

Wolbachia induces reactive oxygen species (ROS)-dependent activation of the Toll pathway to control dengue virus in the mosquito *Aedes aegypti*

Xiaoling Pan^a, Guoli Zhou^a, Jiahong Wu^{a,b}, Guowu Bian^{a,c}, Peng Lu^a, Alexander S. Raikhel^{d,e,1}, and Zhiyong Xi^{a,c,1}

^aDepartment of Entomology and Genetics Program, Michigan State University, East Lansing, MI 48824; ^bDepartment of Parasitology, Guiyang Medical College, Guiyang, Guizhou 550004, China; ^cSun Yat-sen University - Michigan State University Joint Center of Vector Control for Tropical Diseases, Guangzhou, Guangdong 510080, China; and ^dDepartment of Entomology and ^eInstitute for Integrative Genome Biology, University of California, Riverside, CA 92521

Contributed by Alexander S. Raikhel, October 18, 2011 (sent for review August 24, 2011)

***Wolbachia* are maternally transmitted symbiotic bacteria that can spread within insect populations because of their unique ability to manipulate host reproduction. When introduced to nonnative mosquito hosts, *Wolbachia* induce resistance to a number of human pathogens, including dengue virus (DENV), *Plasmodium*, and filarial nematodes, but the molecular mechanism involved is unclear. In this study, we have deciphered how *Wolbachia* infection affects the *Aedes aegypti* host in inducing resistance to DENV. The microarray assay indicates that transcripts of genes with functions related to immunity and reduction-oxidation (redox) reactions are up-regulated in *Ae. aegypti* infected with *Wolbachia*. Infection with this bacterium leads to induction of oxidative stress and an increased level of reactive oxygen species in its mosquito host. Reactive oxygen species elevation is linked to the activation of the Toll pathway, which is essential in mediating the expression of antioxidants to counterbalance oxidative stress. This immune pathway also is responsible for activation of antimicrobial peptides—defensins and cecropins. We provide evidence that these antimicrobial peptides are involved in inhibition of DENV proliferation in *Wolbachia*-infected mosquitoes. Utilization of transgenic *Ae. aegypti* and the RNAi depletion approach has been instrumental in proving the role of defensins and cecropins in the resistance of *Wolbachia*-infected *Ae. aegypti* to DENV. These results indicate that a symbiotic bacterium can manipulate the host defense system to facilitate its own persistent infection, resulting in a compromise of the mosquito's ability to host human pathogens. Our discoveries will aid in the development of control strategies for mosquito-transmitted diseases.**

antiviral resistance | innate immunity | population replacement

Diseases transmitted by hematophagous arthropod vectors, such as mosquito-borne malaria and dengue fever, cause 1.5 million human deaths every year (1). The insufficiency of currently available control strategies, such as vaccines, drugs, and pesticides, has led to a dramatic increase in occurrence of vector-borne diseases (2, 3). Presently, two-fifths of the world's population are at risk from dengue fever, and 50–100 million individuals are infected each year (2). Significant efforts have been devoted to developing novel strategies for dengue control. One such approach involves making the mosquito host inhospitable to dengue virus (DENV) through population replacement, with the goal of impeding or halting disease transmission.

Wolbachia (Rickettsiales) species are reproductive parasites or endosymbionts of arthropods and nematodes. A unique feature of *Wolbachia* biology in many arthropods is their ability to spread through host populations by means of a reproductive mechanism referred to as “sperm–egg cytoplasmic incompatibility” (CI) (4). CI results in early embryonic death when a *Wolbachia*-infected male mates with a female that is uninfected or is harboring a different *Wolbachia* type. Because an uninfected male can mate successfully with an infected female, CI provides a reproductive advantage to a *Wolbachia*-infected female over an uninfected one.

Wolbachia have been explored as a potential tool in the control of mosquito-borne diseases because of their unique ability to invade host populations rapidly (5). *Wolbachia* are found in many mosquito species in nature but surprisingly not in *Anopheles* malaria vectors or the major dengue vector, *Aedes aegypti*. Although a transient somatic infection was made recently in *Anopheles gambiae* (6, 7), *Ae. aegypti* has been stably transfected by several *Wolbachia* strains (8, 9). Previously, we established the *Ae. aegypti* WB1 line carrying the *Wolbachia* strain wAlbB by means of the microinjection transfer of this bacterium from *Aedes albopictus* to the wild-type *Ae. aegypti* Waco strain (8). This WB1 *Ae. aegypti* strain shows strong resistance to DENV compared with the parental Waco strain (10). The wAlbB strain inhibited viral replication in the mosquito midgut as well as its dissemination throughout the mosquito thorax and head, dramatically reducing the mosquito's potential for viral transmission. Resistance to several arboviruses was observed in *Ae. aegypti* infected with the *Wolbachia* strain wMelPop-CLA (11), and in *Culex quinquefasciatus* infected with wPip (12). Development of *Plasmodium* also was inhibited in *An. gambiae* mosquitoes carrying the somatic infection with wMelPOP and wAlbB *Wolbachia* strains (6, 7). These recent discoveries of *Wolbachia*-mediated effects in vector mosquitoes have a great potential for use in population-replacement strategies (5).

Although the molecular mechanism underlying *Wolbachia*-mediated resistance to pathogens is not understood completely, it appears to be associated with boosted mosquito innate immunity (6, 7, 10, 11, 13). A number of immune genes, such as defensins, cecropins, and several Toll pathway genes, were up-regulated by *Wolbachia* in *Ae. aegypti* (10, 11, 13). In agreement with these observations, activation of the Toll pathway by means of RNAi depletion of cactus, an inhibitor of REL1, has been shown to block proliferation of DENV in *Ae. aegypti* (14).

Here, we have deciphered the molecular mechanism underlying *Wolbachia*-mediated resistance to DENV in this mosquito. Microarray and real-time PCR analyses revealed that the Toll pathway and antioxidant genes were induced in *Ae. aegypti* infected with the *Wolbachia* wAlbB strain. This bacterium elevated the expression of NADPH oxidase and of reactive oxygen species (ROS). In turn, ROS up-regulation resulted in activation of the Toll pathway, which mediated both the antioxidant and production of antimicrobial peptides—cecropins and defensins. We used transgenic *Ae. aegypti* mosquitoes with altered immunity and RNAi

Author contributions: X.P., A.S.R. and Z.X. designed research; X.P., G.Z., J.W., G.B., and P.L. performed research; X.P., G.Z., and Z.X. analyzed data; and X.P., A.S.R., and Z.X. wrote the paper.

The authors declare no conflict of interest.

¹To whom correspondence may be addressed. E-mail: alexander.raikhel@ucr.edu or xizy@msu.edu.

See Author Summary on page 13.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1116932108/-DCSupplemental.

depletion of immune factors to demonstrate the involvement of these antimicrobial peptides in anti-dengue responses.

Results

***Wolbachia* Induces the Toll Pathway and Antioxidant Genes in *Ae. Aegypti*.** To determine the physiological responses of the mosquito to *Wolbachia* infection, we conducted microarray assays to compare transcript repertoires in the mosquito midgut and remaining carcass tissues of 7-d-old *Wolbachia*-infected *Ae. aegypti* WB1 females with those of the uninfected Waco strain,

before blood feeding. Similar microarray assays were conducted using *Wolbachia*-infected mosquitoes collected 12 d after additional infection with DENV serotype 2. This time point was chosen because it marks inhibition of DENV replication by *Wolbachia* infection (10). We classified differentially expressed genes that were identified in the two series of microarray assays. The most notable *Wolbachia*-responsive transcripts were related to immunity and the redox/stress/mitochondrion (R/S/M) groups (Fig. 1A). Except for genes belonging to unknown or diverse functions, the immunity and R/S/M groups were the two most

