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The Toll and Imd Pathways Are Not Required for *Wolbachia*-Mediated Dengue Virus Interference

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***Wolbachia* blocks dengue virus replication in *Drosophila melanogaster* as well as in *Aedes aegypti*. Using the *Drosophila* model and mutations in the Toll and Imd pathways, we showed that neither pathway is required for expression of the dengue virus-blocking phenotype in the *Drosophila* host. This provides additional evidence that the mechanistic basis of *Wolbachia*-mediated dengue virus blocking in insects is more complex than simple priming of the host insect innate immune system.**

The common intracellular bacterium *Wolbachia pipientis* (1) is maternally inherited through the eggs of its insect hosts. It is able to successfully invade host populations through a range of reproductive manipulations that either directly or indirectly favor its transmission between insect generations (2–8). In its natural host, it has recently been shown that the presence of *Wolbachia* can block the replication of RNA viruses (9–13). This effect is the basis for the recent development of *Wolbachia* as a biocontrol approach to block dengue virus (DENV) transmission by the mosquito *Aedes aegypti* (14, 15).

The main vector of DENV, the mosquito *Aedes aegypti*, is not naturally infected with *Wolbachia*. However, different strains of *Wolbachia* have recently been artificially introduced from *Drosophila melanogaster* (*Wolbachia* strains *w*Mel and *w*MelPop) or *Aedes albopictus* (*Wolbachia* strain *w*AlbB) into *A. aegypti* and are stably maintained in laboratory and wild mosquito populations (8, 16–18). The expectation is that the negative impact that *Wolbachia* has on DENV replication in the insect will reduce virus transmission to humans and subsequent disease (18–20).

The mechanism(s) that underlies the ability of *Wolbachia* to affect the replication of DENV appears complex. Using the heterologous association of *Wolbachia*-infected *A. aegypti*, transcriptomic and biochemical studies have demonstrated that *Wolbachia* induces the production of reactive oxygen species (ROS); primes the innate immune system of the mosquito, especially the Toll signaling pathway; and induces the production of various antimicrobial effectors (19–23). In addition, the use of RNA interference (RNAi) depletion to partially knock down defensin and cecropin genes in *Wolbachia*-infected *A. aegypti* lowered resistance to DENV and suggested a role for the innate immune system in mediating virus resistance (22). In contrast, in their natural host *D. melanogaster*, the same *Wolbachia* strains do not induce overexpression of immune genes, including the Toll pathway and cecropin- and defensin-encoding genes, yet RNA virus interference, including DENV interference, occurs (23, 24). These results demonstrated that induction of the Toll pathway by *Wolbachia* is not the exclusive mechanism mediating resistance. However, gene expression studies are not sufficient to make a link between a phenotype and a genetic pathway. Since previous studies confirmed the ability of DENV to replicate, and of *Wolbachia* to block its replication in *Drosophila* (23), we took advantage of preexisting and well-characterized mutant fly strains lacking functional Toll or Imd pathways to determine a precise role for these pathways in the *Wolbachia*-mediated viral blocking phenotype.

