Supplementary Materials for

Wolbachia Invades *Anopheles stephensi* Populations and Induces Refractoriness to *Plasmodium* Infection

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Material and Methods

<u>Mosquitoes</u>

A. stephensi Liston strain (LIS) mosquitoes were received from Johns Hopkins Malaria Research Institute. Both *A. stephensi* and *A. albopictus* Houston strain were maintained on sugar solution at 27°C and 85% humidity with a 12-hr light/dark cycle according to standard rearing procedures. Female mosquitoes, 5-7 days post-eclosion, were fed on the blood of anesthetized white mice to initiate egg development.

<u>Embryonic microinjection</u>

Embryonic microinjection was conducted as reported previously (*12, 24*). Briefly, injection needles (Quartz with filament, O.D.: 1.0 mm, I.D.: 0.70 mm, 10 cm length) were pulled with a P-2000 micropipette puller (Sutter Instrument Co.; Novato, CA), and then beveled with Microelectrode Beveler BV-10 (Sutter Instrument Co.; Novato, CA). Cytoplasm was withdrawn from *A. albopictus* Houston embryos and immediately injected into the posterior of 60-90 min old *A. stephensi* embryos using an IM300 microinjector (Narishige Scientific; Tokyo, Japan). After injection, the embryos were transferred to wet filter paper, which was then placed in water. After incubation at 80% relative humidity and 27°C for 2-3 days, larvae were hatched (G_0) and reared using standard maintenance conditions (*25*). Screening for the transinfected line was conducted as described previously (*12, 24*). In brief, G_0 females were isolated as virgins and mated with LIS males. Following oviposition, G_0 females were assayed for *Wolbachia* infection using PCR. G_0 females testing negative for *Wolbachia* infection were discarded along with their progeny. G_1 females from the infected G_0 female were sib mated, blood fed, isolated and allowed to oviposit. Following oviposition, G_1 females were PCR assayed for *Wolbachia* infection. G_1

females testing negative for *Wolbachia* infection were discarded along with their progeny. In one experiment, six larvae were hatched from 400-500 injected eggs. Of the four individuals that survived to adulthood, only one infected female (G_0) successfully produced progeny, resulting in seventeen isofemale (G_1) lines. Four of them were confirmed to be infected by *Wolbachia* *w*AlbB, and one line (designated LB1) with a stable association was chosen for the studies. Beginning in G_8, 30 virgin LB1 females were outcrossed with 30 wild-type LIS males for four generations to establish an outcrossed line (LB1.out). To generate aposymbiotic line LBT, the LB1 strain was fed with 10% sucrose containing 1 mg/ml tetracycline solution for 2 consecutive generations. Removal of *Wolbachia* from mosquito was confirmed by PCR in the subsequent generations. After treatment, mosquitoes were reared in a regular condition (*25*) for at least two generations before used for experiments with the goal to recover and re-colonize gut bacteria (*4*).

Fluorescence *in situ* hybridization (FISH)

Ovaries, fat bodies, midguts and salivary glands were dissected from 5-day-old non-blood-fed females and fixed for 15 min in freshly prepared 4% formaldehyde in PBS with 0.1% Tween 20. Hybridization was conducted following the manufacturer's instructions (GeneDetect, Bradenton, FL) using buffer containing 150 ng.ml^{-1} probes at 37°C for 48 h. Two AlexaFluor 488 5'-end-labeled 16S rDNA *Wolbachia* probes (synthesized by Invitrogen; Carlsbad, CA) with the following sequences were used: W2, 5'-CTTCTGTGAGTACCGTCATTATC -3' (*26*); Wol3, 5'-TCCTCTATCCTCTTTCAATC -3' (*27*). After wash, 0.1 ug/ml 4' 6'-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen, Carlsbad, CA) and 1 ug/ml propidium iodide (PI; Invitrogen, Carlsbad, CA) were applied to counterstain the samples. Samples were viewed with an Olympus FluoView FV1000 confocal microscope.

CI crosses

CI assay was conducted as previously described (*12*). Briefly, ten virgin females were mated with ten virgin males. Mated females were blood fed weekly using mice. Oviposition sites were available constantly to females, and oviposition paper was changed weekly. After egg maturation for 2 days on wet filter paper, eggs were immersed into water. Two days later, the egg hatch rate was determined by counting under a microscope.

Population Cages

The population cage experimental design was as previously described (*1, 10*) with a slight modification. To promote the population replacement and simulate a potential downstream application, 100 LB1 males were released at every generation, to cause CI and suppress the effective mating of LIS females. Each population cages started with fifty LIS females and fifty LIS male adults (both male and female were 2 days old). Three days after cage establishment, cages were provided with mice for blood feeding, followed by release of blood-fed LB1 females into the cages. The number of LB1 females introduced into cages was varied to make the initial female infection frequency as 20% 10% and 5%. There is no further LB1 female release after the generation 0. All cages were maintained identically. Oviposition sites were provided in population cages two days post blood meal. Eggs were collected for two consecutive nights, matured for additional two days, and then hatched. All hatching larvae were reared to adults, and fifty females and fifty males were randomly selected to establish the next generation. After eggs were collected at each generation, approximately 10-20 females were randomly selected in

the cages and examined for *Wolbachia* infection by PCR to determine the female infection frequency.