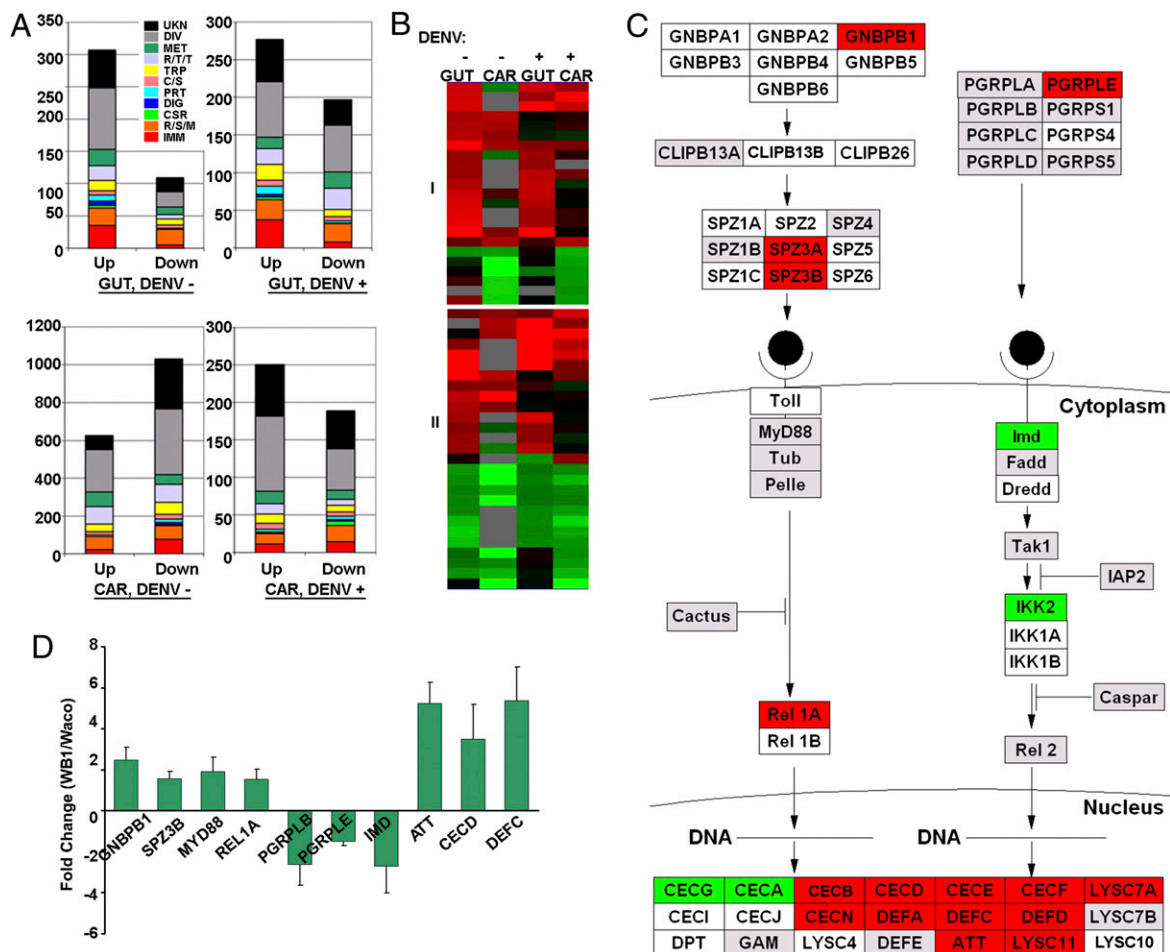


Fig. 1. *Wolbachia*-regulated genes in *Ae. aegypti*. (A) Functional classification of differentially expressed genes in the midgut (GUT) and carcass (CAR) of 7-d-old mosquitoes before a blood meal (DENV-) and 12 d after infection with DENV serotype 2 (DENV+). The graph shows the functional class distributions in real numbers of genes that are regulated by *Wolbachia* ("Up" indicates induced, and "Down" indicates repressed). The *Wolbachia*-regulated gene expression data are presented in Tables S1, S2, S3, and S4. Functional group abbreviations: C/S, cytoskeletal and structural; CSR, chemosensory reception; DIG, blood and sugar food digestive; DIV, diverse functions; IMM, immunity; MET, metabolism; PRT, proteolysis; R/S/M, redox, stress, and mitochondrion; R/T/T, replication, transcription, and translation; TRP, transport; UNK, unknown functions. (B) Cluster analysis of 23 immune genes (I) and 27 R/S/M genes (II) that were regulated in midguts and carcasses of *Wolbachia*-infected female mosquitoes in at least two of four combinations: midgut with (GUT+) or without (GUT-) DENV infection; carcass with (CAR+) or without (CAR-) DENV infection. All genes presented in the cluster are listed in Tables S5 and S6. (C) Regulation of putative Toll signaling pathway genes by *Wolbachia* in the mosquito midgut 12 d after feeding on DENV-infected blood. Red, green, and gray colors indicate *Wolbachia*-responsive up-, down- and nonregulated genes, respectively. White indicates unregulated or filtered signal. The pathway was built with GenMAPP software based on the immunogenomics prediction (49, 50). CECB, Cecropin B; CECE, Cecropin E; CECF, Cecropin F; CECG, Cecropin G; CECI, Cecropin I; CECJ, Cecropin J; CEEN, Cecropin N; CLIPB13A, Clip-domain serine protease 13A; CLIPB13B, Clip-domain serine protease 13B; CLIPB26, Clip-domain serine protease 26; DEFD, Defensin D; DEFE, Defensin E; DPT, Dipterin; Dredd, Death-related ced-3/Nedd2-like protein; Fadd, Fas-Associated Death Domain; GAM, Gambicin; GNBPA1, Gram-negative binding protein A1; GNBPA2, Gram-negative binding protein A2; GNBPB3, Gram-negative binding protein B3; GNBPB4, Gram-negative binding protein B4; GNBPB5, Gram-negative binding protein B5; GNBPB6, Gram-negative binding protein B6; IAP2, Inhibitor of apoptosis 2; IKK1A, I-Kappa-B Kinase 1 A; IKK1B, I-Kappa-B Kinase 1 B; IKK2, I-Kappa-B Kinase 2; LYSC4, C-Type lysozyme 4; LYSC7A, C-Type Lysozyme 7A; LYSC7B, C-Type Lysozyme 7B; LYSC10, C-Type lysozyme 10; LYSC11, C-Type lysozyme 11; Pelle, PGRPLA, Peptidoglycan Recognition Protein (Long) A; PGRPS1, Peptidoglycan Recognition Protein (Short) 1; PGRPLC, Peptidoglycan Recognition Protein (Long) C; PGRPS4, Peptidoglycan Recognition Protein (Short) 4; PGRPLD, Peptidoglycan Recognition Protein (Long) D; PGRPS5, Peptidoglycan Recognition Protein (Short) 5; Rel2, Relish-like protein 2; SPZ2, Spaetzle-like cytokine 2; SPZ4, Spaetzle-like cytokine 4; SPZ5, Spaetzle-like cytokine 5; SPZ1C, Spaetzle-like cytokine 1C; SPZ6, Spaetzle-like cytokine 6; Tak1, TGF-Beta-Activated Kinase-1; Tub, Tube. (D) Real-time PCR validation of *Wolbachia*-regulated genes identified in the microarray assays. Data are shown as fold change in WB1 midguts compared with Waco midguts 12 d after feeding on DENV-infected blood.

highly regulated functional groups in the midgut and comprised the largest number of differently expressed genes in the carcass. Genes within these two groups represented 21.9% (midgut) and 14.4% (carcass) of all of the regulated genes with predicted functions in the non-blood-fed mosquitoes, and 20.2% (midgut) and 13.9% (carcass) in the dengue-infected mosquitoes. Transcripts of 23 immune genes and 27 R/S/M genes exhibited similar patterns in both tissues (midgut and carcass) and with two treatments (with and without dengue infection, Fig. 1B). These genes included defensin C (*DEFC*), attacin (*ATT*), Cu superoxide dismutase (*CuSOD2*), 13 cytochrome P450 genes, two putative NADH dehydrogenase genes, and three heat-shock proteins. Importantly, genes belonging to the Toll pathway—Gram-negative binding protein B1 (*GNBP1*) and Relish-like protein 1A (*REL1A*)—were up-regulated in the midguts of *Wolbachia*-infected mosquitoes, both with and without DENV (Fig. 1C and Fig. S1). The effect of *Wolbachia* on the induction of the Toll pathway was confirmed further using real-time PCR. Transcripts of the antimicrobial peptide genes *ATT*, cecropin D (*CECD*), and *DEFC* were highly elevated in *Wolbachia*-infected *Aedes* mosquitoes (Fig. 1D). Likewise, the components of the Toll pathway—*GNBP1*, Spaetzle 3 (*SPZ3*), myeloid differentiation primary response gene 88 (*MYD88*), and *REL1A*—also were up-regulated (Fig. 1D). In contrast, genes representing the immune deficiency (IMD) pathway—*IMD*, peptidoglycan recognition protein LB (*PGRP-LB*), and peptidoglycan recognition protein LE (*PGRP-LE*)—were down-regulated.