Role of the Toll and Imd pathways in controlling *Wolbachia w*MelPop density and tissue tropism. In order to characterize the role of the Toll and Imd pathways in the phenotype of *Wolbachia*-mediated viral blocking, we introduced the *Wolbachia* strain *w*MelPop into *Drosophila* lines carrying loss-of-function mutations in genes essential to the two pathways. For the Toll pathway, strong hypomorphic mutations of *spätzle* (*spz*) were tested (spz^2 and spz^4). For the Imd pathway, a null allele of *relish* (rel^{E20}) was used (25, 26). These particular alleles of *spz* and *rel* were used as they have been well documented to cause pathogen susceptibility due to reduced production of antimicrobial effector peptides such as cecropins and defensins (25, 26). Prior to introduction of *w*MelPop into the different mutant strains, we tested each for the presence of *Wolbachia* using *Wolbachia*-specific PCR, targeting the *wsp* gene that encodes the major surface protein of the bacterium (27). All strains were determined to be uninfected with the exception of the original spz^4 strain, which was positive for *Wolbachia* infection. Further characterization by sequencing the *wsp* amplicon showed that it was identical to the *wsp* sequence of *w*Mel, a *Wolbachia* strain known to commonly infect *D. melanogaster* (7). Since *Wolbachia* is only maternally transmitted, we could use simple crosses to establish *w*MelPop and *w*Mel single-infected lines in different host genetic backgrounds. To generate *w*MelPop-infected balancer lines, *D. melanogaster* w^{1118}*w*MelPop virgin females were crossed with virgin TM3/TM6B males (w^{1118} background). *w*MelPop-infected TM6B/+ virgin females were crossed with *w*MelPop-infected TM3/+ males to generate the *w*MelPop-infected TM3/TM6B balancer line. The *Wolbachia*-infected mutant lines were established by crossing *w*MelPop-infected TM3/TM6B virgin females with males of the Toll and Imd mutant lines. The spz^2*w*MelPop, spz^4*w*MelPop, spz^4*w*Mel, and

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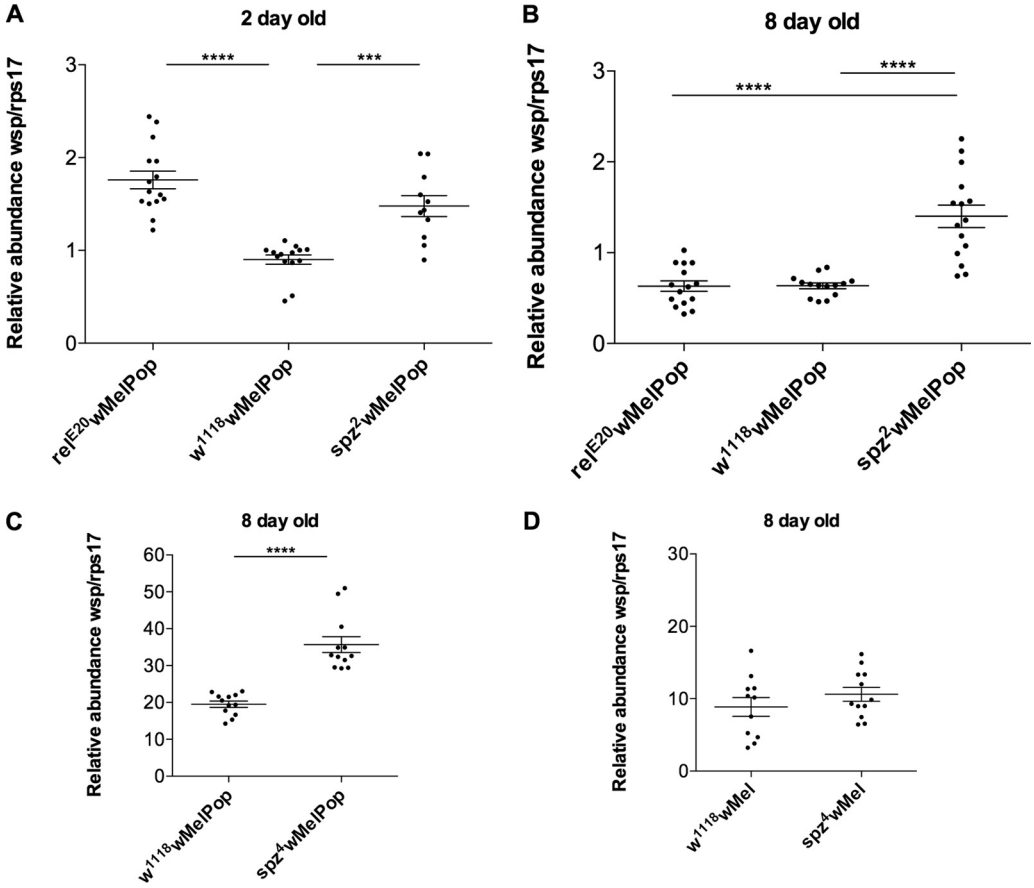