P. falciparum infection assays

To determine the anti-*Plasmodium* effect of *Wolbachia*, the wild type LIS, the transinfected LB1 and the aposymbiotic line LBT were fed on either 0.01% or 0.05% of NF54 *P. falciparum* gametocyte cultures (provided by the Johns Hopkins Malaria Institute Core Facility and Sanaria) (*23, 28*) through membranes at 37 °C. Seven-day-old mosquitoes were starved for 8-10 hr prior to feeding to ensure engorgement. To determine oocyst numbers, unfed mosquitoes were removed after 24 hr, and the rest were incubated for a further 7 to 9 days at 27 °C for *P. falciparum*. Midguts were dissected out in PBS, stained with 0.2% mercurochrome, and examined using a light-contrast microscope (Olympus). At least three biological replicas were performed for each experiment, and parasites from different replicates were pooled for producing the dot-plot through GraphPad Prism5 software.

Ookinete counting in the mosquito guts and lumen was done according to established methodology (*23, 28*), with minor modifications. The guts, including the entire bloodmeal contents, were placed in Corning 96-well plates with 20 μl of sterile PBS and individually homogenized by repeated pipetting; 10 μl of this homogenate was then spotted onto Teflon®-printed microwell glass slides (VWR International), left to air-dry and fixed with methanol. These slides were then stained with Giemsa for 45 min and analyzed under a Nikon E800 microscope. The total number of ookinetes in each spotted sample was counted, and average values for the densities of the ookinetes were calculated from at least two biological replicates.

These average values were then multiplied by the dilution factor of the sample (1 in 2) to give an estimate of the total number of ookinetes present within the entire blood meal.

To determine the sporozoite loads in the salivary glands of the infected mosquitoes, salivary glands were dissected, and individual glands were placed in Eppendorf tubes with 120 μl of PBS, then homogenized (on ice). The homogenate was centrifuged at 8,000 rpm for 10 min, followed by the removal of approximately 90 μl of supernatant. The sporozoites were resuspended in a final volume of 30 μl of PBS, and 10 μl of this suspension was placed in a Neubauer counting chamber and allowed to settle for 5 min before sporozoites were counted using a Leica phase-contrast microscope at 400x magnification.

Wolbachia Quantitative PCR (q-PCR)

qPCR was performed to measure the *Wolbachia* density in midguts, salivary glands, fat bodies and ovaries of LB1 mosquitoes (*29*). After the tissues are dissected and collected in PBS, genomic DNA was extracted as previously. The primers specifically directed to *Wolbachia* surface protein (wsp) of *w*AlbB were used in PCR to measure the *Wolbachia* genome copy, which was normalized with the host ribosomal protein S6 (RPS6) gene of *A. stephensi*. RPS6 was amplified with the primers: For (5'-ACGACCACAAGCTGCGTCAC-3') and Rev (5'-GTCAGCACACCCTGCTTCATG-3'). A standard curve was generated for *w*AlbB by analyzing 10^1 to 10^8 copies/reaction of the pQuantAlb plasmid (*29*). Another plasmid containing the RPS6 fragment was cloned to generate another standard curve for RPS6.

Hydrogen Peroxide assay

Midguts, fat bodies and whole bodies of 7-day-old LB1 and LIS mosquito were collected in 1X

PBS containing 2 mg/ml catalase inhibitor 3-amino-1,2,4-trizole(3-AT) (*30*). After

homogenization, samples were filtered through a 10K MW cut-off spin filter, Corning Spin-XUF

(Corning Incorporated Life Sciences, Lowell, MA, USA). Then, the elution from each

experiment group was collected and tested using Hydrogen Peroxide Assay Kit (BioVision, Inc.,

San Francisco, CA, USA). The fluorescence intensity was detected with Ex/Em = 550/590 using

Flu multi-detection microplate reader (BMG Labtech, Offenburg, Germany) according to the

manufacturer's instruction. The values were normalized by the total amount of proteins in the

sample, as determined by Piece BCA protein assay (Thermo Scientific, Rockford, IL, USA) (*14*).

Fig. S1.

Perfect maternal transmission of the *w*AlbB *Wolbachia* infection in LB1 mosquitoes. (A)
Ten males and 10 females were randomly selected from the transinfected LB1 population cages
at G_9, G_{10} and G_{11}. All were positive by PCR. (**B**) Primers specific for the *Wolbachia w*AlbA and
*w*AlbB strains were used to confirm that all the female LB1 mosquitoes at G_9 carried only a
*w*AlbB infection. +, *A. albopictus* Houston strain; -, *A. stephensi* LIS strain.