Moreover, we observed a number of antioxidant genes being induced by *Wolbachia* in our microarray analysis, including glutathione peroxidase (AAEL012069 and AAEL008397), *CuZnSOD* (AAEL006271 and AAEL011498), manganese superoxide dismutase (*MnSOD*; AAEL004823 and AAEL005108), thioredoxin peroxidase (AAEL013528 and AAEL014548), and glutathione peroxidase (AAEL008397) (Table S7). Transcripts of both ferritin heavy chain (AAEL007385) and light chain (AAEL004335) also were up-regulated by *Wolbachia* in midguts of 7-d-old WB1 mosquitoes before blood feeding or at 12 d postinfection. Such a dramatic induction of antioxidant genes suggests that ROS levels within host cells are elevated because of *Wolbachia* infection.

***Wolbachia* Infection Induces Oxidative Stress in *Ae. Aegypti*.** To confirm that infection with *Wolbachia* induces oxidative stress in

Ae. aegypti, we compared the levels of hydrogen peroxide (H_2O_2) in WB1 and Waco mosquitoes. The level of H_2O_2 was significantly higher in both fat bodies (Student's *t* test, $P < 0.05$) and whole bodies (Student's *t* test, $P < 0.001$) of WB1 mosquitoes than in those of Waco mosquitoes (Fig. 2A). Similar to the oxidative burst in vertebrates, insects generate ROS through the enzymatic activity of NADPH oxidase (NOX) and dual oxidase (DUOX) (15, 16). Therefore, we compared transcript levels of genes encoding two NOX enzymes (NOX5 and NOXM) and two DUOX enzymes (DUOX1 and DUOX2) in WB1 and control Waco females using real-time PCR. There was a 3.1-fold and 4.4-fold increase in NOXM transcript abundance in the midgut and carcass, respectively, in WB1 females compared with Waco controls. Remarkably, we observed a 28.0-fold increase in the DUOX2 transcript in the carcass but only a 1.3-fold increase in the midgut of WB1 mosquitoes compared with Waco controls (Fig. 2B). No significant change in transcript levels of either NOX5 or DUOX1 was found as a result of *Wolbachia* infection. These results support the suggestion that NADPH oxidase-dependent ROS are induced in *Ae. aegypti* as a result of infection with *Wolbachia*.

Increased Level of ROS Is Sufficient to Activate the Toll Pathway in *Ae. Aegypti*. To test whether a high level of ROS could activate the Toll pathway in *Ae. aegypti*, we fed 2-d-old Waco female mosquitoes with a sugar solution containing 1% (vol/vol) H_2O_2 for 5 d and then measured the expression of two Toll pathway marker genes, Serpin-27A (*Spn27A*) and spermatogenic leucine zipper 1 (*SPZ1*) (17), in mosquito midguts. The *Spn27A* transcript level increased by 2.8-fold as a result of H_2O_2 treatment (Fig. 3A), indicating that H_2O_2 alone could activate the Toll pathway. We observed no up-regulation of *SPZ1* in the midgut of Waco mosquitoes in response to 1% (vol/vol) H_2O_2 treatment, suggesting that *SPZ1* is not as sensitive as *Spn27A* to increased ROS. Consistently, *Spn27A* but not *SPZ1* was induced in the WB1 females. To understand better the mechanism of ROS-mediated activation of the Toll pathway, we measured the expression of *SPZ1* and *Spn27A* in fat bodies of Waco mosquitoes after treating with H_2O_2 at three different doses: 1%, 2%, and 4% (vol/vol). *Spn27A* was induced by H_2O_2 at all three doses; the highest fold increase (7.2-fold) was seen with 2% (vol/vol) H_2O_2 treatment. *SPZ1* was induced only with 2% and 4% (vol/vol) H_2O_2 treatments (Fig. 3B). In addition, we measured the expression of two antimicrobial peptide genes, *CECD* and *DEFC*, that are strongly induced by *Wolbachia* in the WB1 mosquitoes. Both were up-regulated by more than 2.5-fold in midguts of Waco mosquitoes as a result of H_2O_2 treatment (Fig. 3A). Thus, an increase in H_2O_2 in uninfected Waco mosquitoes is sufficient to activate the Toll pathway and induce *CECD* and *DEFC*, similar to observations in the WB1 mosquitoes as a result of *Wolbachia* infection.

***Wolbachia*-Induced ROS Activate the Toll Pathway in WB1 Mosquitoes.** To determine whether *Wolbachia*-induced ROS are required to activate the Toll pathway in WB1 mosquitoes, we silenced two *Wolbachia*-induced oxidases, NOXM and DUOX2, using their dsRNA in WB1 females, and then measured the impact of their silencing on the Toll pathway. We consistently found that silencing both oxidases deactivated the Toll pathway in WB1 mosquitoes, as indicated by a dramatic decrease in the expression of *Spn27A* and *SPZ1* (Fig. 3C). Inhibiting ROS production by silencing NOXM and DUOX2 in WB1 also suppressed the expression of *CECD* and *DEFC* (Fig. 3C). To confirm that *Wolbachia* infection induced the expression of *CECD* and *DEFC* in WB1 by activating the Toll pathway, we injected dsRNAs of two genes encoding components of the Toll pathway, *MYD88* and *REL1A*, to turn off this pathway and then tested the impact of this treatment on *CECD* and *DEFC* expression. This silencing

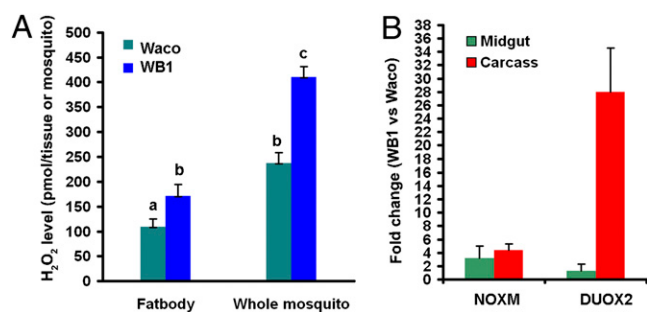


Fig. 2. *Wolbachia* induces ROS formation in the mosquito *Ae. aegypti*. (A) Comparison of H_2O_2 levels in the fat body and whole mosquito in 7-d-old WB1 and Waco mosquitoes before a blood meal. The data shown are means of six replicates, with five fat bodies or three whole mosquitoes for each. The level of H_2O_2 was significantly higher in both fat bodies and whole bodies of WB1 mosquitoes than in those of Waco mosquitoes. Statistical significance is represented by letters above each column, with different letters signifying distinct statistical groups. Student's *t* test: a vs. b, $P < 0.05$; b vs. c, $P < 0.001$. (B) Fold changes in expression of NADPH oxidase (NOXM) and dual oxidase (DUOX2) in the midguts and carcasses of WB1 and Waco mosquitoes. Real-time PCR analysis was performed using midguts and carcasses of 7-d-old, non-blood-fed, female mosquitoes.