FIG 1 *Wolbachia* density in *Drosophila* lines deficient for Toll and Imd pathways. (A and B) *w*MelPop relative density in 2 (A)- and 8 (B)-day-old virgin females deficient for Imd (rel^{E20}*w*MelPop) and Toll (spz^{2}*w*MelPop) pathways and the control line (w^{1118}*w*MelPop). (C and D) *w*MelPop (C) and *w*Mel (D) relative density in 8-day-old virgin females deficient for Toll pathway (allele spz^{4}). $n = 11$ to 15; Mann-Whitney *U* test; ***, $P < 0.001$; ****, $P < 0.0001$.

rel^{E20}*w*MelPop flies were maintained as heterozygotes balanced and as homozygotes for each experiment. The tetracycline-cured lines were derived by the addition of tetracycline (0.3 mg/ml) to the larval diet for two generations and confirmed to be free of *Wolbachia* by PCR (28). The *w*MelPop-infected mutant lines and their uninfected counterparts were confirmed to be homozygous mutant for the different Toll and Imd pathway alleles by bacterial challenge using appropriate pathogens (data not shown; see also reference 29).

Previous studies suggest that the magnitude of virus blocking and *Wolbachia* density are positively correlated (30, 31). Moreover, the *w*MelPop strain of *Wolbachia* is known to overreplicate in host tissues, causing pathology and ultimately reducing the life span of its host (17, 32). Given these observations, we first examined the impact of both the Toll and Imd pathways on *Wolbachia* density and tissue tropism before testing DENV replication in *w*MelPop-infected mutant backgrounds. To assay *Wolbachia* density, 15 virgin females from each line were maintained in vials under controlled conditions, at 26°C with 60% relative humidity and a 12-h light/dark cycle. DNA was extracted from 9 to 15 individual females either 2 or 8 days posteclosion, using the ReliaPrep gDNA Tissue Miniprep system (Promega), according to the manufacturer's instructions. *Wolbachia* density was then determined by relative quantitative PCR (qPCR) by comparing the abundance of the single-copy *Wolbachia* surface protein gene (*wsp*) to that of the single-copy *D. melanogaster rps17* gene as previously described (18). Data were analyzed using Mann-Whitney *U* tests (GraphPad Prism 5). Three independent experimental replicates were performed to confirm the results obtained with rel^{E20} and spz^{2} mutants. Surprisingly, we found a significant increase in *w*MelPop density (approximately 2-fold) in both mutants (rel^{E20} and spz^{2}) in 2-day-old flies compared to the wild-type line w^{1118}*w*MelPop (Fig. 1A). However, in 8-day-old flies only the Toll signaling pathway mutant spz^{2} exhibited a significant difference in *w*MelPop density for all three replicates (Fig. 1B). Similar results were obtained with another genetic background for loss of function of Toll pathway, using 2- and 8-day-old spz^{4} mutants (Fig. 1C). This suggests that Toll and Imd pathways both have a role in modulating *w*MelPop density.

We took advantage of having spz^{4} mutants infected with a different strain of *Wolbachia* to test whether the impact of the Toll signaling pathway on *w*MelPop density extended to other strains of *Wolbachia*. No significant difference in *w*Mel density was observed between 8-day-old virgin females deficient for Toll pathway and the wild-type line w^{1118}*w*Mel (Fig. 1D). This suggests that only the overreplicative strain *w*MelPop is affected.

