pathway: detection of pathogen-associated molecular patterns (PAMPs) of fungi (beta-glucans) and Gram-positive bacteria (Lys-type peptidoglycan) or danger signals (such as proteolytic activity in the hemolymph) triggers a proteolytic cascade, leading to cleavage of the precursor of the dimeric cytokine Spätzle. Mature Spätzle binds to the membrane-anchored Toll receptor, thereby inducing its dimerization. Three intracellular death domain–containing proteins, MyD88, tube, and pelle are then recruited. The kinase pelle is likely responsible for phosphorylation of cactus, thereby directing its degradation by the proteasome. Dif (Dorsal-related immunity factor) is then able to translocate to the nucleus, where it induces transcription of immune genes, including the antimicrobial peptides (AMPs) *Drosomycin* and *Defensin*. Imd pathway: Binding of microbial diaminopimelic (DAP)-type peptidoglycan (PGN) from Gram-negative bacteria to peptidoglycan recognition proteins (PGRP-LC or PGRP-LE) induces the recruitment of the adaptor molecules Imd (Immune deficiency) and *dFADD* (*Drosophila* Fas-associated death domain). This leads to activation of the caspase DREDD (Death related ced-3/Nedd2-like protein) and of TAK1 (Transforming growth factor-β-activated kinase 1). TAK1 and its adaptor TAB2 (TAK1-binding protein 2) activate a complex of IκB Kinase (IKK)-*β* and IKK-*γ* (also known as *ird-5* and *kenny*, respectively), which then directs phosphorylation of Relish, followed by its proteolytic cleavage by DREDD. The inhibitory domain (IκB) of Relish remains stable in the cytoplasm, whereas the *Rel* domain of Relish translocates to the nucleus and induces transcription of immune genes, such as the AMPs *Diptericin* and *Attacin*. PGRP-LE can act as an intracellular receptor, and, in a truncated form, as an extra-cellular receptor. Jak-Stat pathway: upon virus infection, the Janus kinase (Jak) – signal transducers and activators of transcription (Stat) pathway is activated, presumably by binding of an unpaired (Upd) cytokine to the dimeric domeless receptor. The Jak tyrosine kinase Hopscotch (Hop), in association with domeless, then phosphorylates both itself and the cytoplasmic tail of domeless, creating binding sites for Stat92E. Upon binding, Stat92E is phosphorylated, dimerizes and translocates into the nucleus where it induces transcription of target genes that contain stat binding sites in their promoters, such as *virus induced RNA-1* (*vir-1*). RNAi: viral double-stranded RNA (dsRNA) is recognized and processed by Dicer-2 into small interfering (si) RNAs, which are then incorporated in an Argonaute-2 (Ago-2) containing RNA-induced silencing complex (RISC). Within RISC, these siRNAs guide the recognition and cleavage of complementary viral RNA sequences, thereby restricting virus replication. Autophagy: vesicular stomatitis virus binds to the transmembrane receptor Toll-7 and induces autophagy. This is likely mediated through negative regulation of the phosphatidylinositol 3-kinase (PI3K)-Akt kinase pathway. It is likely that cell debris or damaged virus-infected cells release or act as immunostimulatory damage-associated molecular patterns that activate immune signaling pathways via undefined mechanisms.

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Collectively, the Toll and Imd pathways induce factors that participate in killing of microbes, phagocytosis, production of reactive oxygen species, and the melanization cascades that are essential for wound healing and pathogen sequestration (Lemaitre and Hoffmann, 2007). Within minutes after pathogen sensing, a cocktail of AMPs is secreted by the fat body (Lemaitre et al., 1997). As a consequence, the concentration of AMPs in the hemolymph rapidly increases over time, each AMP displaying a specific concentration range and dynamics (Bulet et al., 1999). Although Toll and Imd activate many different genes, induction of AMP expression is traditionally used as a read-out for Toll and Imd activation (De Gregorio et al., 2001; Irving et al., 2001; De Gregorio et al., 2002; Boutros et al., 2002).

A third evolutionary conserved pathway, Jak-Stat (Janus kinase – signal transducers and activators of transcription), originally studied for its role in *Drosophila* development (Zeidler et al., 2000; Luo and Dearolf, 2001; Arbouzova and Zeidler, 2006), was recently shown to participate in antibacterial and antiviral immunity (see Section 3.3) (Agaisse et al., 2003; Agaisse and Perrimon, 2004; Buchon et al., 2009; Goto et al., 2010). Binding of a cytokine from the Unpaired (Upd) family to the receptor Domeless activates the Jak-Stat pathway. *Drosophila* encodes a single Jak kinase (*hopscotch, hop*) and a single Stat transcription factor (*Stat92E*), which upon activation dimerizes and translocates to the nucleus to direct transcription of genes with Stat binding sites in their promoter (Fig. 1) (Agaisse and Perrimon, 2004).

