MOLECULAR BIOLOGY

Versatile transgenic multistage effector-gene combinations for *Plasmodium falciparum* suppression in *Anopheles*

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The malaria parasite's complex journey through the *Anopheles* mosquito vector provides multiple opportunities for targeting *Plasmodium* with recombinant effectors at different developmental stages and different host tissues. We have designed and expressed transgenes that efficiently suppress *Plasmodium* infection by targeting the parasite with multiple independent endogenous and exogenous effectors at multiple infection stages to potentiate suppression and minimize the probability for development of resistance to develop. We have also addressed the fitness impact of transgene expression on the mosquito. We show that highly potent suppression can be achieved by targeting both pre-oocyst stages by transgenically overexpressing either the endogenous immune deficiency immune pathway transcription factor Rel2 or a polycistronic mRNA encoding multiple antiparasitic effectors and simultaneously targeting the sporozoite stages with an anti-sporozoite single-chain antibody fused to the antiparasitic protein Scorpine. Expression of the selected endogenous effector systems appears to pose a lower fitness cost than does the use of foreign genes.

INTRODUCTION

The malaria parasite *Plasmodium falciparum* is transmitted by *Anopheles* mosquitoes and causes malaria, which remains one of the most devastating diseases of humankind. The lack of effective vaccines and rapid development of drug-resistant parasites and insecticide-resistant mosquitoes have underscored the need to develop novel alternative strategies for disease control. Multiple approaches based on the development of engineered refractory mosquitoes incapable of transmitting the parasite have been explored and have gained leverage through the ongoing development of mosquito gene-drive technologies that can enable the replacement of malaria-susceptible mosquito populations with populations refractory to malaria (*1*–*4*).

Malaria parasites have to complete a complex journey in the mosquito vector to accomplish successful transmission to the human host. The insect's innate immune system and physical barriers play important roles in this process, with a variety of host and restriction factors influencing *Plasmodium*'s success in finally moving through the mosquito into the human host (*5*). Restriction factors can be used for *Plasmodium* blocking by being transgenically overexpressed in an appropriate tissue and at an appropriate time point to effectively target the parasites.

Most known *Plasmodium* restriction factors are components of the mosquito's innate immune system, and the extensive study of this field over the past 20 years has generated a plethora of promising candidates for transgenesis [reviewed in (*5*–*9*)]. Blood meal–inducible expression of the mosquito's nuclear factor κB (NF-κB) transcription factor Rel2, the positive regulator of the anti–*P. falciparum* immune deficiency (IMD) immune pathway, in the midgut or fatbody tissue results in potent suppression of multiple *P. falciparum* isolates without measurable fitness cost under laboratory conditions (*10*, *11*). One can also use exogenous restriction factors to suppress *Plasmodium* infection, either by directly killing the parasites or by interfering with their interaction with the midgut or salivary glands (*12*–*18*). A variety of antimicrobial peptides (AMPs) such as Melittin, Scorpine, and Shiva toxin have been shown to exert in vitro and in vivo anti-*Plasmodium* activity. Expression of a modified *P. falciparum* circumsporozoite protein (CSP)–targeting single-chain antibody (m2A10) has been shown to greatly decrease the ability of the parasites to reach and invade the *Anopheles* salivary glands (*19*). An example of small peptides that can block the parasite's interaction with the mosquito midgut is the *Plasmodium* enolase-plasminogen interaction peptide (EPIP), which prevents the binding of plasminogen to the ookinete surface during the parasite's invasion of the midgut epithelium (*16*, *20*). One can also combine parasite-binding single-chain antibodies with antiparasitic peptides to potentiate *Plasmodium* targeting and killing (*19*, *21*). Transgenic mosquitoes expressing a parasite-binding ScFv fused to the anti-*Plasmodium* peptide CecA have been able to achieve almost complete parasite suppression by targeting the sporozoites alone (*19*).

The malaria parasite has a notorious ability to develop resistance to antimalarial drugs, and this feature may also enable it to develop resistance to mosquito-encoded blocking mechanisms. For this reason, it is beneficial to engineer mosquitoes in ways that would suppress *Plasmodium* infection at multiple sporogonic stages through independent mechanisms, akin to using combinatorial drug therapy for pathogen suppression. Here, we have explored the *Plasmodium*-blocking efficacy of single or combinations of versatile endogenous and exogenous transgenic anti-*Plasmodium* restriction factors that can target multiple parasite stages in different mosquito body compartments. For targeting early stages of *Plasmodium* infection in the midgut tissue, we explored the carboxypeptidase promoter (*AgCp*)–driven expression of the endogenous IMD pathway transcription factor Rel2, which controls multiple anti–*P. falciparum* effectors (*10*), and the simultaneous expression of multiple anti-*Plasmodium* effectors (Melittin, TP10, Shiva1, EPIP, and Scorpine) from a single transgene array. For inhibition of post-ookinete infection stages, we used the blood meal–inducible fatbody-specific vitellogenin promoter (*AsVg*) to express either an anti-sporozoite stage single-chain antibody (ScFv of m2A10 targeting CSP) alone or fused with the antiparasitic protein

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Cecropin C (or Cec3, AGAP000694), *Apis mellifera* phospholipase PLA2 (XM_391951), or Scorpine toxin (*16*, *22*, *23*). We also explored the combination of both midgut- and fatbody-expressed anti-*Plasmodium* effector systems to optimize parasite suppression. Last, we have assessed the fitness of the various transgenic mosquito lines, as measured by longevity, size, and fecundity.