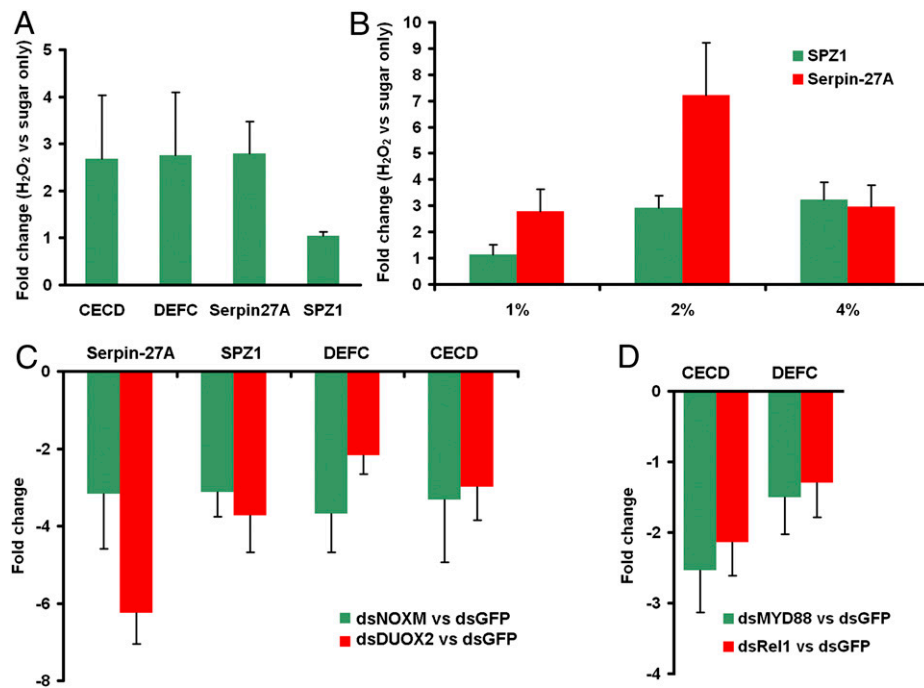


Fig. 3. *Wolbachia*-induced ROS-dependent activation of the Toll pathway. (A) Fold changes in expression of Toll pathway-related genes in midguts of Waco females after H_2O_2 treatment. Two-day-old Waco females were fed with a sugar solution containing 1% H_2O_2 (vol/vol) for 5 d. The total RNA of midguts was extracted to measure the fold change in gene expression compared with a control group fed only a sugar solution. (B) Fold changes in the expression of Toll pathway marker genes in fat bodies of Waco females after H_2O_2 treatment at three different doses—1%, 2%, and 4% (vol/vol)—compared with a control group fed a sugar solution only. (C) Fold changes in the expression of Toll pathway-related genes in WB1 females after silencing of NOXM and DUOX2. Three days after injection with dsRNA, midguts were collected to extract total RNA and measure the fold change in gene expression compared with that in the control group injected with GFP dsRNA. (D) Fold changes in the expression of CECD and DEFC after silencing of the Toll pathway in WB1. Three days after injection with MYD88 or REL1 dsRNA, midguts were collected to extract the total RNA and measure the fold change in gene expression compared with that in the control group injected with GFP dsRNA.

of the Toll pathway caused a significant down-regulation of CECD and DEFC (Fig. 3D). Taken together, our results indicate that *Wolbachia*-induced ROS are required to maintain the activated Toll pathway and induce its downstream expression of CECD and DEFC.

Activation of the Toll Pathway Is Essential for Antioxidant Expression.

Considering that *Wolbachia*-induced ROS activated the Toll pathway, and antioxidants were up-regulated by *Wolbachia* infection in our microarray assays, we hypothesized that the Toll pathway controlled the expression of host antioxidants to combat

damaging effects of ROS. Therefore, we used transgenic *Ae. aegypti* with fat-body-specific ectopic REL1 expression (REL1⁺) to determine whether activation of the Toll pathway is sufficient to induce the expression of antioxidants. Because REL1 in transgenic mosquitoes is driven by the female fat-body-specific vitellogenin (Vg) promoter, the Toll pathway is activated after mosquitoes consume a blood meal (17). We therefore collected WB1 mosquito fat bodies 24 h after a blood meal, a peak time for Vg gene expression, and, using real-time PCR, measured the expression of eight antioxidants and compared their expression with that in wild-type UGAL mosquitoes at the same time point

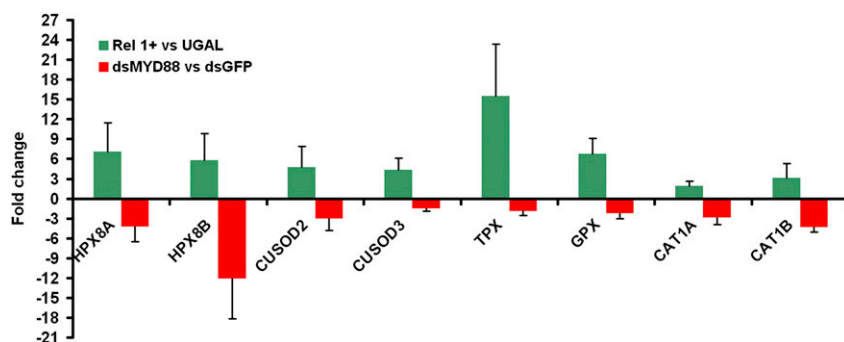


Fig. 4. The Toll pathway controls the expression of antioxidant proteins. Fat bodies of REL1-overexpressing transgenic mosquitoes (Rel1⁺) were collected 24 h after a blood meal, and the expression of eight antioxidants was measured using real-time PCR and compared with that of wild-type UGAL mosquitoes. Midguts of WB1 females injected with MYD88 dsRNA were collected to measure the fold changes in gene expression compared with that of the control group injected with GFP dsRNA. For all experiments, the gene expression data were normalized with RPS6. The primer sequences are listed in Tables S8 and S9. Error bars indicate SE.

(Fig. 4). The eight antioxidants were selected because they are induced by *Wolbachia* infection in WB1 mosquitoes, as shown by the microarray assay (Table S7). We found that the expression of all eight antioxidants was strongly induced when the Toll pathway was activated in the REL1⁺ transgenic mosquitoes. In particular, we saw a 15.5-fold increase in thiol peroxidase (TPX), a 7.5-fold increase in peroxinectin (HPX8A), a 6.8-fold increase in glutathione peroxidase (GPX), and a 4.7-fold increase in CuSOD2 (Fig. 4).

To confirm further that the Toll pathway is required to induce the expression of these antioxidants, we injected *MYD88* dsRNA to knock down the Toll pathway in WB1 mosquitoes and then measured the change in expression of the same eight antioxidants in midguts, comparing the results with those for a control injection of GFP dsRNA. Silencing of the Toll pathway caused a more than twofold decrease in six of the eight antioxidants (Fig. 4). Strikingly, there were 12.0-fold, 4.2-fold, and 3.0-fold decreases in the expression of HPA8B, catalase (CAT1B), and CuSOD2, respectively. Thus, the Toll pathway was shown to regulate the expression of antioxidants in the mosquito.

Knockdown of DEFC and CECD Compromise Resistance to DENV in *Ae. aegypti* WB1 Mosquitoes. We previously reported that the Toll pathway controls dengue infection in *Ae. aegypti* (14), but which downstream effectors restrict DENV has remained unclear. Because DEFC and CECD are strongly induced through the Toll

pathway in WB1 mosquitoes, we investigated whether they play a role as antidengue effectors. We applied RNAi depletion of DEFC and CECD individually or in combination in WB1 mosquitoes and then used plaque assays to measure the titers of DENV serotype 2 in mosquito midguts 7 d after viral infection. Viral titers were increased significantly by individual knockdown of DEFC and by double knockdown of DEFC and CECD compared with the effects of injecting GFP dsRNA (Mann–Whitney *u* test, $P < 0.05$; Fig. 5A). A trend toward an increase in viral titers as a result of single knockdown of CECD was observed also, but the effect was not statistically significant.

Fat-Body-Specific Ectopic Expression of Defensin A and Cecropin A Inhibit Proliferation of DENV in Transgenic *Ae. aegypti*. We used transgenic *Ae. aegypti* mosquito lines with *Vg*-driven fat body ectopic expression of either defensin A (DEFA⁺) or cecropin A (CECA⁺) (18, 19) to study a general role for defensins and cecropins in antidengue defense. In addition, we took advantage of the DEFA/CECA hybrid line produced by crossing these two transgenic mosquito lines, which expressed both *DEFA* and *CECA* (19). In the first experiment, 7-d-old *Ae. aegypti* female mosquitoes, the three transgenic lines, and the wild-type UGAL strain were fed dengue-infected blood, and the level of viral infection was measured in their midguts by indirect fluorescence assay (IFA) 7 d post viral infection. There were significant differences in the midgut infections between the DEFA/CECA