The importance of *Drosophila* as a model for innate immunity relies on the evolutionary conservation of immune signaling pathways. Comparative genomics of *Drosophila* and the major vector mosquitoes *A. gambiae*, *A. aegypti* and *C. quinquefasciatus* suggest a conserved system of immune signaling pathways with the potential for evolutionary plasticity to adapt to new pathogens (Christophides et al., 2002; Waterhouse et al., 2007; Bartholomay et al., 2010). Whereas the core signaling pathways have remained highly conserved, the upstream receptors and downstream effectors have diverged quite significantly. For example, mosquitoes do not possess the Dif transcription factor of the Toll pathway, but rely on the *dorsal* orthologs *Rel1* (*A. gambiae*) or *Rel1A* and *Rel1B* (*A. aegypti*) and the Relish ortholog *Rel2* (*A. gambiae* and *A. aegypti*) for expression of effector genes of the Toll and Imd pathways, respectively (Christophides et al., 2002; Shin et al., 2005; Waterhouse et al., 2007).

Evolutionary conservation of immune pathways even extends to the class of mammals. The Toll and Imd pathways of insects are strikingly similar to the mammalian Toll-like receptor (TLR) and Tumor Necrosis Factor (TNF) pathways, respectively. Mammalian TLRs contribute to innate antiviral immunity by sensing viral nucleic acids in endosomal compartments, leading to the activation of the NF-κB and IRF-3 transcription factors and production of cytokines, among which the potent antiviral type I interferons (Kawai and Akira, 2006, 2010). The TNF signaling pathway regulates basic cellular processes, such as inflammation and cell death, and is essential for innate and adaptive immune functions (Chen and Goeddel, 2002; Aggarwal, 2003). As a consequence, some mammalian viruses evolved strategies to counteract and manipulate TNF signaling (Benedict et al., 2003). Jak-Stat is a central signaling pathway that mediates the response to a wide range of growth factors and cytokines, including type I interferons. Also AMPs seem to be conserved from plants to humans. For example, a *Drosomycin*-like *Defensin* in humans exerts antifungal activity in the skin (Simon et al., 2008).

3. The role of Toll, Imd, and Jak-Stat pathways in antiviral defense

The central role of the Toll and Imd pathways in antimicrobial immunity of *Drosophila* and the importance of the TLR and Jak-Stat pathway in antiviral immunity in mammals (Karst et al., 2003; Dupuis et al., 2003) are strong incentives to analyze the role of these pathways in antiviral immunity of insects. Induction of AMPs is often used as a proxy for Toll and Imd activation. Several studies failed to detect robust induction of AMP expression in virus infection (Table 2). For example, proteomic analyses of flies infected with the positive stranded (+) RNA viruses DCV and FHV did not detect an induction of AMP expression (Sabatier et al., 2003; Go et al., 2006). Microarray analyses and quantitative RT-PCR also failed to detect robust AMP induction in DCV and CrPV infection (Dostert et al., 2005; Costa et al., 2009). Furthermore, a microarray analysis of *A. gambiae* infected through a blood meal with of O'nyong-nyong virus (ONNV) did not detect an induction of components or downstream effectors of the Toll, Imd and Jak-Stat pathways at 14 days after infection (Sim et al., 2005). In the non-Dipteran insect, *Apis mellifera* (the honey bee) neither AMP secretion, nor a cellular melanization response was induced upon Acute bee paralysis virus infection (Azzami et al., 2012). Together, these reports suggest that the Toll, Imd and Jak-Stat pathways do not play major roles in RNA virus infections in *Drosophila* and mosquitoes. Nevertheless, studies using other infection models suggest a more convoluted situation (Table 2).

3.1. Toll pathway

Drosophila infected with the dsRNA virus DXV produced robust levels of the AMPs Drosomycin and Metchnikowin at 24 h after inoculation (Zambon et al., 2005). The level of induction was comparable to that triggered by systemic infection with the Gram-negative bacterium *Escherichia coli*. These AMPs are typically induced in a Toll (Drosomycin) or a Toll and Imd (Metchnikowin) dependent manner. Indeed, a role for the Toll pathway was suggested by a screen for DXV sensitivity among flies with defects in genes with known immune function. Among ~60 tested fly lines, a loss-of-function mutation in *Dif*, the Toll-activated NF-κB transcription factor, and a gain-of-function mutation in *Toll* conferred increased susceptibility to DXV infection. Both mutants succumbed faster to virus infection. Viral titers were higher in the *Dif* mutant, but remained close to control levels in the *Toll* mutant. A direct antiviral activity of the induced AMPs could not be established: over-expression of single AMPs in a Toll and Imd pathway double mutant did not affect viral load or overall survival. Hemocytes, the immune cells that circulate in the hemolymph, are more numerous in the *Toll* gain-of-function mutant (Lemaitre et al., 1995). These results may thus suggest a role for phagocytosis or other hemocyte activities in control of virus infection.

Microarray-based transcriptional profiling of *A. aegypti* mosquitoes identified 240 genes that were up-regulated after DENV infection via an infectious blood meal (Xi et al., 2008). Interestingly, among the induced genes were components of the Toll pathway, such as *Toll, Spätzle*, and *Rel1A*, as well as Toll-dependent antimicrobial peptides such as *Cecropin* and *Defensin*. The importance of this pathway in DENV infection was supported by an increase in viral titers in the midgut after RNAi mediated knockdown of the Toll adaptor protein MyD88 (Xi et al., 2008). Other mosquito infection models also suggest a role for Toll in the response to virus infection. A differential display approach in midguts of *Culex pipiens quinquefasciatus* infected with WNV via a blood meal identified a transcript (*CQ G12A2*) that shares 33% similarity with the *A. aegypti* Toll-like receptor (Smartt et al., 2009). Furthermore, a microarray analysis of SINV infected *A. aegypti* detected a modest 1.8-fold induction of the Toll dependent transcription factor *Rel1* among the 19 genes that we up-regulated at one day after infection (Sanders et al., 2005). However, neither *Rel1*, nor other canonical immune genes were induced at later time points (4 and 8 days after infection).