In summary, we have addressed the following key requirements for transgenic *Anopheles* mosquitoes that would render them suitable for a population suppression–based malaria control strategy: (i) effector transgenes that efficiently suppress *Plasmodium* infection; (ii) targeting of the malaria parasite with multiple independent effectors to potentiate suppression and minimize the probability for the development of resistance; (iii) spatiotemporal specificity in expressing these transgenes for effective targeting of different developmental stages of the parasite; and (iv) transgene selection to allow minimal fitness cost to the mosquitoes.

RESULTS

Targeting the malaria parasite in the midgut with multiple antiparasitic effectors

Several endogenous and exogenous transgenes have been shown to effectively suppress *P. falciparum* infection in the midgut tissue. We have previously shown that blood meal–inducible midgut expression of the IMD immune pathway transcription factor *Rel2* results in an up-regulation of multiple anti–*P. falciparum* effectors and a potent suppression of infection (*10*). The up-regulation of multiple effectors likely potentiates suppression and renders it more difficult for the parasite to develop resistance to the blocking mechanisms. Here, we have explored an alternative approach to unleash a multifactorial attack against the malaria parasite in the midgut tissue at the ookinete to pre-oocyst stage without the use of multiple independent transgenes (Fig. 1A). Previous studies have identified several small anti-*Plasmodium* peptides that suppress the parasites either through directly killing or by interfering with parasitic invasion of the midgut. We explored the blood meal–inducible midgut expression of a polycistronic mRNA encoding a polypeptide containing an array of five different anti–*P. falciparum* effectors separated by self-cleaving viral P2A sequences (Fig. 1B) (*24*). The five effectors are listed in Table 1: Melittin, TP10 dimer $(TP10)_2$, Shiva1, four repeats of the *Plasmodium* $EPIP_4$, and Scorpine (*16*, *20*, *22*, *23*, *25*, *26*). The bee venom AMP Melittin has previously been shown to have in vitro anti-*Plasmodium* killing activity at 50 μM (*27*). The self-cleaving viral P2A sequences may also have enhanced the anti-*Plasmodium* activity of Melittin through the additional C-terminal modification (EENPG) derived from P2A (*28*). The cell-penetrating peptide TP10 has broad-spectrum antiparasitic activity against the blood and mosquito stages of *P. falciparum* and blood-stage *Trypanosoma brucei* (*25*, *26*). Cell-penetrating peptides are molecules that can translocate into cells without causing membrane damage, and TP10 is one of the best characterized peptides of this class that can successfully translocate into various cell types (*25*, *26*). EPIP prevents the binding of plasminogen to the ookinete surface during parasite invasion, thereby also inhibiting the parasite-vector interaction (*16*, *20*). The scorpion venom protein Scorpine has antibacterial activity and can inhibit *Plasmodium* gametogenesis and ookinete formation at low concentrations both in vitro and in vivo (*16*, *22*, *23*). These five anti–*P. falciparum* effectors have previously been tested for anti-*Plasmodium* activity through in vitro or in vivo studies using synthetic peptides or para-transgenic approaches. Rather than developing individual transgenic lines for each effector, here, we explored the utility of expressing multiple antiparasitic effectors, through a single transgene cassette, for blocking the human malaria parasite.

The coding sequences for these five effectors were codon-optimized and synthesized through GenScript Inc. (Supplementary Materials). The transgene cassette (hereafter referred to as *MultiEff*) was fused to the *Anopheles gambiae* Cp (*AgCp*) promoter including its signal peptide sequences and terminated with the *A. gambiae* trypsin gene terminator (*Tryp*T), and then cloned into the piggyBac-based plasmid *pBac[3xP3-EGFPafm]* containing the eye-specific *3xP3* promoter–driven *eGFP* as an eye marker for screening of the positive transgenic mosquitoes at both the larval and adult stages, according to our previously published method (*10*). Through microinjection of the transformation construct plasmid, together with the *phsp-pBac* helper plasmid, into approximately 620 *Anopheles stephensi* embryos, we generated five pools of mosquitoes that were crossed with the wild type (AsWT), resulting in three *AgCp*-driven *MultiEff* transgenic mosquito lines (#2, #3, and #4) that showed stable expression of the GFP eye fluorescence in both larval and adult stages (Fig. 1C). Chromosomal integration of transgenes was confirmed by polymerase chain reaction (PCR) (Fig. 1D and table S1). The heterozygous G2 generation of the three transgenic lines was screened for *P. falciparum* suppression, allowing the selection of the MultiEff-2 line demonstrating the most potent anti–*P. falciparum* activity. The temporal expression specificity of the transgene was monitored through quantitative reverse transcription PCR (qRT-PCR), showing a >22-fold induction within a 6- to 12-hour time period after blood feeding (Fig. 1E).

To assess the resistance of homozygous transgenic MultiEff mosquitoes to *P. falciparum,* we fed them on both medium- and low-concentration gametocyte cultures (gametocytemia at 0.05 and 0.01%, respectively) and then investigated the infection phenotype. MultiEff transgenic mosquitoes displayed significant lower permissiveness to pre-oocyst stage *P. falciparum* when fed on blood with a medium gametocytemia (0.05%), resulting in an infection intensity unnaturally higher than that observed for field-caught mosquitoes. The midguts of the WT (AsWT) mosquitoes had a median of 19 oocysts and an infection prevalence of 88% (Fig. 1F and table S2). The prevalence at the oocyst infection stage decreased by 17.1% (Fisher's exact test, $P < 0.05$; Fig. 1F and table S2), and the median infection intensity was reduced to 39.5% of AsWT control mosquitoes (Mann-Whitney test, $P = 0.0034$; Fig. 1F and table S2). When the mosquito cohorts were fed on a fivefold lower gametocytemia (0.01%) to mimic a natural infection system, the MultiEff transgenic mosquitoes displayed strong parasite suppression, with a median oocyst count of 0, whereas the AsWT control mosquitoes had a median of 3.5 oocysts per midgut (Fig. 1G). The MultiEff transgenic mosquitoes displayed an infection prevalence of 45%, in contrast to 83% for the AsWT (Fig. 1G and table S2). Under natural infection intensity conditions, the MultiEff transgenic mosquitoes displayed a profound suppression that resulted in a median sporozoite count of zero in the salivary glands. The sporozoite infection prevalence showed a 1.6-fold decrease in the MultiEff transgenic mosquitoes (46% in the MultiEff versus 75% in the control mosquitoes) (Fig. 1H and table S2).