Fig. S2.

Oocytes of LB1 and LIS female stained with *Wolbachia*-specific FISH probes. The oocytes
were collected 3 days after females took a blood meal. FISH staining was carried out as
previously described (*12*).

Fig. S3.

A reduction in egg hatch rate associated with *w*AlbB. LB1.out is the mosquito line derived by outcrossing the transinfected LB1 females with the wild-type LIS males for four generations. LBT is the mosquito line generated by tetracycline treatment of the LB1 strain to remove *Wolbachia.* Statistical significance is represented by letters above each column, with different letters signifying distinct statistical groups. There is no significant difference in egg hatch rate between the LB1 and LB1.out strains. However, the egg hatch rate of the LBT strain is significantly higher than those of both LB1 and LB1.out strains (P<0.0001, Chi-square test).

Table S1.

Ookinetes, ooycsts, and sporozoites in wild-type LIS, LB1 and LBT mosquitoes. *Pf, Plasmodium falciparum.*

Fig. 2A (Pf ookinetes)	LIS (wt)	LB1	LBT
n=	36	36	36
range	0-454	0-302	24-290
prevalence	94.4%	97.2%	100.0%
Chi-square test p-value		0.4977	0.0289
median (with zeros)	115	103	113
mean (with zeros)	161.6	118.6	119.7
% to control (median)		89.6%	98.3%
Kruskal-Wallis p-value		0.3562	
Dunn's Multiple Comparison Summary		ns	ns
Mann-Whitney test p-value		0.2303	0.2051
Fig. 2B (Pf oocysts)	LIS (wt)	LB1	LBT
n=	152	142	110
range	0-182	0-128	0-126
prevalence	96.7%	86.6%	92.7%
Chi-square test p-value		0.0165	0.3311
median (with zeros)	20	9	12
mean (with zeros)	26.3	16.7	21.2
% to control (median)		45.0%	60.0%
Kruskal-Wallis p-value		< 0.0001	

	LIS (wt)		
Dunn's Multiple Comparison Summary		***	ns
Mann-Whitney test p-value		<0.0001	0.1162
Fig. 2C (Pf sporozoites)	LIS (wt)	LB1	LBT
n=	33	25	32
range	0-107100	0-18225	0-23025
prevalence	97.0%	96.0%	100.0%
Chi-square test p-value		1.0000	0.2462
median (with zeros)	8250	2400	8887.5
mean (with zeros)	14745.5	4911.0	9438.3
% to control (median)		29.1%	107.7%
Kruskal-Wallis p-value		0.0002	
Dunn's Multiple Comparison Summary		***	ns
Mann-Whitney test p-value		0.0001	0.9008

LB1

LIS
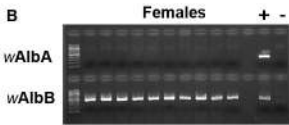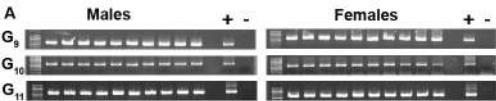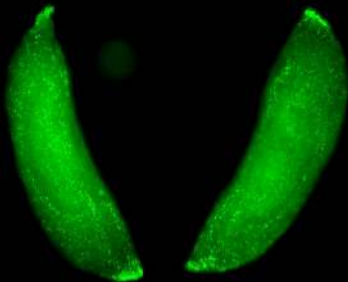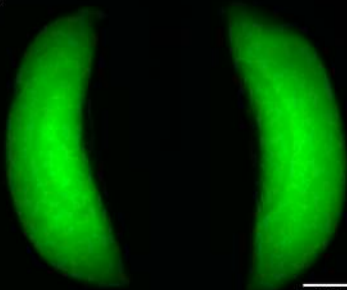
100µm

100µm
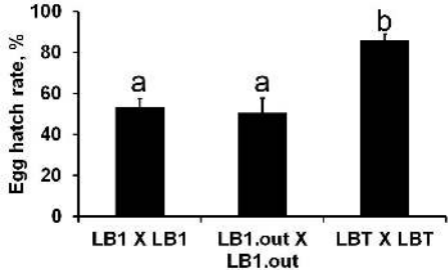
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identified as inhibitory for heart field specification (19, 20).

To determine whether Wnt signaling is required to promote PC fate, we microinjected cells expressing the soluble Wnt antagonist Crescent (19, 20) adjacent to PC precursors before their specification. After 8 hours, PC precursors were explanted and allowed to differentiate ex vivo. Exposure to Crescent decreased the slope of PC phase 4 depolarization by 65% relative to control injections (Fig. 4, B and D). These experiments could not rule out the possibility that Crescent is interacting with factors not associated with canonical Wnt signaling. Therefore, to further demonstrate that Wnt signaling was capable of inducing PC fate, we injected Wnt-expressing cells into the presumptive heart fields. This resulted in a 69% increase in phase 4 slope (Fig. 4, C and D). We then used Bio, a pharmacological inhibitor of glycogen synthase kinase 3 (GSK3) that has been shown to stabilize β-catenin (21, 22) to activate Wnt signaling in the heart field. Consistent with the findings above, 10 μM Bio increased diastolic slope in heart field explants relative to control cells (fig. S10).