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Table 2
Inducible immune pathways implicated in antiviral defense in *Drosophila* and mosquito.

Pathway	Organism	Virus	Inducible immune response				References
			Genetic evidence[a]	Transcriptional response			
				Pathway components[b]	AMP expression[c]	Other humoral factors[d]	
Toll	*Drosophila melanogaster*	DXV	+	n.t.	Up	n.a.	Zambon et al. (2005)
	Aedes aegypti	DENV	+	Up	Up	n.a.	Xi et al. (2008)
		DENV	n.t.	Down	–	n.a.	Colpitts et al. (2011)
		SINV	n.t.	Up	n.t.	n.a.	Sanders et al. (2005)
		YFV	n.t.	Down	–	n.a.	Colpitts et al. (2011)
	Aedes albopictus	SFV[e]	n.t.	n.t.	Down[f]	n.a.	Fragkoudis et al. (2008)
Imd	*Drosophila melanogaster*	CrPV	+	n.t.	–	n.a.	Costa et al. (2009)
		DmelSV[g]	n.t	Up[g]	Up[g]	n.a.	Tsai et al. (2008)
		SINV[h]	+	n.t.	Up	n.a.	Avadhanula et al. (2009)
	Aedes albopictus	SFV[e]	n.t.	n.t.	Down[f]	n.a.	Fragkoudis et al. (2008)
	Anopheles gambiae	ONNV	-	Down	–	n.a.	Waldock et al. (2012)
Jak-Stat	*Drosophila melanogaster*	DCV	+	–	–	Up	Dostert et al. (2005) Deddouche et al. (2008)
		FHV	n.t.	n.t.	–	Up	Dostert et al., 2005
	Aedes aegypti	DENV	+	Up	Up	Up	Souza-Neto et al., 2009
		DENV	n.t.	–	Down[i]	–	Colpitts et al. (2011)
		WNV	n.t.	–	Down[i]	–	Colpitts et al. (2011)
		YFV	n.t.	–	Down[i]	–	Colpitts et al., 2011
	Aedes albopictus	SFV[e]	n.t.	n.t.	Down[f]	n.t.	Fragkoudis et al. (2008)

n.t., not tested; n.a., not applicable.
[a] +, increased virus replication or virus-induced mortality upon inactivation of key components of these pathways using genetic mutants in *Drosophila* or RNAi in mosquitoes. –, no change detected.
[b] Transcriptional changes of components of the pathways of at least 2-fold. Up, increased expression; down, reduced expression; –, no or less than 2-fold change in expression.
[c] Changes in AMP expression of at least 5-fold. Up, increased expression; down, reduced expression; –, no or less than 5-fold change in expression.
[d] Other humoral factors are defined here as Jak-Stat inducible humoral factors, such as Thioester-containing proteins (TEPs), Turandot proteins, or *vir-1*. Up, increased expression; down, reduced expression; –, no or less than 5-fold change in expression.
[e] This study was performed in the *Aedes albopictus* derived U4.4 cell line; all other reported studies were performed in the adult insect.
[f] Reduced expression of a luciferase reporter driven by Toll, Imd and Jak-Stat responsive promoters after ectopic activation of these pathways.
[g] The results obtained by Tsai et al. (2008) are in conflict to those obtained by Carpenter et al. (2009), who did not observe an induction of the Imd, Toll and Jak-Stat pathways.
[h] This study analyzed a Sindbis virus replicon encoded in the genome of a transgenic fly.
[i] Reduced expression of *Cecropin*-A like genes and *Defensin* I-like genes that were previously shown to be Jak-Stat dependent (Souza-Neto et al., 2009).

3.2. Imd pathway

Two studies suggested a role for the Imd pathway in the defense against RNA viruses in *Drosophila*. Several isogenic mutants of the Imd pathway displayed higher levels of viral RNA and increased mortality after CrPV infection (Costa et al., 2009). Mutants for the receptor *PGRP-LC*, as well as downstream signaling components *Tak1*, *ird5*, *kenny*, and *Relish* were more sensitive to infection. Strikingly however, mutants of the adaptor protein Imd and *dFADD* did not show an increased CrPV susceptibility. Imd is located at a branch-point in the Imd pathway and transduces a signal into two distinct arms of the pathway. Moreover, an interaction between PGRP-LC and Imd does not seem to be essential for signal transduction (Kaneko et al., 2006). The authors thus conclude that distinct branches of the Imd pathway contribute differently to antiviral immunity. Despite the genetic support for Imd in control of CrPV infection, no AMP induction could be detected, suggesting that activation of the Imd pathway and AMP induction can be uncoupled in the response to virus infection.