Mosquitoes harbor a microbiota, including an intestinal flora comprising a variety of bacteria and fungi that can influence vector competence for *Plasmodium* by stimulating the mosquito's innate immune system or through direct effects on the parasite (*29*). We

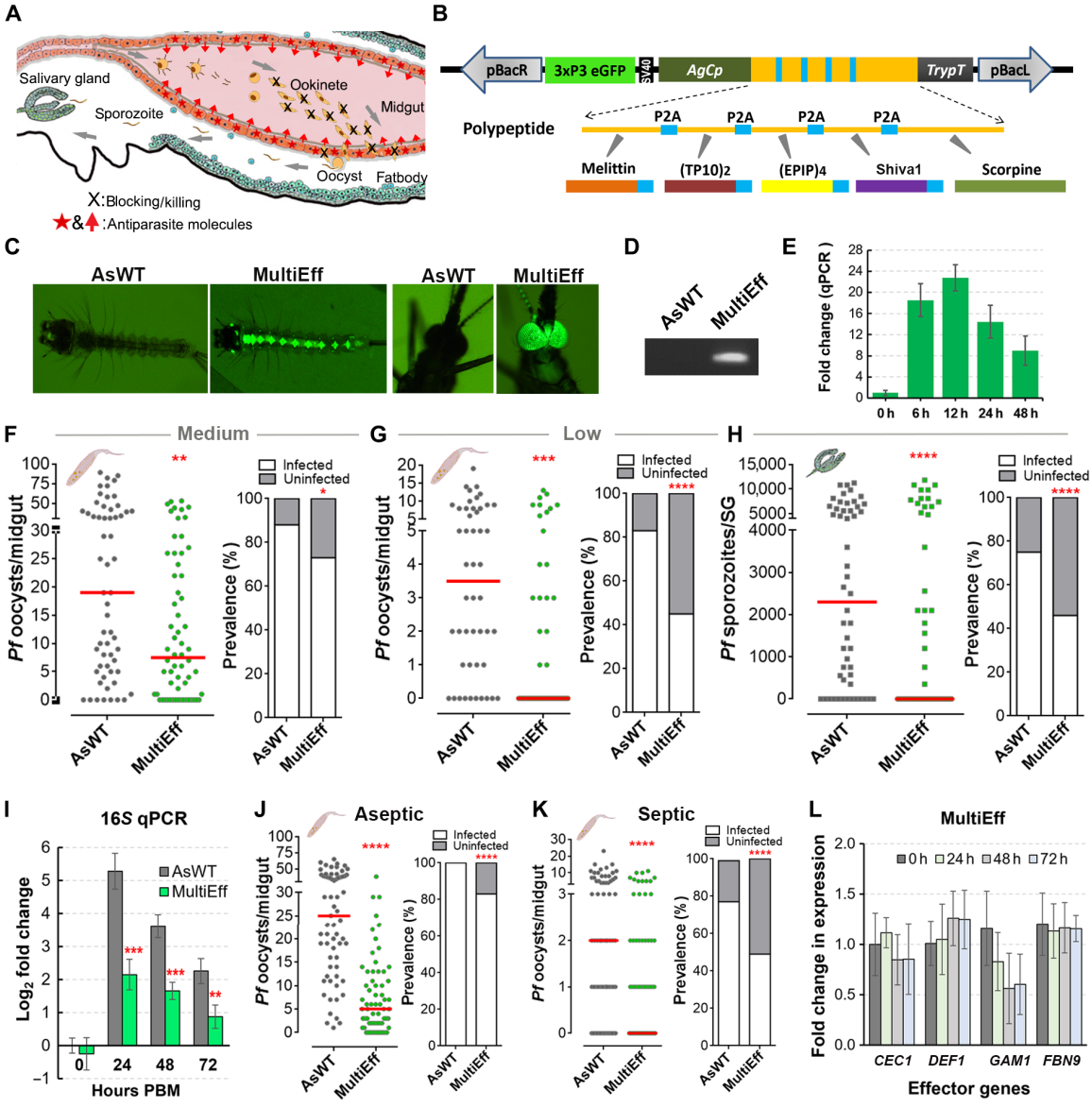

Fig. 1. Generation of transgenic mosquitoes (MultiEff) using an *AgCp*-driven transgene array with five anti-*Plasmodium* effectors targeting the malaria parasite in the midgut, and *P. falciparum* infection phenotypes at both oocyst and sporozoite stages. (**A**) Schematic illustration of transgenic targeting of parasite midgut infection stages and the design of the transgenes to target the parasites at this stage. (**B**) Five known anti-*Plasmodium* effectors (Melittin, TP10, EPIP, Shiva1, and Scorpine) were selected. The gene array with these five anti-*Plasmodium* effector genes was synthesized through GenScript Inc. and cloned under the *AgCp* promoter, followed by the trypsin terminator (*TrypT*) in the *pBac[3xP3-EGFPafm]* vector. These five antiparasitic effector genes were transcribed on one cassette separated by viral P2A sequences, translated into one polypeptide, and self-spliced into five individual peptides with an extra viral P2A amino acid tag on the first four peptides. (**C**) Fluorescent images of a positive larva and an adult transgenic mosquito. (**D**) Polymerase chain reaction (PCR) validation of the partial [500 base pairs (bp)] transgene cassette of *MultiEff* in the transgenic mosquitoes. (**E**) Transcript abundance of the transgene in the gut of MultiEff transgenic mosquitoes at various time points post-blood meal (PBM). Each bar represents the relative fold change in the transgene as compared to the control at time 0 hour. The *S7* ribosomal gene was used to normalize the complementary DNA (cDNA) templates. Error bars indicate SEM. (**F** to **H**) *P. falciparum* (NF54) oocyst and sporozoite infection intensities and prevalence at 8 days post-infection (dpi) in the gut or 14 dpi in the salivary glands (SG) when fed on blood with a medium (0.05%) (F) or low (0.01%) (G and H) gametocytemia. At least three biological replicates were pooled for the dot plots. Each dot represents the number of parasites in an individual gut or a pair of salivary glands, with the median values indicated by red bars. *P* values were calculated by a Mann-Whitney test. Detailed statistical analysis is presented in table S2. (**I**) Midgut microbial flora of female transgenic MultiEff and WT control (AsWT) mosquitoes at 0-, 24-, 48-, and 72-hour PBM (mean ±SEM). (**J** and **K**) *P. falciparum* oocyst infection intensities and prevalence in the aseptic (antibiotic-treated) and septic (non–antibiotic-treated) transgenic and AsWT mosquitoes at 8 dpi. (**L**) Expression of AMP and anti-*Plasmodium* effector as fold change in expression through quantitative reverse transcription PCR (qRT-PCR). Error bars indicate SEM. *CEC1*, *Cecropin* 1; *DEF1*, *Defensin* 1; *GAM1*, *Gambicin* 1; *FBN9*, *Fibrinogen-related protein 9*.