We then verified whether the observations made with *w*MelPop were linked with its tissue tropism in *Drosophila* strains deficient for Toll and Imd pathways. Fluorescence *in situ* hybridizations (FISH) to detect *Wolbachia* in tissues were made on paraffin sec-

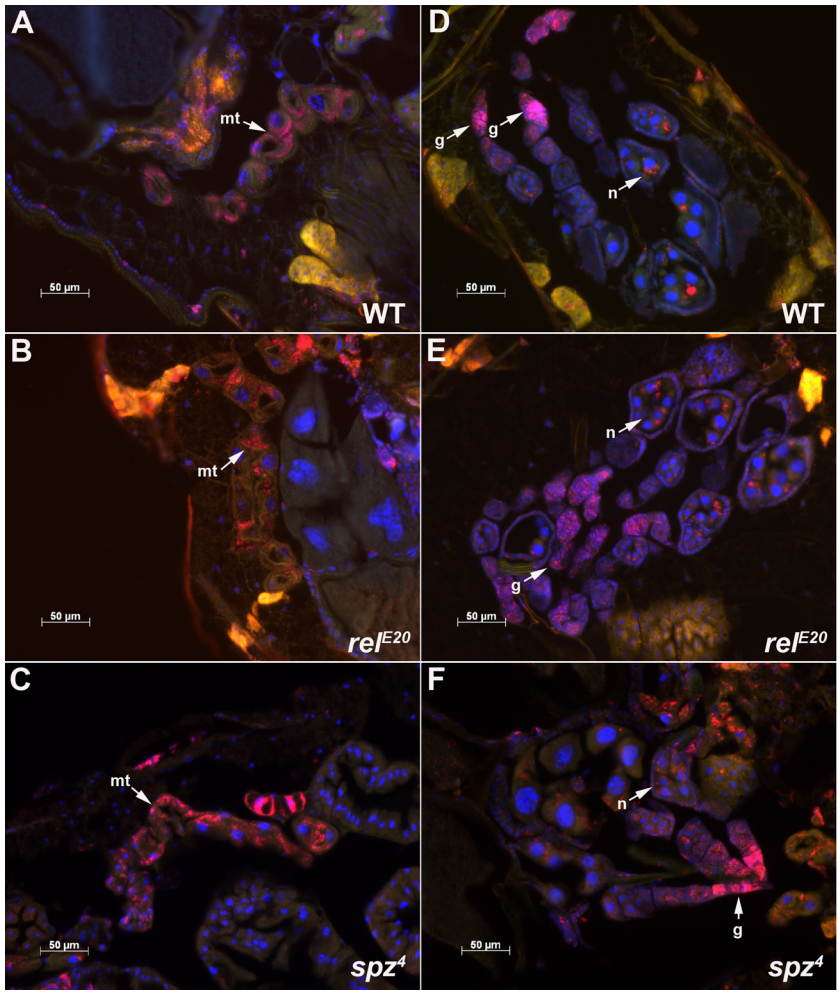

FIG 2 *Wolbachia* tissue tropism in *Drosophila* lines deficient for Toll and Imd pathways. *Wolbachia* is shown in red and DNA in blue. (A to C) *Wolbachia* localization in Malpighian tubules (mt) from w^{1118}*w*MelPop (WT), rel^{E20}*w*MelPop, and spz^{4}*w*MelPop flies. (D to F) Ovary sections, showing the localization of *Wolbachia* in the germarium (g) and in nurse cells (n) in developing oocytes.

tions of spz^{2}*w*MelPop, spz^{4}*w*MelPop, rel^{E20}*w*MelPop, and wild-type w^{1118}*w*MelPop females, as described previously (19). No difference in *Wolbachia* localization was observed between the different lines in 8-day-old flies (Fig. 2).

Role of the Toll and Imd pathways in the *Wolbachia*-mediated DENV-blocking phenotype. *D. melanogaster* has already been shown to be a good model for studying interactions between human viruses or parasites (including DENV) and innate immunity (33, 34). In a previous study, we artificially infected *D. melanogaster* with DENV, showing that the virus can replicate in *Drosophila* and, secondly, that *Wolbachia* interferes with this replication in flies (23). Using *Drosophila* as a model for DENV infection allows both utilization of the natural *Wolbachia* host and access to the genetic tools of this model species to gain a deeper understanding of the complexity of the *Wolbachia*-induced phenotype. After confirmation that the different *Drosophila* mutants used for this study retained *Wolbachia* infection with a cellular tropism similar to that for the wild type, we measured the impact of *spätzle* and *relish* loss of function on the DENV-*Wolbachia* interaction.