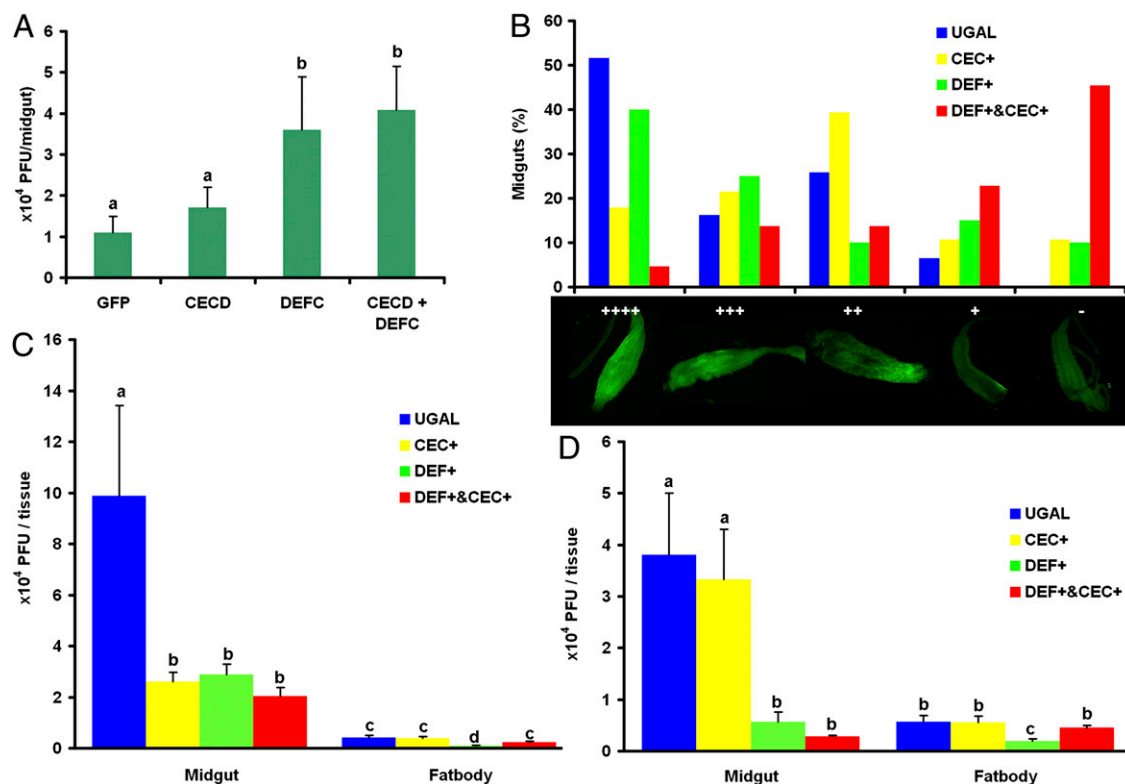


Fig. 5. Roles of defensins and cecropins in the inhibition of DENV proliferation. Viral infection was detected by plaque assay in C6/36 cells (A, C, and D) or by immunofluorescence staining of the DENV-2 envelope protein (B). (A) DENV loads were measured in the midguts of mosquitoes after RNAi depletion of CECD, DEFC, a double depletion (CECD + DEFC), or in GFP dsRNA-treated control mosquitoes. a vs. b, $P < 0.05$ (Mann–Whitney *U* test). (B) Midgut infection rates were measured at 7 d after viral infection in transgenic *Ae. aegypti* with fat body ectopic expression of either defensin A (DEF⁺) or cecropin A (CEC⁺) and in their hybrid strain (DEF⁺ and CEC⁺). Midgut infection levels were recorded as uninfected (–) or infected in one of four increasing levels (+, ++, +++, +++++). There were significant differences in the levels of midgut DENV infection in the hybrid strain and UGAL controls (χ^2 test, $P < 0.0001$) and in levels in CECA transgenic mosquitoes and UGAL controls (χ^2 test, $P < 0.05$). DENV loads were measured in the midguts and fat bodies of three transgenic mosquitoes and the wild-type UGAL strain 10 d after infection with DENV-2 during the first blood feeding (C) or 10 d after infection with DENV-2 during the second blood feeding (the first blood meal was blood without DENV to activate the transgenes) (D). For all figures, error bars represent SE. Statistical significance is represented by letters above each column, with different letters signifying distinct statistical groups. For C and D, $P < 0.05$ for a vs b, b vs c, and c vs d in Student's *t* test.

hybrid strain and UGAL control mosquitoes (χ^2 test, $P < 0.0001$; Fig. 5B) and between CECA⁺ transgenic and UGAL control mosquitoes (χ^2 test, $P < 0.05$; Fig. 5A). Strong inhibition of DENV proliferation was observed in the CECA⁺ and DEFA/CECA transgenic mosquitoes compared with UGAL controls 7 d post viral infection; 45.5% of the DEFA/CECA hybrids and 10.7% of the CECA⁺ transgenic mosquitoes were negative for DENV envelope protein in the midgut, compared with 0% of the UGAL mosquitoes. Of the DEFA⁺ transgenic mosquitoes, 10% also were negative for DENV, but this effect was not statistically significant (Fig. 5B).

We conducted another comparison in a similar manner, instead measuring the viral titers in both the midgut and fat body by plaque assay 10 d after viral infection. The DENV titers in the midgut were significantly lower in all three transgenic mosquito lines than in the UGAL wild-type mosquitoes (Student's t test, $P < 0.05$; Fig. 5C). A significantly lower viral titer also was observed in the fat bodies of DEFA⁺ transgenic mosquitoes compared with the other three groups (Student's t test, $P < 0.05$; Fig. 5C).

CECD and DEFC were induced by *Wolbachia* infection before viral invasion in the WB1 mosquitoes, whereas the CECA and DEFA transgenes were induced in fat bodies after DENV entry with a blood meal into the midgut of the transgenic mosquitoes. Immune factors produced in the preinvasion and postinvasion phase have different effects on pathogens (20). To mimic the effect of CECD and DEFC on DENV in WB mosquitoes more closely, we fed 7-d-old transgenic and control female mosquitoes with uninfected blood to induce the expression of DEFA and CECA in their fat bodies. Five days later, female mosquitoes were fed with DENV-containing blood. Ten days after viral infection, mosquito midguts were dissected to measure viral titer. Again, we observed a significantly lower viral titer in the midguts of both the DEFA/CECA hybrid and DEFA⁺ transgenic mosquitoes than in the midguts of the UGAL controls (Student's t test, $P < 0.05$; Fig. 5D). Similarly, a significant inhibition of DENV was observed only in the fat bodies of the DEFA⁺ transgenic mosquitoes (Student's t test, $P < 0.05$; Fig. 5D). This result was consistent with the RNAi knockdown assay (Fig. 5A) showing that defensin had a stronger inhibitory effect against DENV than cecropin. Thus, we demonstrated that antimicrobial peptides (defensins and cecropins) induced by *Wolbachia* infection in *Ae. aegypti* are downstream effectors that inhibit DENV proliferation. Although both defensins and cecropins contributed to viral inhibition, utilization of the DEFA/CECA transgenic mosquitoes clearly indicated that up-regulation of both these antimicrobial peptides produces a synergistic inhibitory effect against DENV.

Discussion

In this study, we deciphered how *Wolbachia* infection affects the mosquito host in inducing resistance to DENV. Our microarray assay indicates that transcripts of genes with functions related to immunity and R/S/M are up-regulated in *Ae. aegypti* infected with *Wolbachia*. Infection with this bacterium leads to induction of oxidative stress and an increased level of ROS in its mosquito host. ROS elevation is linked to the activation of the Toll pathway, which is essential in mediating the expression of antioxidants to counterbalance oxidative stress. This immune pathway also is responsible for activation of antimicrobial peptides, e.g., defensins and cecropins. We provide evidence that these antimicrobial peptides are involved in inhibition of DENV proliferation in *Wolbachia*-infected mosquitoes. Utilization of transgenic *Ae. aegypti* and the RNAi depletion approach have been instrumental in proving the role of defensins and cecropins in resistance to DENV in *Wolbachia*-infected *Ae. aegypti* (Fig. 6).

As facultative symbionts, *Wolbachia* can be transferred artificially to novel host species and form stable associations. Within

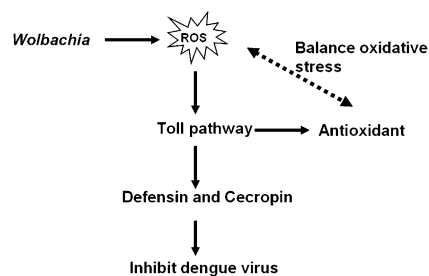


Fig. 6. A current model of *Wolbachia*-mediated resistance to DENV in *Ae. aegypti*.

their new host *Ae. aegypti*, *Wolbachia* elicit a strong immune response (10, 11, 13). Such responses have been observed in *An. gambiae* with transient somatic *Wolbachia* infections (6, 7). In addition to DENV, *Wolbachia* have been shown to induce resistance to several other pathogens—*Plasmodium*, Chikungunya, and filarial nematodes—in *Ae. aegypti* (10, 11, 13) and to *Plasmodium* in *An. gambiae* (6, 7). However, the vector competence of mosquito species with naturally occurring *Wolbachia* is not significantly affected by these bacteria. For example, the *Wolbachia* wAlbB strain lacks the ability to induce resistance to DENV in the naturally infected *Ae. albopictus* (10). Previous studies reported that *Wolbachia* neither induces nor suppresses transcripts encoding antimicrobial peptides in *Ae. albopictus* (21). Thus, resistance to pathogens in *Wolbachia*-infected *Ae. aegypti* and *An. gambiae* mosquitoes is likely a consequence of the elevated host immunity to *Wolbachia*, which is aimed at counterbalancing the adverse effects of this newly acquired parasite.