In another study, a transgenic fly line expressing a SINV replicon was crossed to Toll, Imd and Jak-Stat mutants to screen for host factors modifying virus replication (Avadhanula et al., 2009). Mutants for intracellular components of the Imd pathway, such as *Relish*, *imd*, *dFADD* and *Dredd*, and the Jak-Stat mutant *Stat92E* showed higher viral RNA loads. No difference was observed in Toll pathway mutants. In accordance, the Imd-dependent AMP *Diptericin* and the partially Imd-dependent *Metchnikowin* were induced by virus replication. Viral RNA loads remained normal in mutants for *PGRP-LC* and *PGRP-LE*, the receptors that bind peptidoglycans to initiate the Imd pathway in a bacterial infection. Thus, it is likely that yet undefined receptors detect intracellular viral RNA and trigger the Imd pathway.

Transcriptional profiling of the immune response of *A. gambiae* to another alphavirus, ONNV, revealed altered expression of Imd pathway genes early after systemic infection by injection. The IκB Kinase *IKK-1*, a component of the Imd pathway, was down-regulated at 4 days after inoculation. In contrast, the AMPs *Diptericin* and *Cecropin* were modestly up-regulated (Waldock et al., 2012). However, silencing of the Imd dependent transcription factor *Rel2* did not significantly affect virus titers, suggesting a limited contribution of this pathway to antiviral defense in *Anopheles*. This observation is also consistent with the lack of a significant induction of immune genes in ONNV-infected *A. gambiae* at 14 days after infection in another study (Sim et al., 2005). Analyses of the transcriptome of *Drosophila* infected with the negative stranded (−) RNA Sigma virus revealed that two PGRPs and four Imd dependent AMPs were potently induced (Tsai et al., 2008). Nevertheless, another study found no activation of the Toll, Imd and Jak-Stat pathways (Carpenter et al., 2009). The discrepancies between these reports may be due to differences in experimental set-up, including the genetic background of the *Drosophila* strains analyzed.

3.3. Jak-Stat

A microarray analysis on DCV-infected *Drosophila* identified 138 genes that were upregulated at 24 and 48 h after infection (Dostert et al., 2005). Two thirds of these genes did not overlap with those induced upon bacterial or fungal infection. Several of the virus-induced genes contained Stat binding sites in their promoters, for example *virus induced RNA-1* (*vir-1*), the antimicrobial peptide

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Listericin/*CG9080*, and a putative GNBP-like receptor *CG12780* (Dostert et al., 2005). Different DCV strains could activate these genes, but UV-inactivated, non-infectious DCV particles or dsRNA did not (Hedges and Johnson, 2008), suggesting that active virus replication or virus-induced cellular damage and cell debris is required for activation of Jak-Stat signaling. Mutants for the Jak kinase *hop* expressed lower levels of *vir-1*, harbored higher viral loads, and succumbed faster to infection (Dostert et al., 2005). *Vir-1* null mutants, however, did not show an enhanced sensitivity to DCV infection, suggesting that *vir-1* is not a direct antiviral effector. Interestingly, the tissues with the highest *vir-1* expression were not the same as the ones with the highest virus levels, suggesting that a putative cytokine alerts non-infected cells to the presence of virus via Jak-Stat activation. The Gram-positive intracellular bacterium *Listeria monocytogenes* also induces *Listericin* expression, in a Jak-Stat and PGRP-LE dependent manner (Goto et al., 2010), suggesting that this pathway plays a role in both intracellular bacterial and viral infections.

Comparative genomic analyses of *Drosophila*, *A. gambiae*, and *A. aegypti* identified orthologs for core components of the Jak-Stat pathway (*domeless*, *hop*, *Stat*, *PIAS*, *SOCS*) in these insects (Waterhouse et al., 2007; Souza-Neto et al., 2009). The role of the Jak-Stat pathway in antiviral defense in *A. aegypti* was suggested by a transcriptome analysis that detected an up-regulation of the receptor *domeless* and three other Jak-Stat genes at 10 days after DENV infection via an infectious blood meal (Xi et al., 2008; Souza-Neto et al., 2009). In accordance, RNAi mediated knockdown of *hop*, a positive regulator of the Jak-Stat pathway, resulted in increased virus titers in the mosquito. Furthermore, knockdown of the Jak-Stat dependent, DENV-induced genes *Q1HR00/Dengue virus restriction factor 1* (*DVRF1*) and *AAEL000896/DVRF2* resulted in higher DENV replication in the midgut. Notably, Colpitts et al. (2011) demonstrated consistent down-regulation of expression of four *Cecropin* A-like AMPs and one *defensin* l-like AMPs, previously suggested to be Jak-Stat dependent (Souza-Neto et al., 2009), in DENV, WNV, and Yellow fever virus infection of *A. aegypti*.

3.4. Do Toll, Imd and Jak-Stat mediate antiviral defense in insects?

Together, the studies discussed in Sections 3.1–3.3 imply that the Toll, Imd, and Jak-Stat pathways, classically implicated in antimicrobial responses, can also contribute to antiviral defense in some virus infections in *Drosophila* and mosquito. Nevertheless, the literature remains fragmentary and in some cases even contradictory. For example, two studies obtained conflicting results regarding the induction of AMPs in Sigma virus infections of *Drosophila* (Tsai et al., 2008; Carpenter et al., 2009). Moreover, for viruses within the same family, different model systems yielded different conclusions. Within the alphavirus genus of the *Togaviridae*, a study that used transgenic flies expressing a SINV replicon implicated the Imd pathway in antiviral defense (Avadhanula et al., 2009). In contrast, neither a transcriptome analysis of SINV in blood-fed *A. aegypti* nor a functional assay in systemic ONNV infected *A. gambiae* supported a major function for Imd in the defense against alphaviruses (Sanders et al., 2005; Waldock et al., 2012). It remains unclear whether the observed differences are due to the different experimental approaches, to the unique properties of these viruses, or to the different host species. More generally, the limited consistency between infection models, inoculation routes, and other experimental conditions makes it difficult to identify consistent patterns in the induction of specific immune pathways in response to virus infection.