When we allowed injected embryos to develop to late looping stages, aberrant Wnt signaling led to severe morphological defects, consistent with previous reports (Fig. 4, F and H) (23). Crescent injection adjacent to PC precursors led to the ectopic expression of Nkx2.5 in PC at St18, which is in agreement with a conversion of PC into a more working myocardial fate (24) (Fig. 4, E and F). About 35% of Wnt-injected embryos survived to heart looping stages. Wnt introduction into the primary and secondary heart field mesoderm resulted in irregularly contracting hearts, with decreased Nkx2.5 expression on the injected side of the embryo (Fig. 4, G and H). To confirm that these Nkx2.5-negative regions were still electrically active, we performed optical mapping. Consistent with a Wnt-based conversion of working myocardium into PC-like cells, we detected retrograde propagation (outflow toward inflow) as well as ectopic pacemaker sites (movie S8). These ectopic sites were restricted to the Wnt-injected side of the embryo and displayed AP shapes similar to those of control PCs (Fig. 4, I and J, and movie S8).

These findings suggest that early mesodermal Wnt-mediated cues are sufficient to induce pacemaker-like fates that do not manifest until late looping stages. However, Wnts are broadly and bilaterally expressed in the posterior mesoderm, so it is likely that additional cues are required to restrict PC fate, including laterality genes (25, 26). The early diversification of PC fate from the working myocardium suggests that fate specification is assigned directly in the lateral plate mesoderm, and is not the result of the specialization of an already functional embryonic myocyte. These data establish a framework through which PC development should be viewed, thereby providing a foundation for tissue engineering and stem cell–based approaches for PC generation.

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Wolbachia Invades Anopheles stephensi Populations and Induces Refractoriness to Plasmodium Infection

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Wolbachia is a maternally transmitted symbiotic bacterium of insects that has been proposed as a potential agent for the control of insect-transmitted diseases. One of the major limitations preventing the development of Wolbachia for malaria control has been the inability to establish inherited infections of Wolbachia in anopheline mosquitoes. Here, we report the establishment of a stable Wolbachia infection in an important malaria vector, Anopheles stephensi. In A. stephensi, Wolbachia strain wAlbB displays both perfect maternal transmission and the ability to induce high levels of cytoplasmic incompatibility. Seeding of naturally uninfected A. stephensi populations with infected females repeatedly resulted in Wolbachia invasion of laboratory mosquito populations. Furthermore, wAlbB conferred resistance in the mosquito to the human malaria parasite Plasmodium falciparum.

The ability of Wolbachia to spread through cytoplasmic incompatibility (CI) (1, 2) and render mosquitoes resistant to a variety of human pathogens (3–6) has instigated the development of Wolbachia-based strategies for both suppression and replacement of disease vector populations (2, 7). Given their medical importance, there have been considerable efforts to extend this approach to anopheline malaria vector mosquitoes, which are not naturally infected by Wolbachia spp. (8). Over the past two decades, various attempts to artificially generate stably infected Anopheles spp. have failed, raising concern that the Anopheles germ line is inhospitable to Wolbachia or that Wolbachia infection might cause reproductive ablation in Anopheles mosquitoes (6). Studies based on a transient somatic infection have recently indicated that Wolbachia can inhibit the development of the malaria parasite in the Anopheles mosquito, possibly by stimulating a mosquito antiparasitic immune response (5, 6). These results reinforced the potential of a Wolbachia-based intervention for malaria vector control, but only if the bacterium could be made to form a stable association with this mosquito.

Anopheles stephensi is the major vector of human malaria in the Middle East and South Asia. We

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infected *A. stephensi* [Liston strain (LIS)] by embryonic microinjection of the *w*AlbB *Wolbachia* strain derived from *Aedes albopictus* (Houston strain) (*9*). Cytoplasm was withdrawn from *A. albopictus* embryos and directly injected into the posterior of *A. stephensi* early embryos (*1*). After oviposition, we used polymerase chain reaction (PCR) to test females (G$_0$) developed from surviving embryos for *Wolbachia* infection. We observed a stable *w*AlbB infection in one isofemale line (designated LB1) at G$_1$ with a 100% infection frequency maintained through G$_{34}$ (the last generation assayed thus far). At G$_9$, G$_{10}$, and G$_{11}$, we randomly selected 20 individuals (10 males and 10 females) from the LB1 cage population and tested them by diagnostic PCR (*10*). All

individuals (*n* = 60) were infected with *w*AlbB (fig. S1).