Table 1. Selected AMPs and anti-*Plasmodium* peptides for transgenic expression in the genetically modified mosquitoes.

Effectors	Characterization	Parasite stage(s)	Function or mechanism	Expression method	Inhibition (%)	Reference
Melittin	Bee antimicrobial peptides	Ookinete	Lysis of parasites	Synthesized	100.0%	(*27*)
TP10 dimer	Wasp antimicrobial peptides	Ookinete	Lysis of parasites	Synthesized	100.0%	(*25*–*27*)
Shiva1	Cecropin-like synthetic peptide	Gametes Ookinete	Lysis of parasites	Para-transgenesis	94.3%	(*27*)
EPIP four repeats	Enolase-plasminogen interaction peptide	Ookinete	Block midgut invasion	Para-transgenesis	97.7%	(*20*)
Scorpine	Scorpion venom	Gametogenesis Ookinete	Cecropin and defensin-like lytic peptide	Para-transgenesis	97.8%	(*23*)

have previously shown that transgenically immune enhanced mosquitoes have an altered midgut flora (*10*). Considering that some of our five selected anti-*Plasmodium* effectors are also AMPs and thereby might influence the midgut microbiota, we assayed the bacterial loads of transgenic and WT mosquitoes at 24- to 72-hour post-blood meal (PBM) as described in (*11*). As expected, the MultiEff transgenic line harbored a lower microbiota load (2 to 3 $\log_2$ decrease in 16*S* ribosomal RNA abundance) than the AsWT mosquitoes (Fig. 1I). To investigate whether the MultiEff-mediated suppression of the microbiota could counteract the antiparasitic activity of the recombinant effectors (*30*), we performed identical infection assays with MultiEff transgenic and AsWT mosquitoes under aseptic (antibiotic-treated) and septic (without antibiotic treatment) conditions. In the absence of midgut microbiota, both mosquito groups are more susceptible to *P. falciparum* infection, but the MultiEff-mediated suppression of the parasite, in terms of infection prevalence and intensity, remained at the same significance levels as that of the septic mosquitoes ($P < 0.0001$; Fig. 1, J and K, and table S2). The difference in infection prevalence between the septic transgenic and WT mosquito cohorts was greater than that between aseptic transgenic and WT cohorts, suggesting that the anti-*Plasmodium* activity exerted by the recombinant effectors is greater than that exerted by the bacteria. The mosquito microbiota has been shown to influence immune gene expression, including AMPs and anti-*Plasmodium* effectors (*30*). We therefore investigated whether the expression of the *MultiEff* transgene could influence the expression of the endogenous AMPs and anti–*P. falciparum* effector genes *Cecropin 1* (*CEC1*), *Defensin 1* (*DEF1*), *Gambicin 1* (*GAM1*), and *Fibrinogen-related protein 9* (*FBN9*) (*7*, *31*). This assay only showed an insignificant reduction of endogenous *GAM1* gene expression at 24- to 72-hour PBM (Fig. 1L; qRT-PCR primers are presented in table S1), suggesting that the modulation of *Plasmodium* infection is attributed to the expression of the recombinant effectors rather than the regulation of the mosquito immune system.