Despite numerous immune responses mounted against pathogens, *Wolbachia*-uninfected *Ae. aegypti* and *An. gambiae* are highly competent vectors for DENV and *Plasmodium*, respectively (14, 22–24). The *Wolbachia*-boosted immunity differs from pathogen-induced immune responses in that immune factors are induced by *Wolbachia* before pathogen invasion, whereas the immune factors induced by pathogens occur after pathogen invasion. It was reported that knockdown of the inhibitor protein Cactus of the Toll pathway before infection renders mosquitoes totally refractory to the *Plasmodium* parasites, whereas Cactus depletion after infection has no effect on oocyst development (20). Because *Wolbachia* boost Toll pathway activity before DENV invasion, *Wolbachia* can play more crucial roles in killing invading virus than the late activation of Toll pathway by DENV (14).

Wolbachia infection of *Ae. aegypti* leads to a high elevation of transcripts of genes encoding NOXM and DUOX2 enzymes, which are involved in production of ROS (15, 16). We also observed an increased H₂O₂ level in WB1 relative to Waco mosquitoes. This increase is similar to that seen in closely related parasitic *Rickettsia* bacteria, in which H₂O₂ represents a major oxidant and causes cell injury. High concentrations of ROS damage cellular components, creating a state of oxidative stress in a host (25). Such a negative effect of ROS on an organism is controlled through an antioxidant defense system. We found that a number of antioxidant genes were up-regulated in the presence of *Wolbachia* in WB1 mosquitoes. Previous studies also have shown induction of ROS and expression of antioxidant proteins in an *Ae. albopictus* cell line infected by *Wolbachia* (26). It is likely that the existence of an antioxidant regulatory feedback plays an important role in counteracting a potential damaging effect of ROS on the infected host cells and permitting *Wolbachia* to maintain a persistent infection within their newly acquired host, *Ae. aegypti*.

In *Ae. aegypti*, *Wolbachia* infection activates the Toll pathway, and our data suggest that ROS mediate this activation. In both *Wolbachia*-infected WB1 and H₂O₂-treated Waco mosquitoes,

the levels of transcripts Spn27A, DEFC, and CECD were elevated, whereas RNAi depletion of either NOXM or DUOX2 resulted in a significant decrease of these transcripts in WB1 mosquitoes. In vertebrates, multiple links exist between ROS and NF- κ B signaling pathways (27, 28). ROS mediate activation of NF- κ B, which is central to immunity, inflammation, and cell survival (27, 29). Exposure to H₂O₂ or the other oxidants triggers nuclear translocation of NF- κ B in certain cell types (30, 31). The other side of the cross-talk between ROS and the NF- κ B pathway is the activation of antioxidant gene expression, which mediates protection from ROS (27). A similar up-regulation of the antioxidant genes—*MnSOD*, *CuZnSOD*, ferritin heavy and light chains, *GPX*, and catalase—is observed in WB1 mosquitoes. We further show that the Toll pathway is essential for this antioxidant gene expression. Eight antioxidant genes are strongly up-regulated in transgenic *Ae. aegypti* with ectopic expression of REL1, the Toll pathway NF- κ B factor. Furthermore, silencing the Toll pathway in WB1 mosquitoes by means of RNAi *MYD88* depletion results in down-regulation of antioxidant genes. Taken together, our data suggest that *Wolbachia*-induced oxidative stress activates the Toll pathway, which then elevates the expression of mosquito antioxidants to counteract damaging effects of ROS.

Insects use powerful immune mechanisms—siRNA, JAK-STAT, Toll, IMD, and autophagy—to regulate viral infections (32–36). Insect RNA viruses, including DENV, are targets of the RNAi antiviral pathway (22, 32, 36). Involvement of other immune pathways in antiviral defense appears to be virus specific, but their precise modes of action in response to each specific viral infection are not clear (35). Interestingly, viral infections by DENV and *Drosophila* X virus induce genes of the Toll pathway (14, 34), whereas DENV and *Drosophila* C virus activate the JAK-STAT pathway in *Ae. aegypti* and *Drosophila*, respectively (37, 38). Activation of both these pathways likely is related to cellular signaling from virus-damaged cells and proliferation and function of hemocytes (34, 37). Similar mechanisms also have been suggested to be active in the IMD-mediated antiviral response against cricket paralysis virus (33). Although up-regulation of antimicrobial peptides has been documented in all these studies, their role in antiviral defense has remained unclear. In our current study, we have demonstrated that *Aedes* defensins and cecropins are strongly induced by the *Wolbachia*-mediated Toll pathway and confer resistance to DENV in the *Ae. aegypti* WB mosquitoes. RNAi depletion of either DEFC or CECD compromises *Wolbachia*-induced resistance to DENV in WB1 mosquitoes. Moreover, ectopic expression of DEFA and CECA inhibits proliferation of DENV in transgenic *Ae. aegypti*. The antiviral activity of defensins was reported first in vertebrates, and defensins were thought to target enveloped viruses by disrupting the viral envelope membrane in a manner similar to defensin antibacterial action (39). Recent results indicate that human defensins inhibit infections of both enveloped and non-enveloped viruses through multiple mechanisms: preventing viral membrane attachment and cellular entry, disrupting the viral envelope, and blocking viral nuclear import (40). The exact mechanism of action of the mosquito antimicrobial peptides (cecropins and defensins) on DENV proliferation in the mosquito host remains to be determined.

In transgenic *Aedes* mosquitoes, CECA and DEFA are produced in the fat body under the control of the *Vg* promoter and are secreted to the hemolymph (18, 19). Thus, their antiviral effect is extracellular. Interestingly, the fat-body-specific expression of DEFA and CECA affects DENV loads in the midgut, indicating their humoral effect on midgut virus proliferation. Our previous study showed that *Wolbachia* infection inhibits DENV replication in the *Aedes* midgut (10). However, the sites of *Wolbachia*-elicited antimicrobial peptides in the mosquito have yet to be determined. A synergistic antidenegue activity of

CECA and DEFA was observed when these antimicrobial peptides were coexpressed in the DEF/CEC hybrid transgenic mosquitoes. This synergistic effect is similar to the previously reported strong anti-*Plasmodium* activity through cooperative action of CECA and DEFA in the DEF/CEC hybrid transgenic mosquitoes (19). Given the activation of numerous antimicrobial peptides in *Wolbachia*-infected mosquitoes, it is likely that these peptides act synergistically in limiting DENV proliferation, as has been demonstrated using the DEF/CEC transgenic *Ae. aegypti* (19).

Although our results indicate that ROS function as signaling molecules to activate the Toll pathway-mediated antiviral defense, this function may represent one of multiple mechanisms underlying *Wolbachia*-mediated viral interference. ROS also may function as final effectors of antiviral immunity. A 28-fold increase in the DUOX2 transcript in WB1 mosquitoes provides a rationale for characterizing the role of ROS generated by this enzyme. Furthermore, we cannot exclude the possibility that the other physiological changes caused by *Wolbachia* also may contribute to viral interference. *Wolbachia* may perturb the host metabolic network that provides DENV the energy and macromolecule subunits necessary for replication (41). Future studies should include the identification of each key factor and the determination of their associations and relative contributions to the overall resistance effect.

In conclusion, we have shown that *Wolbachia* can induce ROS-dependent activation of the Toll pathway to control DENV. The ability of *Wolbachia* to boost immunity and block DENV proliferation in a newly acquired host, *Ae. aegypti*, makes it a potential “mosquito vaccine” that could be used efficiently to prevent pathogen transmission. More detailed understanding of molecular mechanisms of *Wolbachia* effects on DENV proliferation in *Ae. aegypti* is of paramount importance for refining the utilization of a *Wolbachia/Aedes* system approach for the future control of DENV transmission.

Materials and Methods

Mosquito Rearing and Cell-Culture Maintenance. The *Wolbachia*-uninfected *Ae. aegypti* Waco strain and *Wolbachia*-infected *Ae. aegypti* WB1 strain were maintained in sugar solution at 27 °C and 85% humidity, with a 12-h/12-h light/dark cycle, according to standard rearing procedures. The same conditions were used to maintain transgenic *Ae. aegypti* lines and the wild-type Rockefeller/UGAL strain. We used the following transgenic *Ae. aegypti* lines: Vg-REL1-A (Rel1⁺), Vg-DefA (DEF⁺), Vg-CecA (CEC⁺), and Vg-CecA/Vg-DefA (DEF⁺ and CEC⁺) (17–19). Female mosquitoes, 3–5 d after eclosion, were fed on the blood of anesthetized white mice to initiate egg development. This study was carried out in strict accordance with the recommendations set out in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The protocols were approved by Michigan State University and the University of California, Riverside Institutional Animal Care and Use Committees, and all efforts were made to minimize suffering.