The 100% maternal transmission efficiency was also confirmed by fluorescence in situ hybridization (FISH) of LB1 mosquito ovaries showing heavy *w*AlbB infection of all ovarian egg chambers. In the ovaries of the 5-day-old non–blood-fed females, *w*AlbB was mainly found in the oocytes of the egg chambers, with a low-level presence in nurse cells (Fig. 1A). This observation is consistent with a previous model showing *Wolbachia* migration from nurse cells to the oocytes through the ring canals during oogenesis (*11*). As in *A. albopictus* and the transinfected *Aedes aegypti* WB1 line (*1*, *12*), *Wolbachia* was concentrated in the anterior and posterior part of LB1 mos-

quito oocytes 3 days after a blood meal (fig. S2), indicating that the *w*AlbB distribution pattern in the ovaries is conserved between mosquito species.

Of 8087 eggs resulting from crosses between LB1 males and the naturally uninfected LIS females, only 1.2% (95% confidence interval = 0.15 to 2.16) hatched (Fig. 1B), indicating a typical CI pattern. We observed a >50% egg-hatch rate in the other cross types. The egg hatches resulting from LB1 self-crosses (52.4%) were significantly lower than those observed in compatible crosses of wild-type individuals (91.0%; *P* < 0.01, χ^2 = 2016.4). Outcrossing of the LB1 females with LIS males for four generations did not improve the egg-hatch rate, but tetracycline treatment of the outcrossed line increased the rate to 85.9 ± 5.3% (fig. S3), supporting the hypothesis that the *w*AlbB infection is responsible for the reduced hatch rate.

To assess the ability of the *w*AlbB infection to invade a natural uninfected population, we seeded LB1 females at ratios of 5, 10, and 20% into uninfected LIS cage populations composed of 50 females and 50 males. To promote population replacement, we also released 100 LB1 males at every generation to suppress the effective mating of LIS females. In all populations, *w*AlbB increased to 100% infection frequency within eight generations and remained fixed in subsequent generations (Fig. 1C). These results support the potential for *Wolbachia* to mediate population replacement in a public health intervention strategy. Specifically, the *w*AlbB infection was able to invade and replace the naturally uninfected cytotype within eight generations after an introduction rate as low as 5% and continued inundative release of males at a rate of two times the male population size each generation. These results also raise the challenge in application that large-scale programs for breeding and releasing male infected mosquitoes might be necessary, perhaps in conjunction with short-term intensive mosquito abatement.

A transient *Wolbachia* infection in *Anopheles gambiae* mosquitoes is known to inhibit *Plasmodium falciparum* development (*5*, *6*). To assess the possible anti–*P. falciparum* activity of *w*AlbB in the transinfected LB1 mosquitoes, we fed them on a gametocyte culture, along with LIS
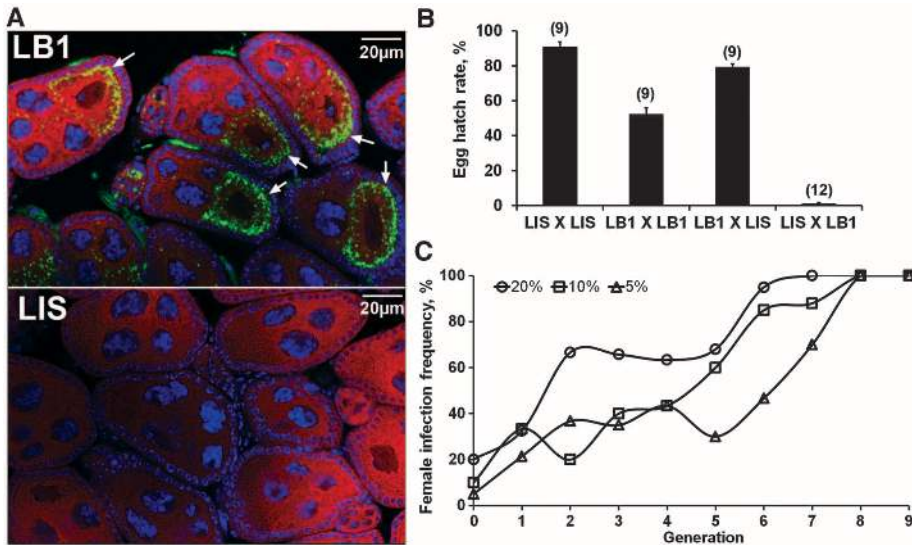