Targeting the sporozoite-stage malaria parasite in the hemolymph with single-chain antibodies fused to antiparasitic effectors

Moving forward, we hypothesized that incorporating another mechanism that would target the sporozoite stage would result in a greater resistance to *P. falciparum* (Fig. 2A). Previous studies have shown that transgenic expression of *Cecropin* A (*CecA* or *CEC1*) fused to a CSP-binding single-chain antibody (m2A10 ScFv) effectively blocks *P. falciparum* sporozoite-stage infection (*19*). We explored the three *A. stephensi* vitellogenin (*AsVg*) promoter–driven transgenes comprising the *CSP-ScFv* fused to either Cecropin C (*CecScFv*), PLA2 (*PLAScFv*), or Scorpine (*ScorpScFv*) for efficacy in blocking *Plasmodium* at the sporozoite stage (Fig. 2B). The transgene cassette, including the *AsVg* promoter, *CecScFv*, and *AsVg* 3′-*UTR* and terminator sequences, was codon-optimized (for ScFv-CSP), synthesized through GenScript Inc. (Supplementary Materials), and cloned into the *pUC57-Kan* cloning vector. The *CecC* signal peptide sequences and the coding sequences were flanked with Pme I and Spe I restriction sites for ease in cloning other effector genes. The *PLA2* and *Scorpine* sequences were synthesized through GenScript Inc. (Supplementary Materials) with the same restriction sites for *CecC*, and *PLA2* and *Scorpine* sequences were separately cloned to replace *CecC* on the *pUC57-Kan* vector. The three constructs were separately incorporated into the piggyBac-based plasmid *pBac[3xP3-dsRed]* containing the eye-specific *3xP3* promoter–driven *dsRed* as an eye marker to identify transgenic larvae and mosquitoes (Fig. 2, B and C). About 800 embryos were injected together with the helper plasmid *phsp-pBac* for each construct, and from the 20% of larvae that hatched, about 90% survived to adulthood and were outcrossed to control mosquitoes as described by Dong *et al.* (*10*). This outcrossing resulted in four, two, and two lines for *CecScFv*, *ScorpScFv*, and *PLAScFv*, respectively. All the insertions of the transgenes into the chromosomes of transgenic G2 larval offspring were confirmed through PCR on larval genomic DNA (gDNA) and the fluorescence markers in both larvae and adult mosquito eyes (Fig. 2, C and D, and table S1). The heterozygotes of the G3 generation of all the transgenic lines were screened for *P. falciparum* suppression. The CecScFv-4 and ScorpScFv-2 lines, but none of the PLAScFv lines, showed strong anti–*P. falciparum* activity (fig. S1) and were therefore selected for further study. Because the ScFv targets the sporozoite stage, whereas the transgenes are driven by the *AsVg* promoter that is induced several days (12- to 24-hour PBM) before sporozoite formation (reaching a >20-fold increase in recombinant transcript abundance) (Fig. 2E), we blood-fed the mosquitoes 5 and 9 days after the *P. falciparum*–infected blood meal to ensure an enrichment of recombinant protein at the time of sporozoite release from the oocysts (fig. S1). Positive transgenic mosquitoes were outcrossed with WT (AsWT) control