Two-day-old *Drosophila* females, lines spz^{2}*w*MelPop and rel^{E20}*w*MelPop and their uninfected counterparts, spz^{2}tet and rel^{E20}tet, were intrathoracically injected with 69 nl of DENV-2 suspension (strain ET300, 2.7×10^{7} PFU/ml). Virus propagation and injection were performed as described previously (23, 35). Each experiment was repeated independently 2 to 3 times with 15 females injected per line. After injection, flies were maintained under identical controlled conditions; low density (15 females per vial), 26°C, 60% relative humidity, and 12-h light/dark cycle. Insects were collected 6 days postinjection and kept at −80°C until RNA extraction. Accumulation of genomic (+RNA) RNA strands was assessed by quantitative real-time PCR (qRT-PCR) using hydrolysis probes (TaqMan) specific to the 3′ untranscribed region (UTR) of the four serotypes of DENV (36). Only individuals with detectable levels of DENV infection were used to examine the effect of *w*MelPop on virus titer using Mann-Whitney *U* tests (GraphPad Prism 5). Regardless of the loss of function of Toll (spz^{2}) and Imd (rel^{E20}) pathways, *Wolbachia* still dramatically reduced DENV replication in flies (Fig. 3A). The Toll pathway result was further confirmed with a second mutant allele, spz^{4} (Fig. 3B). The number of DENV copies is approximately 6 times higher in spz^{4}tet than in w^{1118}tet, in which the Toll pathway is intact