Fig. 1. Establishment and invasion of *Wolbachia* *w*AlbB in *A. stephensi* populations. (**A**) *w*AlbB distribution in the ovarian egg chambers of 5-day-old non–blood-fed LB1 females with LIS females as controls. *Wolbachia*, cytoplasm, and nuclear DNA were stained with 16S ribosomal DNA *Wolbachia* probes (green), propidium iodide (red), and 4′,6-diamidino-2-phenylindole (blue), respectively. White arrows indicate *Wolbachia*. (**B**) *w*AlbB induces nearly complete CI in *A. stephensi* when infected males are crossed with uninfected females. Error bars indicate SE. The number of replicates for each of the four cross types is shown in parentheses. (**C**) *w*AlbB invades the *A. stephensi* laboratory populations. Female infection frequency was measured by PCR after a single release of LB1 females into LIS populations and continued inundative release of LB1 males at a rate of twice the male population size for each generation.
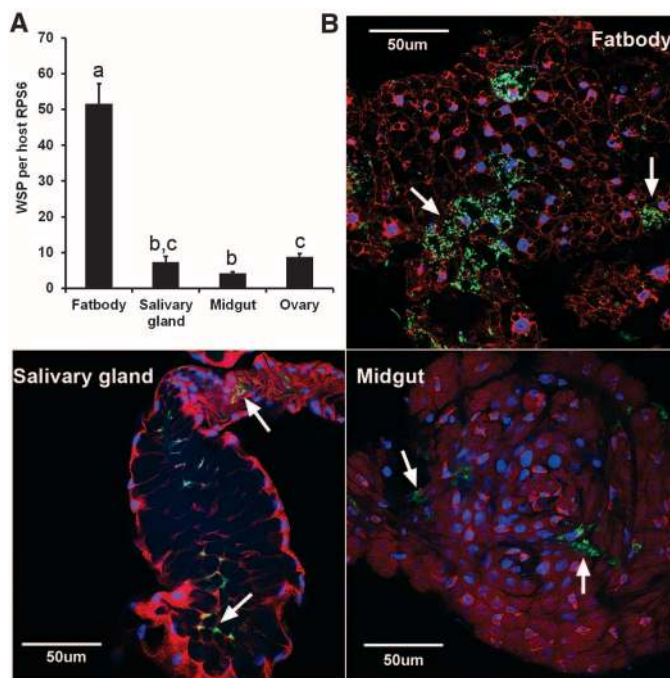
Fig. 2. *Wolbachia* *w*AlbB-mediated inhibition of *Plasmodium* development. *P. falciparum* ookinete (**A**), oocyst (**B**), and sporozoite (**C**) loads in midgut lumens, midguts, and salivary glands, respectively, of *A. stephensi* LIS, LB1, and LBT strains. Points represent the number of parasites from an individual mosquito; horizontal lines indicate the median number of para-

sites per tissue. Different letters above each column signify distinct statistical groups [(**B**) *P* < 0.0001 for LB1 versus LIS and *P* < 0.01 for LB1 versus LBT; (**C**) *P* < 0.001 for both LB1 versus LIS and LB1 versus LBT; Mann-Whitney test].
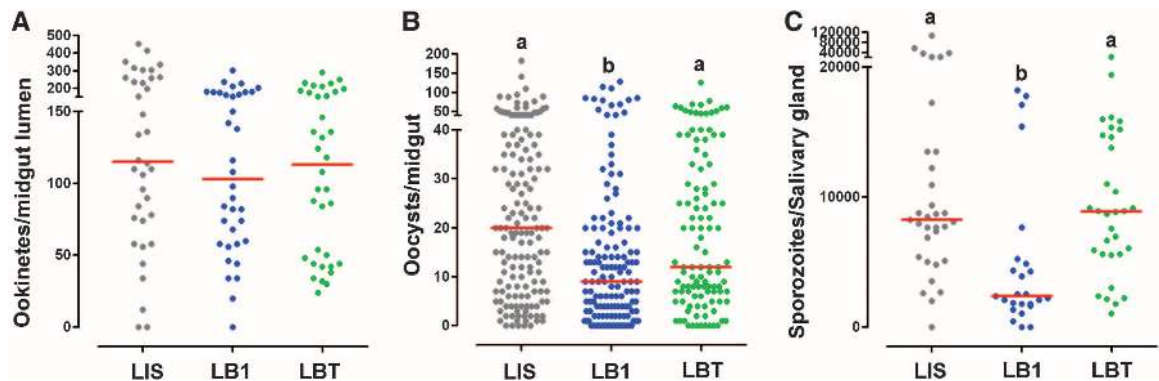
and the aposymbiotic line LBT mosquitoes (generated by tetracycline treatment of the LB1 strain to remove wAlbB) as controls. Although the presence of wAlbB had no impact on the ookinete stage parasites before midgut invasion (Fig. 2A and table S1), it did result in a significantly reduced prevalence and mean intensity of the oocyst stage parasite on the basal side of the midgut, as assayed at 7 days postinfection (dpi). Specifically, the LB1 strain displayed significantly lower infection prevalence and intensity than the LIS strain (Mann-Whitney U test, $P < 0.0001$) and the aposymbiotic LBT strain (Mann-Whitney U test, $P < 0.01$), whereas no difference was observed between the LIS and LBT strains (Fig. 2B and table S1). We also investigated the impact of wAlbB on the salivary gland sporozoite stage infection at 14 dpi and observed a greater inhibition than at the oocyst stage (Fig. 2C and table S1). wAlbB infection resulted in a 3.4- and 3.7-fold reduction in the sporozoite loads in salivary glands of LB1 mosquitoes when compared with LIS and LBT mosquitoes, respectively (Mann-Whitney U test, $P < 0.001$) (Fig. 2C and table S1). These data suggest that wAlbB inhibit P. falciparum development between the preinvasion lumenal ookinete and oocyst stages and between the oocyst and salivary gland sporozoite stages.