The *Ae. albopictus* cell line C6/36 was grown in Eagle’s minimal essential medium (MEM) with 10% (vol/vol) heat-inactivated FBS, 100 units/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, and 100 μ M nonessential amino acids at 32 °C and 5% (vol/vol) CO₂.

DENV Infection in Mosquitoes. The New Guinea C strain of DENV-2 was propagated in C6/36 cells, according to standard procedures (42). In short, 180- μ L aliquots of virus stock were used to infect 25-cm² flasks of C6/36 cells at 80% confluence with a multiplicity of infection of 1.0 virus particle per cell. Infected cells were incubated at 32 °C and 5% (vol/vol) CO₂ for 7 d. Cells were harvested with a cell scraper and lysed to release the virus particles by repeated freezing and thawing using dry ice and a 37 °C water bath. The virus suspension was mixed 1:1 with commercial ox defibrinated blood (Colorado Serum Company). The blood meal was maintained at 37 °C for 30 min before 7-d-old mosquitoes were fed.

Microarray Analysis. Seven-day-old WB1 (treatment) and Waco (reference) mosquitoes before blood feeding and 19-d-old WB1 (treatment) and Waco (reference) mosquitoes at 12 d postinfection were dissected to collect the midguts and remaining carcass tissues. Four biological replicates, with 80

tissue samples for each, were used for each treatment or reference group. Total RNA was extracted using RNeasy Mini Kit (QIAGEN), according to the manufacturer's instruction. A 4 × 44K microarray (Agilent) was designed based on the V1.2 version annotation of the *Ae. aegypti* full genome sequence. A microarray assay was conducted as reported previously (43). Briefly, 3 μg total RNA was used for probe synthesis of cy3- and cy5-labeled cRNA. Hybridizations were carried out using an Agilent Technologies In Situ Hybridization kit (Agilent Technologies) at 65 °C, according to the manufacturer's instructions. Hybridization intensities were determined using an Axon GenePix 4000B scanner, and images were analyzed using GenePix software. The expression data were processed and analyzed as described previously (43).

H₂O₂ Assay. Fat bodies and whole bodies of 7-d-old Waco and WB1 mosquitoes were collected in 1 × PBS containing 2 mg/mL of the catalase inhibitor 3-amino-1,2,4-triazole (44). After homogenization, samples were filtered through a 10K molecular weight cutoff spin filter (Corning Spin-XUF; Corning Incorporated Life Sciences). The elution from each experimental group then was collected and tested using a Hydrogen Peroxide Assay Kit (BioVision). The fluorescence intensity was detected with Excitation/Emission = 550/590 using a fluorescence multidetector microplate reader (BMG Labtech), according to the manufacturer's instructions. The values were normalized by the total amount of proteins in the sample, as determined by the Pierce bicinchoninic acid protein assay (Thermo Scientific). Six biological replicates, with five fat bodies or three whole bodies for each, were used for each treatment.

Effect of Exogenous H₂O₂ on Mosquito Toll Pathway. Two-day-old Waco mosquitoes were fed with either the 10% (wt/wt) sugar meal containing 1%, 2% or 4% (vol/vol) H₂O₂ or the 10% (wt/wt) sugar meal only (control). Five days later, mosquito midguts and fat bodies were dissected to extract the total RNA. The expression of the Toll pathway-related genes—*Spn27A*, *SPZ1*, *CECD*, and *DEFC*—were measured using real-time PCR.

RNAi-Mediated Gene Silencing. The dsRNA was synthesized in vitro using the Megascript T7 high-yield transcription kit (Ambion). The primers designed to amplify *Ae. Aegypti* *MYD88*, *REL1*, *DEFC*, *CECD*, *NOXM*, *NOX5*, *DUOX1*, and *DUOX2* are listed in Tables S8 and S9. An RNAi-based assay was conducted according to standard methodology (45). Approximately 69 nl dsRNA (4 μg/μL) in water was injected into the thorax of CO₂-anesthetized 2-d-old female mosquitoes using a nano-injector, as previously described (46). To measure gene expression, the midguts of mosquitoes were dissected 3 d after injection and used for RNA extraction.

RNA Extraction, cDNA Synthesis, and Real-Time PCR Assays. Total RNA was extracted from mosquito tissues using the RNeasy Mini Kit (QIAGEN Sciences), and the cDNA transcript was produced using QuantiTect Reverse Transcription Kit (QIAGEN Sciences). Real-time PCR was performed using the QuantiTect SYBR Green PCR Kit (QIAGEN Sciences) and the ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The ribosomal protein S6 (*RPS6*) gene was used for normalization of cDNA templates. All primers used for real-time PCR are listed in Table S9.

IFA. The viral antigen in mosquito midguts was detected using an IFA7 d postinfection. IFAs were performed as previously described (47). The midgut samples were dissected and fixed in 4% (vol/vol) paraformaldehyde overnight and washed with PBS. Then the samples were incubated in PBS, 0.1% (vol/vol) Triton X-100, and 1% (wt/wt) BSA at room temperature for 1 h. To detect the antigen, a mouse anti-Dengue complex monoclonal antibody (obtained from the Centers for Disease Control, Atlanta, GA) and a fluorescein-conjugated affinity-purified secondary antibody (Invitrogen) were used in all IFA assays. Specimens were examined under an Olympus IX71 fluorescence microscope (Olympus).

Mosquito Tissue Collections. To measure the virus titer in mosquito tissues, transgenic mosquitoes [Vg-DefA (DEF⁺), Vg-CecA (CEC⁺), and Vg-CecA/Vg-DefA (Cx^D)] at 10 d postinfection were washed briefly in 70% (vol/vol) ethanol and then were rinsed in sterile distilled water. The midguts and fat bodies were dissected in sterile PBS, transferred to microcentrifuge tubes containing 150 μL MEM and then were homogenized using a Kontes pellet pestle motor in a sterile environment. In the knockdown experiment, 2-d-old WB1 mosquitoes were injected with the dsRNA of GFP, CECD, DEFC, or a mixture of CECD and DEFC. Five days later, mosquitoes were fed with the dengue-infected blood. Seven days after infection, the midguts were dissected and collected as described in the above.

Plaque Assays. The DENV titers in the mosquitoes' midgut and fat-body homogenates were measured using a plaque assay, as previously reported (48). The virus-containing homogenates were serially diluted and inoculated into C6/36 cells in 24-well plates. After incubation at 32 °C and 5% (vol/vol) CO₂ for 5 d, the plates were assayed for plaque formation by peroxidase immunostaining, using mouse hyperimmune ascitic fluid (specific for DENV-2) and a goat anti-mouse HRP conjugate as the primary and secondary antibody, respectively.

Accession Numbers. Genes mentioned in this study are listed in the Entrez Gene database: *Cactus* (ID no. 5565922), *REL1A* (ID no. 5569526), *SPZ3B* (ID no. 5565708), *MYD88* (ID no. 5569574), *PGRPLB* (ID no. 5572949), *PGRPLE* (ID no. 5577250), *IMD* (ID no. 5572865), *DEFC* (ID no. 5579094), *CECD* (ID no. 5564826), *attacin* (ID no. 5578028), *NOX5* (ID no. 5573122), *NOXM* (ID no. 5572965), *DUOX1* (ID no. 5569378), *DUOX2* (ID no. 5569376), *Spn27A* (ID no. 5566833), *SPZ1* (ID no. 5577953), *HPX8A* (ID no. 5564683), *HPX8B* (ID no. 5564679), *CUSOD2* (ID no. 5567695), *CUSOD3* (ID no. 5574885), *TPX* (ID no. 5564123), *GPX* (ID no. 5570587), *catalase* (*CAT1A* and *CAT1B*, ID no. 5577893), and *GNBPB1* (ID no. 5579192).

ACKNOWLEDGMENTS. We thank the Arbovirus Diseases Branch at the Centers for Disease Control for providing the anti-DENV antibodies (mouse hyperimmune ascitic fluid) and Dr. Deborah McClellan for editorial assistance. This work was supported by the National Institutes of Health/National Institute of Allergy and Infectious Disease Grants R01AI080597 and R37AI24716. J.W. is supported in part by a fellowship from the China Scholarship Council.