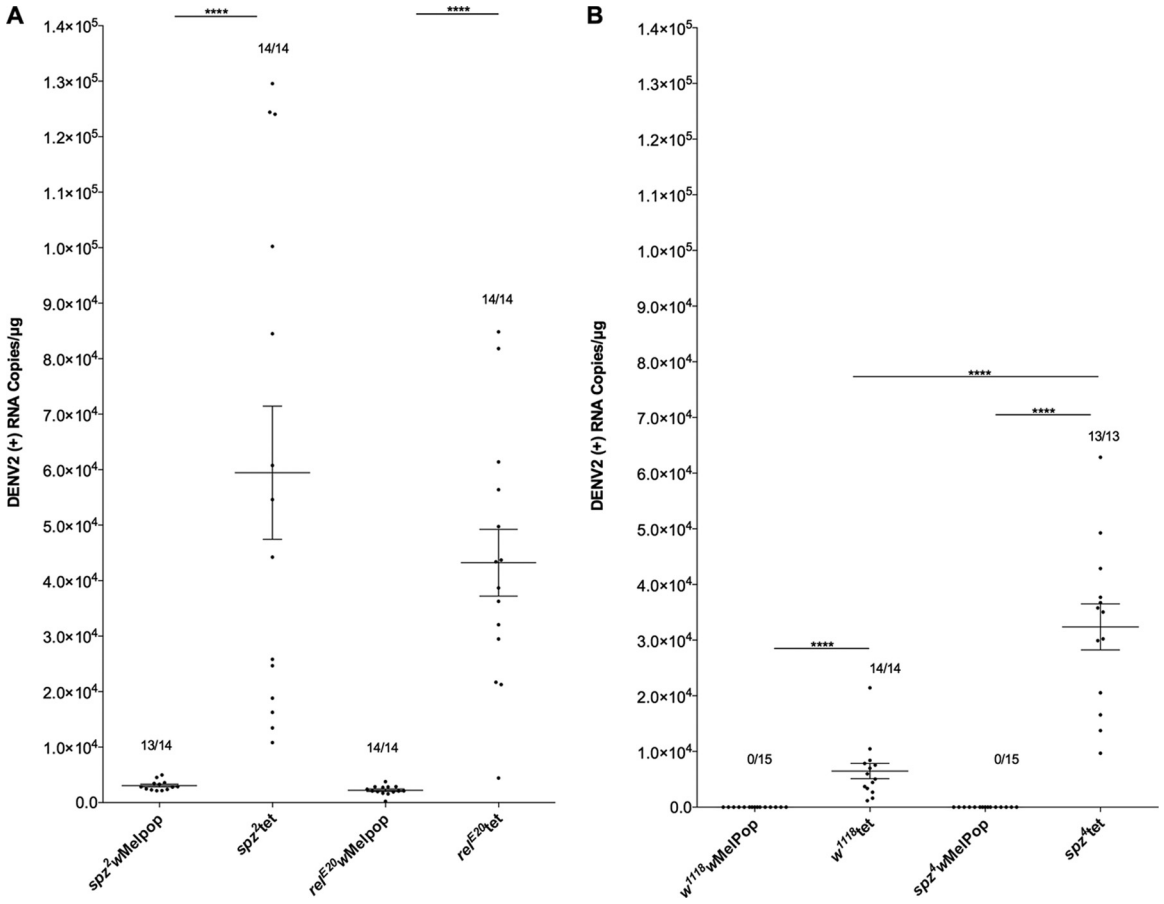

FIG 3 Dengue virus blocking in *Wolbachia*-infected *Drosophila* lines deficient for Toll and Imd pathways. (A) DENV-2 was injected into 2-day-old female flies, spz^2*w*MelPop and rel^{E20}*w*MelPop, and their tetracycline-treated uninfected counterparts, spz^2tet and rel^{E20}tet. (B) DENV-2 was injected into 2-day-old females, spz^4*w*MelPop and w^{1118}*w*MelPop control flies, and their tetracycline-treated counterparts, spz^4tet and w^{1118}tet. Total DENV-2 in whole female *Drosophila* (n = 13 to 15) was measured 6 days postinjection by qRT-PCR using a TaqMan assay specific to DENV in 1 μg of total RNA. The fraction of flies that had detectable DENV-2 infections is shown above each set of data points. Mann-Whitney U test; ****, $P < 0.0001$.

(Fig. 3B). These results indicate that the Toll pathway, independently of *Wolbachia* infection status, influences DENV replication in *Drosophila*, as has been described previously in *A. aegypti* (37), reinforcing the relevance of this model to studying DENV-*Wolbachia* interactions. This study clearly demonstrates that both Toll and Imd pathways are not required for viral replication blocking by *Wolbachia*.

Conclusion. Utilizing *Drosophila* mutants for key regulatory genes of both the Toll and Imd pathways, we showed a clear interaction with infection density of the pathogenic *Wolbachia* strain *w*MelPop. Considering that this effect was not shared with the nonpathogenic *w*Mel strain that grows to lower densities within the fly, it is possible that this effect is a response of the fly to the pathology and overreplication associated with this infecting *Wolbachia* strain. This is the first evidence to suggest an active role by the host insect in regulating *Wolbachia* densities. Our results show that the mutant genetic backgrounds do not negatively impact on *Wolbachia* densities in the host, by decreasing *Wolbachia* infection, which could confound interpretation of virus-blocking effects. Our results clearly demonstrate that functional Toll and Imd pathways are not required for the DENV interference phenotype to be expressed in *w*MelPop-infected flies. Other work done in *Drosophila* has shown that the antiviral small interfering RNA pathway is not involved either (38). It would be interesting to look at the impact of *Wolbachia* on dengue virus replication using *Drosophila* mutants for other immune pathways such as the JAK-STAT pathway (39).

This study provides further evidence that the mechanism of DENV blocking is likely to be more complicated than a simple priming of the insect innate immune system (23). Two recent studies support this notion: *Wolbachia* and *Drosophila* C virus compete for cholesterol, resulting in a delay in virus-induced mortality for *Wolbachia*-infected flies (40), and upregulation of an *A. aegypti* methyltransferase gene by *Wolbachia* contributes to dengue virus inhibition (41). These results are important in an applied context of utilizing *Wolbachia* infections for dengue virus control. If the mechanism of interference has a complex basis, then there is a reasonable expectation that the development of resistance by the virus may be slower than if a single interference mechanism is involved.

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