A local distribution of wAlbB and wMelPop strains in mosquito somatic tissues, especially those in which pathogens replicate, develop, and travel, is important for Wolbachia to induce pathogen interference (4, 13). We examined the wAlbB density in midguts, salivary glands, and fat bodies from 7-day-old LB1 non–blood-fed females by real-time PCR. We detected Wolbachia wAlbB in all tissues, with a marked 5.9-fold higher density in the fat bodies than in the ovaries (Fig. 3A).

Salivary glands and ovaries contained similar levels of wAlbB, whereas midguts had lower infection than ovaries, a distribution confirmed by FISH assay (Fig. 3B). This result is similar to observations made in the transiently infected A. gambiae, in that Wolbachia resided primarily within cells of the fat bodies and had a low affinity for midgut cells (6).

We have previously shown that wAlbB induces the production of reactive oxygen species (ROS) in Aedes (14), and other work has shown that ROS can inhibit Plasmodium infection in Anopheles (15, 16). To explore whether wAlbB-induced ROS could play a role in the mosquitoes' resistance to Plasmodium, we compared the levels of H_2O_2 in midguts, fat bodies, and whole bodies of LB1 and LIS mosquitoes. The levels of H_2O_2 were significantly higher in tissues of LB1 mosquitoes than in those of LIS mosquitoes (Student's t test, $P < 0.01$) (Fig. 4) and nearly twofold higher in whole LB1 than in LIS mosquitoes.

In conclusion, we show that the Wolbachia wAlbB strain can form a stable symbiosis with A. stephensi, invade laboratory mosquito populations through CI, and confer elevated resistance to Plasmodium infection, potentially through ROS generation. Previous failures in establishing a stable Wolbachia infection in Anopheles mosquitoes may be due to the Wolbachia strains used. To form a symbiosis, the Wolbachia strain should be sufficiently invasive to establish an infection in germ tissues but without being lethal to the host. The success of wAlbB may be attributed to its ability to confer a fitness advantage to its host (10) and its high infectivity to Anopheles germ tissues (17). We used a previously described embryo microinjection technique (1) but observed a lower survivor rate, possibly due to the greater sensitivity of Anopheles eggs to desiccation. The

low egg-hatch rate associated with the wAlbB infection in A. stephensi, which is not observed in wAlbB-infected A. aegypti, may be related to the use of mouse (an unnatural host) blood in this study. A previous study has reported suppression of egg hatch after a long-distance transfer of wMelPop into A. aegypti feeding on nonhuman blood sources, but only mild decreases when the mosquitoes fed on human blood (18).

The recent success of a field trial has demonstrated that Wolbachia can be deployed as a practical dengue intervention strategy, with the potential for area-wide implementation (2). The design of Wolbachia-based malaria control strategies would have to accommodate the fact that Plasmodium is vectored by multiple and frequently sympatric Anopheles species in different parts of the world (19, 20). However, this complication can be resolved by integrating a Wolbachia-based approach with other vector control strategies and by targeting the dominant malaria vectors that are the most difficult to control. For example, Wolbachia could be used to target outdoor-biting and -resting species that can evade current vector control methods, such as insecticide-treated nets and residual insecticide sprays (21). In our studies, we used a laboratory P. falciparum infection model that results in unnaturally high infection intensities, reaching a median of 20 oocysts per midgut, whereas infection levels in nature rarely exceed 2 to 3 oocysts (22). As we have shown in other studies comparing natural and laboratory infection intensities (23), it is quite likely that a stable wAlbB infection would confer complete refractoriness under natural field conditions. Our success in rendering A. stephensi resistant to P. falciparum by stable introduction of wAlbB offers a potential approach to permanently reduce the vectorial capacities of dominant malaria vectors in sub-Saharan Africa, one of the most challenging goals in current malaria vector control (21). However, it is still unknown whether Plasmodium will develop resistance to ROS or other Wolbachia-mediated inhibitory mechanisms in mosquitoes.

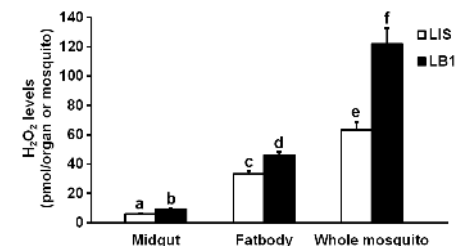**Fig. 3. Wolbachia wAlbB distribution in somatic tissues of LB1 mosquitoes (G_{27}). (A)** The genome copy of Wolbachia surface protein (WSP) was measured by real-time PCR and normalized by A. stephensi ribosomal protein S6 (RPS6). Different letters above each column signify distinct statistical groups ($P < 0.05$ for comparison between a, b, and c; Student's t test). Error bars indicate SEM of at least 10 biological replicates. **(B)** Wolbachia wAlbB distribution in fat body, midgut, and salivary gland of an LB1 mosquito, assayed by FISH as described in Fig. 1A. White arrows indicate Wolbachia.