- Hill CA, Kafatos FC, Stansfield SK, Collins FH (2005) Arthropod-borne diseases: Vector control in the genomics era. *Nat Rev Microbiol* 3:262–268.
- Halstead SB (2007) Dengue. *Lancet* 370:1644–1652.
- Kyle JL, Harris E (2008) Global spread and persistence of dengue. *Annu Rev Microbiol* 62:71–92.
- Serbus LR, Casper-Lindley C, Landmann F, Sullivan W (2008) The genetics and cell biology of *Wolbachia*-host interactions. *Annu Rev Genet* 42:683–707.
- Iturbe-Ormaetxe I, Walker T, O'Neill SL (2011) *Wolbachia* and the biological control of mosquito-borne disease. *EMBO Rep* 12:508–518.
- Hughes GL, Koga R, Xue P, Fukatsu T, Rasgon JL (2011) *Wolbachia* infections are virulent and inhibit the human malaria parasite *Plasmodium falciparum* in *Anopheles gambiae*. *PLoS Pathog* 7:e1002043.
- Kambris Z, et al. (2010) *Wolbachia* stimulates immune gene expression and inhibits plasmodium development in *Anopheles gambiae*. *PLoS Pathog* 6:e1001143.
- Xi Z, Khoo CC, Dobson SL (2005) *Wolbachia* establishment and invasion in an *Aedes aegypti* laboratory population. *Science* 310:326–328.
- McMeniman CJ, et al. (2009) Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. *Science* 323:141–144.
- Bian G, Xu Y, Lu P, Xie Y, Xi Z (2010) The endosymbiotic bacterium *Wolbachia* induces resistance to dengue virus in *Aedes aegypti*. *PLoS Pathog* 6:e1000833.
- Moreira LA, et al. (2009) A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue, Chikungunya, and *Plasmodium*. *Cell* 139:1268–1278.
- Glaser RL, Meola MA (2010) The native *Wolbachia* endosymbionts of *Drosophila melanogaster* and *Culex quinquefasciatus* increase host resistance to West Nile virus infection. *PLoS ONE* 5:e11977.
- Kambris Z, Cook PE, Phuc HK, Sinkins SP (2009) Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. *Science* 326:134–136.
- Xi Z, Ramirez JL, Dimopoulos G (2008) The *Aedes aegypti* Toll pathway controls dengue virus infection. *PLoS Pathog* 4:e1000098.
- Kumar S, Molina-Cruz A, Gupta L, Rodrigues J, Barillas-Mury C (2010) A peroxidase/dual oxidase system modulates midgut epithelial immunity in *Anopheles gambiae*. *Science* 327:1644–1648.
- Kawahara T, Quinn MT, Lambeth JD (2007) Molecular evolution of the reactive oxygen-generating NADPH oxidase (Nox/Duox) family of enzymes. *BMC Evol Biol* 7:109.
- Bian G, Shin SW, Cheon HM, Kokoza V, Raikhel AS (2005) Transgenic alteration of Toll immune pathway in the female mosquito *Aedes aegypti*. *Proc Natl Acad Sci USA* 102:13568–13573.
- Kokoza V, et al. (2000) Engineering blood meal-activated systemic immunity in the yellow fever mosquito, *Aedes aegypti*. *Proc Natl Acad Sci USA* 97:9144–9149.
- Kokoza V, et al. (2010) Blocking of *Plasmodium* transmission by cooperative action of Cecropin A and Defensin A in transgenic *Aedes aegypti* mosquitoes. *Proc Natl Acad Sci USA* 107:8111–8116.
- Frolet C, Thoma M, Blandin S, Hoffmann JA, Levashina EA (2006) Boosting NF-κB-dependent basal immunity of *Anopheles gambiae* aborts development of *Plasmodium berghei*. *Immunity* 25:677–685.
- Bourtzis K, Pettigrew MM, O'Neill SL (2000) *Wolbachia* neither induces nor suppresses transcripts encoding antimicrobial peptides. *Insect Mol Biol* 9:635–639.
- Blair CD (2011) Mosquito RNAi is the major innate immune pathway controlling arbovirus infection and transmission. *Future Microbiol* 6:265–277.

23. Cirimotich CM, Dong Y, Garver LS, Sim S, Dimopoulos G (2010) Mosquito immune defenses against *Plasmodium* infection. *Dev Comp Immunol* 34:387–395.
24. Ramirez JL, Dimopoulos G (2010) The Toll immune signaling pathway control conserved anti-dengue defenses across diverse *Ae. aegypti* strains and against multiple dengue virus serotypes. *Dev Comp Immunol* 34:625–629.
25. Fridell YW, Sánchez-Blanco A, Silvia BA, Helfand SL (2005) Targeted expression of the human uncoupling protein 2 (hUCP2) to adult neurons extends life span in the fly. *Cell Metab* 1:145–152.
26. Brennan LJ, Keddie BA, Braig HR, Harris HL (2008) The endosymbiont *Wolbachia pipientis* induces the expression of host antioxidant proteins in an *Aedes albopictus* cell line. *PLoS ONE* 3:e2083.
27. Morgan MJ, Liu ZG (2011) Crosstalk of reactive oxygen species and NF- κ B signaling. *Cell Res* 21:103–115.
28. Bubici C, Papa S, Dean K, Franzoso G (2006) Mutual cross-talk between reactive oxygen species and nuclear factor-kappa B: Molecular basis and biological significance. *Oncogene* 25:6731–6748.
29. Schreck R, Rieber P, Baeuerle PA (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J* 10:2247–2258.
30. Gloire G, Legrand-Poels S, Piette J (2006) NF-kappaB activation by reactive oxygen species: Fifteen years later. *Biochem Pharmacol* 72:1493–1505.
31. Bowie A, O'Neill LA (2000) Oxidative stress and nuclear factor-kappaB activation: A reassessment of the evidence in the light of recent discoveries. *Biochem Pharmacol* 59:13–23.
32. Ding SW (2010) RNA-based antiviral immunity. *Nat Rev Immunol* 10:632–644.
33. Costa A, Jan E, Sarnow P, Schneider D (2009) The Imd pathway is involved in antiviral immune responses in *Drosophila*. *PLoS ONE* 4:e7436.
34. Zambon RA, Nandakumar M, Vakharia VN, Wu LP (2005) The Toll pathway is important for an antiviral response in *Drosophila*. *Proc Natl Acad Sci USA* 102:7257–7262.
35. Sabin LR, Hanna SL, Cherry S (2010) Innate antiviral immunity in *Drosophila*. *Curr Opin Immunol* 22:4–9.
36. Kemp C, Imler JL (2009) Antiviral immunity in *Drosophila*. *Curr Opin Immunol* 21:3–9.
37. Dostert C, et al. (2005) The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of *Drosophila*. *Nat Immunol* 6:946–953.
38. Souza-Neto JA, Sim S, Dimopoulos G (2009) An evolutionary conserved function of the JAK-STAT pathway in anti-dengue defense. *Proc Natl Acad Sci USA* 106:17841–17846.
39. Daher KA, Selsted ME, Lehrer RI (1986) Direct inactivation of viruses by human granulocyte defensins. *J Virol* 60:1068–1074.
40. Ding J, Chou YY, Chang TL (2009) Defensins in viral infections. *J Innate Immun* 1:413–420.
41. Guo X, et al. (2010) Response of the mosquito protein interaction network to dengue infection. *BMC Genomics* 11:380.
42. Troyer JM, et al. (2001) A live attenuated recombinant dengue-4 virus vaccine candidate with restricted capacity for dissemination in mosquitoes and lack of transmission from vaccinees to mosquitoes. *Am J Trop Med Hyg* 65:414–419.
43. Nene V, et al. (2007) Genome sequence of *Aedes aegypti*, a major arbovirus vector. *Science* 316:1718–1723.
44. Molina-Cruz A, et al. (2008) Reactive oxygen species modulate *Anopheles gambiae* immunity against bacteria and *Plasmodium*. *J Biol Chem* 283:3217–3223.
45. Dong Y, et al. (2006) *Anopheles gambiae* immune responses to human and rodent *Plasmodium* parasite species. *PLoS Pathog* 2:e52.
46. Garver L, Dimopoulos G (2007) Protocol for RNAi assays in adult mosquitoes (*A. gambiae*). *J Vis Exp* (5):230.
47. Salazar MI, Richardson JH, Sánchez-Vargas I, Olson KE, Beaty BJ (2007) Dengue virus type 2: Replication and tropisms in orally infected *Aedes aegypti* mosquitoes. *BMC Microbiol* 7:9.
48. Das S, Garver L, Ramirez JR, Xi Z, Dimopoulos G (2007) Protocol for dengue infections in mosquitoes (*A. aegypti*) and infection phenotype determination. *J Vis Exp* (5):220.
49. Waterhouse RM, et al. (2007) Evolutionary dynamics of immune-related genes and pathways in disease-vector mosquitoes. *Science* 316:1738–1743.
50. Dahlquist KD, Salomonis N, Vranizan K, Lawlor SC, Conklin BR (2002) GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways. *Nat Genet* 31:19–20.