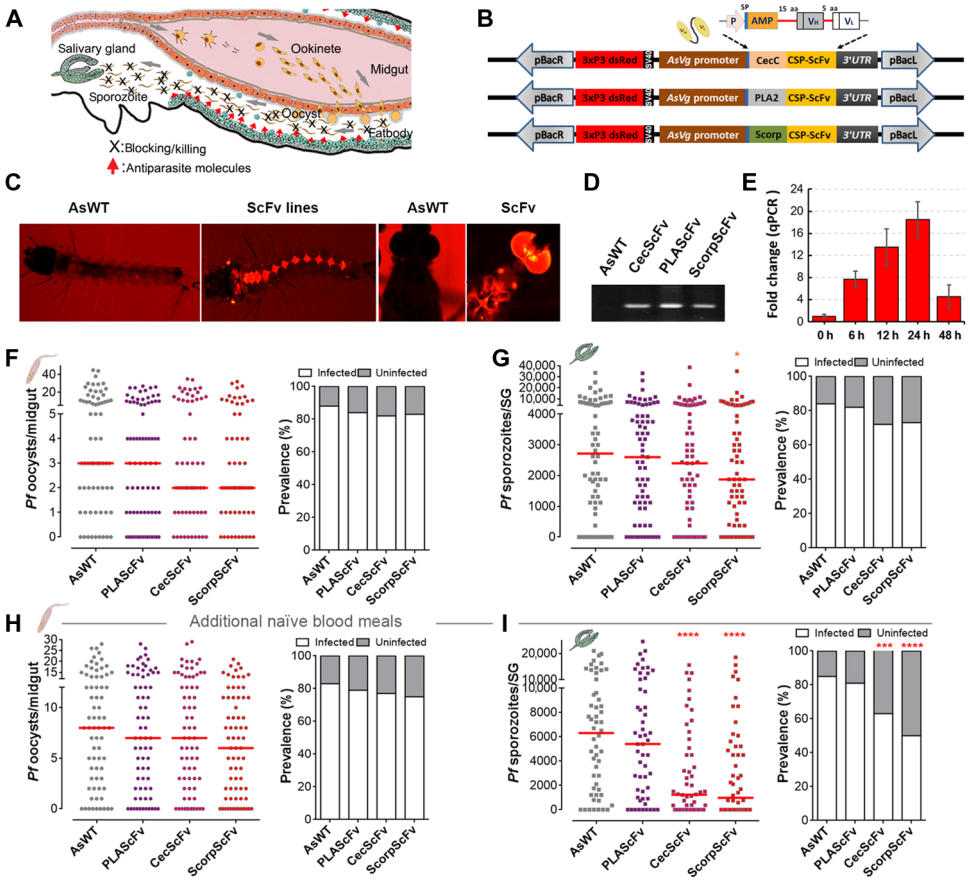

Fig. 2. Generation of transgenic mosquitoes targeting the sporozoite-stage malaria parasite in the hemolymph with single-chain antibodies fused to antiparasitic effectors and *P. falciparum* infection phenotypes at both the oocyst and sporozoite stages. (**A**) Schematic illustration of transgenic targeting of parasite sporozoite infection stages and the design of *AsVg*-driven transgenes to be expressed in the fatbody after the blood meal, to specifically target the parasites at this stage. (**B**) Schematic representation of single-chain antibody (ScFv) targeting the CSP protein fused to AMP. The single-chain antibodies consist of variable regions of the V_H heavy and V_L light chains. Each transgene encodes a short 5–amino acid polypeptide linker between V_H and V_L and a long 15–amino acid (aa) sequence linking the V_H to the AMP peptides (CecC, PLA2, and Scorpine), including the *CecA* signal peptide sequence (SP). Three individual transformation plasmids, *pBAC-AsVg-CecC* (or *PLA2*, *Scorpine*)*–ScFv* (with the red fluorescent eye reporter gene *3xP3 dsRed*), were used for the germline transformation. *AsVg* promoter with the same *AsVg* endogenous terminator (*AsVg 3′-UTR*) was used. (**C**) Fluorescent images of a positive larva and an adult transgenic *ScorpScFv* mosquito. (**D**) PCR validation of the partial transgene cassette (~500 bp) of *CecScFv*, *PLAScFv*, and *ScorpScFv* in the transgenic mosquitoes. (**E**) Transcript abundance of the transgene in the fatbody of *ScorpScFv* transgenic line at various time points PBM. Each bar represents the relative fold change in the transgene as compared to the control at time 0 hour. The *S7* ribosomal gene was used to normalize the cDNA templates. Error bars indicate SEM. (**F** to **I**) *P. falciparum* (NF54) oocyst and sporozoite infection intensities and prevalence of the three transgenic ScFv lines (PLAScFv, CecScFv, and ScorpScFv) at 8 dpi in the gut or 14 dpi in the salivary glands (SG) without (F and G) or with (H and I) additional naïve blood meals at days 5 and 9 post-infectious blood meal (PIBM). Each dot represents the number of parasites in an individual midgut or salivary glands, and the horizontal lines (red) indicate the median values. Detailed statistical analysis is presented in table S2.

mosquitoes for the first four generations, and homozygotes were obtained for the most potent *Plasmodium*-resistant lines.

Homozygous transgenic CecScFv (line 4), PLAScFv (line 2), and ScorpScFv (line 2) mosquitoes were fed on an NF54 *P. falciparum* gametocyte culture, and infection prevalence and intensity at both the oocyst and sporozoite stages were assayed (Fig. 2, F to I). As in earlier observations (*19*), the oocyst loads in the midguts of CecScFv and ScorpScFv mosquitoes showed a nonsignificant decrease either without or with additional naïve blood meals at 5 and 9 days after feeding on the infectious blood meal (PIBM) ($P > 0.05$; Fig. 2, F and H, and table S2). Without additional naïve blood meals, the salivary gland infection intensity of ScorpScFv decreased significantly ($P < 0.05$; Fig. 2G and table S2), whereas when the mosquitoes had been given additional blood meals, both the sporozoite load and infection prevalence of CecScFv and ScorpScFv, but not PLAScFv, mosquitoes were significantly reduced (Fig. 2I). The salivary gland infection prevalence decreased by 25.9 and 41.2% (χ^2 test, $P < 0.001$, $P < 0.0001$; Fig. 2I and table S2), and the median infection intensity was reduced by 80.5 and 84.6% for CecScFv and ScorpScFv, respectively, when compared to the AsWT control mosquitoes (Mann-Whitney test, $P < 0.0001$; Fig. 2I and table S2).

Targeting the malaria parasite with multiple transgenes at multiple infection stages results in near-complete refractoriness

We then investigated whether expression of multiple anti-*Plasmodium* transgenes in both the midgut and fatbody compartments would potentiate resistance to *P. falciparum* infection. Dual transgene-expressing

lines were generated by crossing virgin ScorpScFv homozygote females with the CpRel2 homozygote males (*10*, *11*). The larval offspring were screened for both red and green fluorescent eye markers. These dual-transgene mosquitoes (referred to as hybrids, or Rel2ScFv) were fed on gametocyte cultures, along with the AsWT controls and the single-transgene mosquito lines ScorpScFv and CpRel2 (Fig. 3, A and B). The oocyst infection intensity and prevalence at 8 days post-infection (dpi) for the single-transgenic CpRel2 and hybrid line Rel2ScFv were significantly reduced (Fig. 3A and table S3). At 14 dpi, the sporozoite infection intensity and prevalence of the ScorpScFv, CpRel2, and hybrid (Rel2ScFv) lines were significantly suppressed, with the median sporozoite number in the Rel2ScFv salivary glands being zero, indicating near-complete refractoriness (Fig. 3, A and B). We also generated and tested a dual-transgene mosquito line expressing *MultiEff* in the midgut and *ScorpScFv* in the fatbody by crossing virgin ScorpScFv homozygote females with MultiEff homozygote males to obtain the hybrid line denoted MultiEffScFv. Feeding mosquitoes on a low-concentration gametocyte culture resulted in a significantly reduced oocyst infection intensity and prevalence in both the MultiEff and hybrid MultiEffScFv transgenic mosquitoes (Fig. 3C and table S3). The sporozoite infection intensity and prevalence in the salivary glands were also significantly suppressed when compared to AsWT, with the median infection intensity being zero for all three lines (ScorpScFv, MultiEff, and MultiEffScFv) (Fig. 3D and table S3). The sporozoite loads were significantly lower in both hybrid lines (Rel2ScFv and MultiEffScFv) compared to the corresponding individual transgenic lines CpRel2/ScorpScFv and MultiEff/ScorpScFv, respectively (Mann-Whitney test, $P < 0.05$; Fig. 3, B and D, and table S3). These data suggest that the spatiotemporal expression of multiple antiparasitic effectors targeting different stages of the parasite produces an elevated resistance level in the transgenic mosquitoes, as illustrated in Fig. 3E.