Fig. 4. Wolbachia-induced ROS production in LB1 mosquitoes. This figure shows a comparison of H_2O_2 levels in the fat body, midgut, and whole mosquito in 7-day-old LB1 and LIS females before a blood meal. The data shown are means of 6 (fat body and midgut) or 10 (whole-mosquito) replicates. Different letters above each column signify distinct statistical groups ($P < 0.01$ for each pair of comparison between a, b, c, d, e, and f; Student's t test). Error bars indicate SEM.

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Delineating Antibody Recognition in Polyclonal Sera from Patterns of HIV-1 Isolate Neutralization

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Serum characterization and antibody isolation are transforming our understanding of the humoral immune response to viral infection. Here, we show that epitope specificities of HIV-1–neutralizing antibodies in serum can be elucidated from the serum pattern of neutralization against a diverse panel of HIV-1 isolates. We determined "neutralization fingerprints" for 30 neutralizing antibodies on a panel of 34 diverse HIV-1 strains and showed that similarity in neutralization fingerprint correlated with similarity in epitope. We used these fingerprints to delineate specificities of polyclonal sera from 24 HIV-1–infected donors and a chimeric siman-human immunodeficiency virus–infected macaque. Delineated specificities matched published specificities and were further confirmed by antibody isolation for two sera. Patterns of virus-isolate neutralization can thus afford a detailed epitope-specific understanding of neutralizing-antibody responses to viral infection.

Upon infection or vaccination, the adaptive immune system typically generates polyclonal antibody responses that recognize multiple epitopes (*1–3*). The serologic characterization of such polyclonal responses can inform vaccine design by elucidating which epitopes on the antigen are immunodominant and/or targets of pathogen-specific neutralizing antibodies. Such serologic analysis can further lead to the isolation of new monoclonal antibodies that may be of therapeutic value. As a result of extensive effort to understand the antibody response to viral infection, recent years have seen a surge in the isolation of monoclonal antibodies against HIV-1, influenza, hepatitis C, and other viruses (*4–15*). The link between polyclonal sera and component monoclonal antibodies, however, remains complex and difficult to decipher, in part, because of the extraordinary diversity of circulating antibodies. Viral genetic diversity can be an integral mechanism of immune evasion (*16–22*); this same diversity may, however, also provide a means by which to understand antibody responses (*23, 24*). Specifically, monoclonal antibodies targeting the same epitope on an antigen are likely to be affected in a similar way by diversity in that epitope region. When presented with a diverse set of viral isolates, monoclonal antibodies may thus exhibit characteristic neutralization patterns or "neutralization fingerprints" (Fig. 1). Furthermore, neutralization patterns of a polyclonal serum could be viewed as the combined effect of the neutralization fingerprints of component monoclonal antibodies, and, if this relationship could be deconvoluted, then serum neutralization would serve as a predictor of component-antibody specificity.

To test this conjecture, we selected HIV-1 because of its high viral sequence diversity, the availability of well-characterized sera and antibodies, and the limited number of sites of vulnerability targeted by neutralizing antibodies on the HIV-1 spike (Env). These sites encompass the CD4-binding site (CD4bs), a variable loop V1/V2 site, and a glycan-V3 site on glycoprotein gp120, and the membrane-proximal external region (MPER) on gp41 (*4–7, 13, 14, 25–35*). The same site of vulnerability may encompass multiple epitopes and, as a result, can be targeted by antibodies with diverse specificities. To determine whether the neutralization fingerprints of HIV-1 monoclonal antibodies are a reflection of their epitope specificities, we utilized neutralization data for a panel of 34 diverse HIV-1 isolates (table S1), for 30 monoclonal antibodies recognizing diverse epitopes on HIV-1 Env, and for two variants of the CD4 receptor (table S2). Neutralization fingerprints for antibodies known to target similar epitopes correlated significantly better (Spearman correlation) than fingerprints of antibodies targeting different epitopes (fig. S1). On the basis of the neutralization-correlation values, antibodies were grouped into 10 clusters (Fig. 2A) (*36*), by using a clustering cutoff chosen to agree with known antibody structures and epitope-mapping (*4–6, 13–15, 25–27, 37–40, 41*). Two antibodies, 8ANC195 and HJ16, whose precise epitopes are currently unknown, clustered separately (*5, 15*), whereas all of the other antibody clusters could be mapped to known sites of Env vulnerability.

Overall, neutralization fingerprints appeared to exhibit sufficient specificity to successfully distinguish between antibodies targeting different

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