Standardized, systematic approaches will be required before firm conclusions can be drawn about the antiviral activity of specific pathways, especially in relation to host species, routes of inoculation, virus family, and pathogenicity. One such study analyzed the transcriptional response of *A. aegypti* to infection with three flaviviruses, DENV, WNV, and Yellow fever virus, under the same experimental conditions (Colpitts et al., 2011). A set of 35 genes were differentially expressed at 1 day after infection by all three viruses. Overlap between these viruses was more limited or even absent at later stages, suggesting that the specific response of the host may be unique for each virus. Finally, we note that although transcriptomic approaches are essential to detect inducible responses, they should be extended with functional assays using genetic mutants or other gene inactivation methods before conclusions can be drawn about the importance of specific pathways in antiviral defense.

3.5. Open questions

Many important questions remain to be addressed. First, the cellular receptors that detect the presence of a virus infection and the detected viral molecular patterns remain to be identified. Double-stranded RNA is readily detectable in cells infected with (+) RNA, dsRNA, and DNA viruses, but not in non-infected cells (Weber et al., 2006). It is therefore not surprising that this viral PAMP is a potent inducer of innate antiviral responses in mammals. However, injection of dsRNA into insects does not induce an antiviral state (Keene et al., 2004; Sanchez-Vargas et al., 2009; Saleh et al., 2009). Thus in insects, dsRNA alone does not seem to suffice to initiate an inducible immune response and additional signals may be required for a robust immune response. Indeed, induction of the antiviral gene *Vago* seems to require dsRNA sensing by Dicer-2, in addition to other virus-induced signals (Deddouche et al., 2008) (see Section 4.1). For instance, damaged cells from virus-infected tissues may act as or release danger signals that could activate an immune response (Fig. 1).

Second, potent AMP induction has only been reported in some virus infections of *Drosophila* and mosquito. Differences in replication strategy, viral tropism, route of inoculation, or other experimental conditions may partially explain the discrepancy in AMP induction between these studies. Alternatively, some viruses may encode specific antagonistic activities that inhibit the activation or signaling of the Toll and Imd pathways. Many mammalian viruses encode interferon antagonists that interfere with the pathways that lead to the production of type I interferon or with downstream effector mechanisms (Randall and Goodbourn, 2008). As a consequence, robust interferon production may not be detectable unless the viral interferon antagonist is inactivated. Therefore, a lack of AMP production should be interpreted with caution, as it could be due to viral interference with immune signaling. Indeed, there is evidence that arboviruses are able to suppress immune pathways in insects. For instance, DENV is capable of repressing the Toll and Imd-dependent induction of AMPs in response to bacterial challenge in an *A. aegypti* cell line (Sim and Dimopoulos, 2010). Similarly, Semliki Forest virus (SFV) inhibited reporter gene expression under Toll, Imd and Jak-Stat responsive promoters in *Aedes albopictus* derived cells after activation by a constitutively active Toll construct (Toll) or by heat-inactivated *E. coli* (Imd and Jak-Stat), respectively (Fragkoudis et al., 2008). SFV induces a global inhibition of host gene expression in these cells, which may contribute to the inhibition of inducible immune responses (Fragkoudis et al., 2008). Whether suppression of inducible immune responses is a general strategy of arboviruses and how these activities contribute to virus transmission by mosquitoes remains to be established.

Interestingly, viral antagonism of NF-κB activity has also been reported in non-Dipteran insects. Polydnaviruses, such as *Microplitis demolitor* bracovirus that infects the parasitoid wasp *M. demolitor*, encode inhibitory proteins (H4 and N5) that are homologous to the IκB factors that inhibit of NF-κB activation. These viral IκB

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mimics bind to Dif and Relish and thereby inhibit Toll and Imd-dependent AMP production (Thoetkiattikul, 2005; Falabella et al., 2007; Bitra et al., 2012). These results imply that NF-κB dependent pathways are activated in the parasitized host, but that polydnaviral NF-κB antagonists prevent expression of downstream effectors.

Third, the downstream antiviral effector of the Toll, Imd and Jak-Stat pathways remain to be identified. It is unclear whether AMPs directly contribute to antiviral defense in *Drosophila*. Several studies, however, have addressed this question in mosquitoes. High-throughput sequencing of the *A. aegypti* salivary gland transcriptome in response to DENV infection revealed a strong induction of a gene belonging to the *Cecropin* family (*AAEL000598*). The corresponding (chemically synthesized) peptide inhibited DENV replication in *A. albopictus* cells and Chikungunya virus replication in a human cell line (Luplertlop et al., 2011). Ectopic expression of *Defensin-A* and *Cecropin-A* in the fat body of transgenic *A. aegypti* mosquitoes also inhibited replication of DENV, both in midgut and fat body (Luplertlop et al., 2011; Pan et al., 2011). An antiviral activity of AMPs or other downstream targets is also suggested by the observation that pretreatment of *A. albopictus* cells with heat-inactivated bacteria, which activate the Imd and Jak-Stat pathways, inhibited replication of SFV (Fragkoudis et al., 2008). Thus, AMPs seem to participate in antiviral responses in mosquitoes, yet, their mechanism of action remains unclear. AMPs in *Drosophila* directly target microbial invaders by disrupting bacterial and fungal membranes. This mechanism is unlikely to be active against non-enveloped viruses, such as CrPV and DCV, but may be activate against enveloped viruses. Vertebrate AMPs (Defensins) can inhibit virus replication through various other means, such as inhibiting cell attachment or virus entry (Ding et al., 2009), and similar mechanisms may also play a role antiviral immunity in insects.