Fitness impact of *CecScFv*, *ScorpScFv*, and *MultiEff* effector gene expression

Because trade-offs between the expression of foreign genes and fitness parameters are likely to occur, we compared the longevity, mosquito body size, egg production, and hatch rate of the transgenic

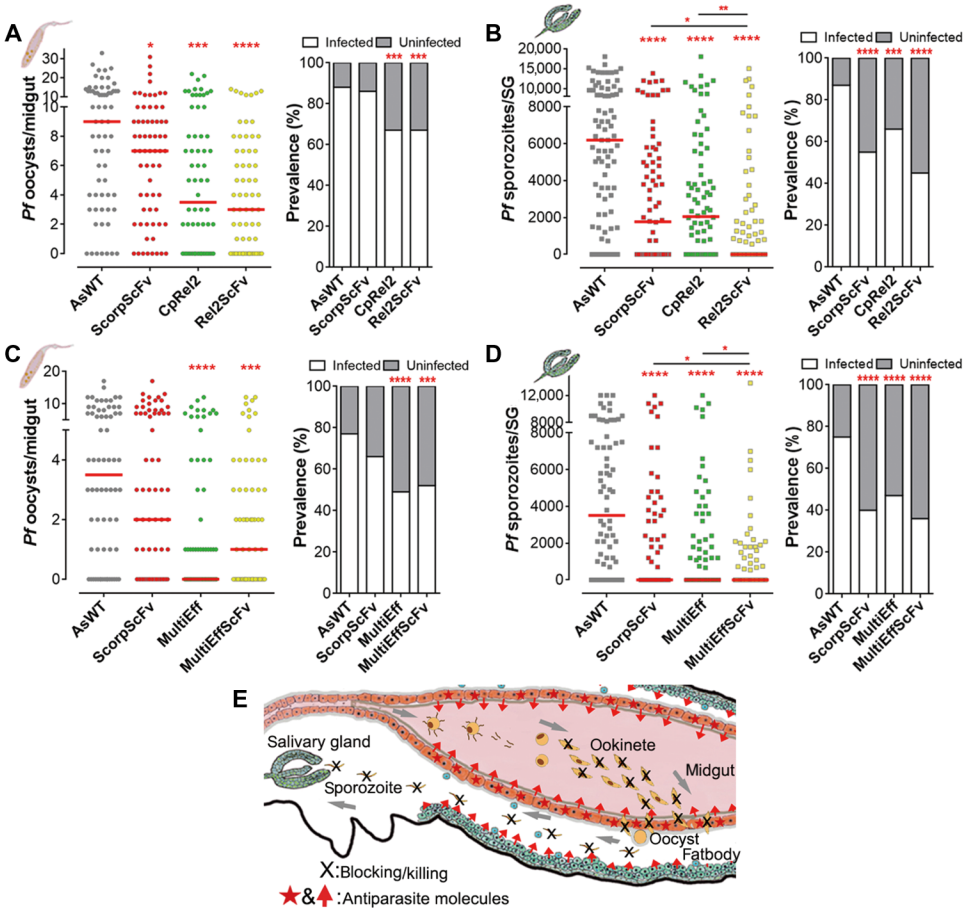

Fig. 3. The hybrid lines ScorpScFv with CpRel2 or with MultiEff targeting the malaria parasite at multiple infection stages result in near-complete refractoriness. (**A** and **B**) *P. falciparum* (NF54) oocyst and sporozoite infection intensities and prevalence of two transgenic lines (ScorpScFv and CpRel2) and the hybrid line of the two transgene cassettes (Rel2ScFv) at 8 dpi in the gut (A) or 14 dpi in the salivary glands (SG) (B) with additional blood meals at days 5 and 9 PIBM. (**C** and **D**) *P. falciparum* (NF54) oocyst and sporozoite infection intensities of two transgenic lines (ScorpScFv and MultiEff) and the MultiEffScFv hybrid transgenic mosquitoes at 8 dpi in the gut (C) or 14 dpi in the salivary glands (SG) (D), with additional blood meals on days 5 and 9 PIBM. Assays were performed with at least four biological replicates, and the horizontal lines (red) indicate the median values. Mann-Whitney test was used to calculate *P* values and determine the significance of parasite numbers. A χ^2 test was used to compare infection prevalence values. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Detailed statistical analysis is presented in table S3. (**E**) Schematic illustration of transgenic targeting of parasite midgut and sporozoite infection stages using carboxypeptidase and vitellogenin promoter-driven transgenes.

and AsWT control mosquitoes (Fig. 4). We measured the longevity of the transgenic lines when maintained on 10% sugar alone and when provided one naïve blood meal. The life span of the transgenic mosquitoes did not differ from that of the AsWT mosquitoes when maintained on a 10% sugar solution (Fig. 4A). Provision of a single naïve blood meal resulted in a significantly decreased longevity in the ScorpScFv and MultiEff mosquitoes but not in the CecScFv transgenic mosquitoes (Fig. 4B).