4. Alternative inducible antiviral responses

4.1. Vago

Vago is one of the genes with the highest induction upon DCV infection of *Drosophila*, but it does not contain a Stat-binding site in its promoter (Dostert et al., 2005). This gene encodes a 160 amino acid peptide that is mainly expressed in the fat body, the main target organ for DCV (Dostert et al., 2005). Viral replication is enhanced in the fat body of *Vago* null mutants, which suggests a direct role for *Vago* in restricting virus infection (Deddouche et al., 2008). Strikingly, *Vago* induction depends on the DExD/H-box helicase domain of Dicer-2, but not on the Toll, Imd and Jak-Stat signaling pathways. Dicer-2 is the ribonuclease that processes viral dsRNA into small interfering (si) RNAs to initiate an antiviral RNAi pathway. Yet, Argonaute 2 and R2D2, other central actors of the RNAi pathway, are not involved in *Vago* induction. These results suggest that Dicer-2 is a sensor for viral dsRNA that not only initiates an antiviral RNAi response, but also activates an inducible antiviral response. Strikingly, the DExD/H-box helicase domain of Dicer-2 is similar to the helicase domain of the RIG-I like helicases RIG-I and MDA-5 that sense viral RNA and initiate an interferon response in mammals. This is likely due to convergent evolution, as *Vago* does not seem to be evolutionary conserved in other insects, such as *Aedes*, *Anopheles*, or *Culex* species.

4.2. Autophagy pathway

Autophagy is a mechanism for degradation of cell components by lysosomes that is essential for growth and homeostasis. The interaction between viruses and the autophagy pathway is complex. Some viruses exploit the pathway for production of membranous replication organelles or for non-lytic virus secretion (Kirkegaard, 2009). In plants, autophagy is a well-known defense mechanism (Seay et al., 2009), but its role in antiviral immunity in animals has only recently been demonstrated in Herpes simplex virus and SINV infection in mice, and in VSV infection in *Drosophila* (Dreux and Chisari, 2010; Yordy and Iwasaki, 2011). VSV is a (−) RNA arbovirus from the *Rhabdoviridae* family that, under experimental conditions, also infects *Drosophila* (Shelly et al., 2009). Adult flies deficient for components of the autophagy pathway support increased levels of VSV replication. As a consequence, mutant flies succumb to VSV infection, whereas wild-type control flies do not. The detection of the VSV-G glycoprotein by an unknown receptor results in downregulation of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway that under normal nutritional conditions represses autophagy. Thereby, VSV mimics a starvation state that favors autophagy in virus-infected cells. More recently, it was demonstrated that VSV binds Toll-7, a member of the *Drosophila* Toll family, at the cell surface, thereby activating autophagy (Nakamoto et al., 2012).

A role for autophagy is also suggested by the observation that a polymorphism in the *Drosophila ref(2)p* gene confers resistance against Sigma virus, another member of the *Rhabdoviridae* family (Dru et al., 1993; Contamine et al., 1989). Intriguingly, *ref(2)p* is involved in autophagy (Nezis et al., 2008; Taillebourg et al., 2012) but also in Toll dependent signaling (Avila et al., 2002). Nevertheless, direct evidence that the antiviral effect of *ref(2)p* is mediated by its role in autophagy is thus far lacking. In contrast to the observations in VSV-infected flies, the PI3K-Akt-Tor pathway seems to be proviral in SINV infection of *Drosophila* and *A. albopictus*. Moreover, SINV infection activates this pathway, thereby increasing viral replication and promoting cell survival (Patel and Hardy, 2012).

4.3. Heat-shock response

The alphavirus O'nyong nyong virus (ONNV) is the only known arbovirus that is transmitted by *A. gambiae*. A microarray analysis of blood-fed ONNV infected *A. gambiae* did not detect an induction of the Toll, Imd, and Jak-Stat pathways, but identified *Hsc70B* as the gene with the strongest induction at 14 days after infection (Sim et al., 2005). Hsp70B is a chaperone protein of the heat-shock protein family that can be induced by a variety of stresses. Knockdown of Hsp70B increased ONNV replication in head, thorax, and abdomen, suggesting an important role in control of virus infection (Sim et al., 2007). Interestingly, a proteomic analysis on FHV infected *Drosophila* S2 cells also revealed robust induction of the heat-shock proteins Hsp23 and Hsp27 (Go et al., 2006). Heat-shock responses are known to play various roles in virus infections in mammals (reviewed by Santoro et al., 2010). For example, heat-shock proteins may constitute a danger signal that initiates innate immune responses (Matzinger, 2002). The role of the heat-shock response in insect immunity awaits further characterization. Strikingly, the Hsp70/Hsp90 machinery is critical in loading of siRNAs into the RNAi effector complex RISC (RNA-induced silencing complex) in flies (Iwasaki et al., 2010). An intriguing and testable hypothesis therefore is that the induction of Hsp70 upon virus infection facilitates loading of RISC with viral siRNAs, thereby strengthening the antiviral RNAi response.

5. Contribution of symbionts to antiviral immune defenses

5.1. Wolbachia

Wolbachia is an obligate intracellular Gram-negative bacterium from the Rickettsiales order that infects ~65% of invertebrate species, including nematodes, crustaceans and insects (Werren et al.,

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2008). Its high prevalence is based on the remarkable ability to manipulate the reproductive system of the host via cytoplasmic incompatibility. This mechanism provides a selective reproductive advantage to infected females ensuring efficient spread of *Wolbachia* in the population (Serbus et al., 2008). Many wild populations, as well as laboratory stocks of *D. melanogaster* carry *Wolbachia* (Clark et al., 2005). Strikingly, *Wolbachia* infected flies are less sensitive than *Wolbachia*-free flies to infection with the RNA virus DCV, which is associated with a reduction in viral titers (Hedges and Johnson, 2008; Taillebourg et al., 2012). *Wolbachia* also provides protection against FHV and Nora virus-induced mortality, but this is not associated with a decrease in viral titers (Teixeira et al., 2008). *Wolbachia* does not increase resistance to experimental infection with the DNA virus Invertebrate iridescent virus 6 (Teixeira et al., 2008), but it remains to be established whether the same holds true for a natural DNA virus pathogen of *Drosophila*. Additionally, some, but not all *Wolbachia* strains protect *Drosophila simulans* against DCV and FHV infection, suggesting an evolutionary conserved, but strain specific mechanism for *Wolbachia* mediated antiviral protection (Osborne et al., 2009).

Whereas *Wolbachia* induced viral resistance may partially explain the evolutionary success of the symbiont, its mechanism remains unclear. An intriguing hypothesis is that *Wolbachia* instructs the immune system to respond faster and more potently to a microbial challenge (immune priming). However, the lack or low induction of AMP expression in *D. melanogaster* (Wong et al., 2011) or *D. simulans* (Bourtzis et al., 2000) infected with *Wolbachia* argues against this hypothesis. In accordance, two different strains of *Wolbachia* (*w*MelPop or *w*Mel) inhibited DENV replication in *Drosophila*, but did not induce consistent changes in expression of AMPs and other immune genes (Rancès et al., 2012). Moreover, *Wolbachia* did not alter survival after challenge with several bacteria, including *Pseudomonas aeruginosa*, *Serratia marcescens* and *Erwinia carotovora* (Wong et al., 2011). These results suggest that the Toll and Imd pathways and downstream AMPs do not mediate *Wolbachia* induced viral resistance in *Drosophila*.

Surprisingly, *Wolbachia* does not naturally infect *A. gambiae* and *A. aegypti*, although it is found in other mosquito species. Nevertheless, *Wolbachia* inhibited DENV midgut replication and subsequent dissemination to thorax and head in *A. aegypti* (Bian et al., 2010). Infection of *A. aegypti* with the more virulent strain, *w*MelPop-CLA, reduced the lifespan of the mosquitoes by 50% (McMeniman et al., 2009) and limited the replication of DENV, Chikungunya virus, and *Plasmodium gallinaceum* (Moreira et al., 2009). As the specific responses against pathogens as diverse as RNA viruses and eukaryotic parasites are likely to be very dissimilar, these results suggest that *Wolbachia* interferes with global immune responses. Indeed, transcriptome analyses showed that *Wolbachia* induces several immune genes in *A. aegypti*, including components of the Toll (*PGRP-SA*, *GNBP*, *Rel1*), Imd (*Rel2*), Jak-Stat (*SOCS36E*), and melanization pathways, as well as their downstream effectors, such as AMPs (*Cecropin* and *Defensin*) and thio-ester containing proteins (Xi et al., 2008; Kambris et al., 2009; Moreira et al., 2009; Rancès et al., 2012). Induction of the Toll, Imd and Jak-Stat pathways by *Wolbachia* is not well understood. Toll activation seems to be mediated through the production of reactive oxygen species (ROS) and activation of the NADPH oxidase upon infection by the bacterium (Pan et al., 2011). These results are in striking contrast to those obtained in *Drosophila*, in which these pathways were not activated (Bourtzis et al., 2000; Wong et al., 2011). The lack of co-evolution with the symbiont may provide a partial explanation for activation of inducible immune pathways by *Wolbachia* in mosquitoes.

Competition between bacterium and virus for essential resources may provide an alternative explanation for the antiviral effect of *Wolbachia*. Indeed, *Wolbachia* invades several tissues in *A. aegypti*, including brain and fat body, which are also target organs for DENV. In *Wolbachia*-infected mosquitoes, DENV levels in these tissues were strongly reduced, which may suggest a cell-autonomous antiviral effect (Moreira et al., 2009). Moreover, *Wolbachia* is unable to synthetize cholesterol and has to obtain it from host cells (Lin and Rikihisa, 2003; Wu et al., 2004). Since cholesterol is also required for flavivirus and alphavirus replication, the presence of *Wolbachia* could compete for cholesterol, thereby restricting DENV infection and pathogenesis (Lu et al., 1999; Mackenzie et al., 2007). Two lines of evidence exclude a role for the RNAi pathway in *Wolbachia* mediated protection. *Wolbachia* inhibits DENV replication in the *A. albopictus* derived C6/36 cell line that lacks functional *Dicer-2* expression (Frentiu et al., 2010). Furthermore, *Wolbachia* infection renders *Argonaute-2* mutant flies more resistant to WNV infection (Glaser and Meola, 2010).

Wolbachia mediated protection from arbovirus infection inspired the development of a new DENV control strategy that is based on a population replacement approach. In 2011, a groundbreaking field trial demonstrated the successful replacement of a wild *A. aegypti* population with *Wolbachia* infected mosquitoes with reduced susceptibility to DENV (Hoffmann et al., 2011; Walker et al., 2011). Other organisms that boost the mosquito's immune response, such as the entomopathogenic fungus *Beauveria bassiana*, might be exploited in a similar fashion to limit transmission of arboviruses (Dong et al., 2011).

5.2. Gut microbiome

The gut lumen of mammals and insects contains a dense population of mutualistic bacteria, referred to as the gut microbiome. This bacterial community plays crucial roles in host physiology, such as nutrition and digestion (Dillon and Dillon, 2004), immune system maturation (Heller, 2011; Weiss et al., 2011), insulin-signaling and metabolic homeostasis (Shin et al., 2011; Storelli et al., 2011; Leulier and Royet, 2009), and mating preference (Sharon et al., 2010). In *Drosophila*, the resident gut bacteria activate Imd signaling in intestinal epithelial cells. As a consequence, the transcription factor Relish localizes to the nucleus; nevertheless, only very low levels of AMPs are produced. The homeobox gene *Caudal* represses NF-κB dependent AMP expression, preventing clearance of the microflora from the gut lumen (Ryu et al., 2008). Knockdown of *Caudal* results in AMP up-regulation, a modification of the resident bacterial population, and a predominance of a pathogenic bacterium, resulting in gut apoptosis and pathology. This and other studies imply that negative regulation of the Imd pathway is crucial for maintenance of the equilibrium of the gut microbiome (Zaidman-Remy et al., 2006; Lhocine et al., 2008; Leulier and Royet, 2009; Lee and Ferrandon, 2011; Storelli et al., 2011).

Several studies suggest a role for the gut microbiome in arbovirus infection and transmission by mosquitoes. Elimination of the gut bacteria by antibiotic treatment results in higher DENV loads in *A. aegypti* midguts (Xi et al., 2008). Several bacterial species of the microbiome contribute to virus control in the midgut, possibly through activation of immune pathways and induction of AMPs. In return, DENV indirectly diminishes the overall bacterial load, likely through up-regulation of AMP expression, creating an intricate tripartite relationship between mosquito, virus, and gut bacteria (Ramirez et al., 2012). Two recent reports highlight the importance of the gut microbiome in virus infection in mice, although in these cases, the presence of gut bacteria enhanced virus transmission, replication and pathogenesis (Kane et al., 2011; Kuss et al., 2011).

Together, these studies imply that the gut microbiome is not merely a passive commensal population with limited functions in digestion, but that it also contributes to local and, perhaps, systemic immune responses. Gut bacteria influence local AMP production by epithelial cells, as well as systemic AMP secretion by the fat

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body (Ryu et al., 2008; Wu et al., 2012). This suggests the existence of cross-talk between these organs, possibly mediated by motile cells or secreted factors (Rodrigues et al., 2010; Wu et al., 2012). Understanding these interactions may provide opportunities to manipulate virus infection in insect vectors.

6. Concluding remarks

In recent years, important progress has been made in our understanding of antiviral immunity in insects. RNAi seems to be the major antiviral strategy of *Drosophila* and mosquitoes. Nevertheless, several lines of evidence suggest that insects also mobilize other immune defenses to fight virus infection. Unlike the RNAi pathway, these immune responses are inducible and seem tailored to each virus infection, perhaps depending on route of infection, viral tropism, and pathogenicity. This adds an unexpected and poorly understood layer of complexity to antiviral innate immunity in insects.

The immune system is historically defined by its ability to detect and eliminate pathogens, thereby protecting the host from excessive pathology. Medzhitov et al. (2012) recently proposed a complementary view on host defense that is based on the reduction of the pathogenic effects of microbes without an associated reduction in pathogen burden, which the authors defined as tolerance. Arboviruses are considered to be non-pathogenic to their vector. The notion of tolerance may provide a fruitful conceptual framework for understanding arbovirus infection of the vector.

Obtaining a more comprehensive view of inducible antiviral immune responses, understanding the mechanisms underlying their induction, identifying downstream effector molecules, and evaluating their contribution to vector competence are major challenges of the field. Moreover, the immunomodulatory activity of symbionts, such as intracellular bacteria and gut microflora, awaits further investigation and may provide important leads towards understanding antiviral immunity in Diptera. A further dissection of insect immunity through a combination of fly genetics and functional analyses in arbovirus infected mosquitoes will hopefully provide novel opportunities to control the spread of arboviruses.

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