None of the three transgenic mosquito lines had a body size different from that of the AsWT mosquitoes, as measured by the wing lengths of female and male mosquitoes (Fig. 4, C and D). Fecundity, measured as the number of eggs laid after a blood meal, was significantly lower for ScorpScFv and MultiEff transgenic mosquitoes than for the controls (Mann-Whitney test, $P < 0.05$ and $P < 0.01$, respectively; Fig. 4E). The egg hatch rate did not differ from the AsWT mosquitoes to any of the transgenics (Fig. 4F).

None of the transgenic lines showed any change in male longevity compared to the AsWT controls when reared at insectary conditions with a 10% sugar solution (Fig. 4G). The fitness of males is important to ensure spread of the transgene to offspring.

We also investigated the fitness of the hybrid transgenic line that targets multiple infection stages through expression of *MultiEff* and *ScorpScFv* in the midgut and fatbody tissues, respectively. We selected this hybrid line because both MultiEff and ScorpScFv transgenic lines showed significant decreased life spans after one blood meal (Fig. 4B). As shown in Fig. 4 (G and H), when the transgenic mosquitoes were maintained on the regular 10% sugar solution, neither the hybrid line nor the individual transgenic lines showed impaired life spans, and the hybrid transgenic line (MultiEffScFv) did not show a shorter life span after one naïve blood meal compared to the corresponding individual transgenic lines (MultiEff and ScorpScFv).

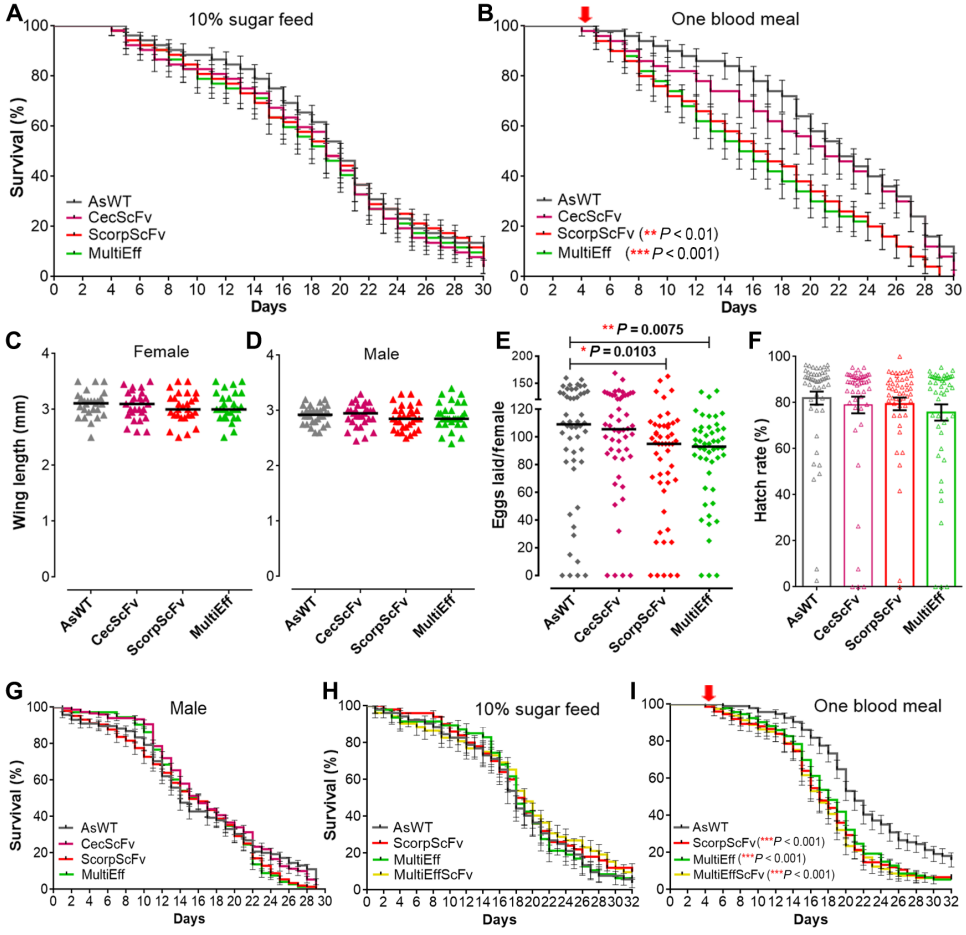

Fig. 4. Fitness effects of three transgenic lines with anti-*Plasmodium* activity. (**A** and **B**) Life spans of the female mosquitoes of the three transgenic *A. stephensi* lines maintained on 10% sucrose solution (A) or after one blood meal on mice (B) with WT control (AsWT). The life spans of ScorpScFv and MultiEff mosquitoes were significantly shorter than those of the controls when the mosquitoes were fed once on the naïve mice ($P < 0.01$ and $P < 0.001$, respectively). The pooled values from three replicates are shown, with SE. Survival rates were analyzed by Kaplan-Meier survival analysis with Wilcoxon test to determine the significance. (**C** and **D**) The wing lengths of the three anti-*Plasmodium* transgenic mosquito (CecScFv, ScorpScFv, and MultiEff) females or males did not differ from those of the control mosquitoes (AsWT). (**E**) Numbers of eggs laid by female homozygous transgenic ScorpScFv and MultiEff were significantly lower than those of the control AsWT mosquitoes. Each dot represents the eggs laid by an individual female after a single blood meal on mice. The median values (black horizontal bars) are shown. The *P* values were calculated with a Mann-Whitney test. (**F**) Hatch rates indicate the average percentage of eggs giving rise to first- and second-instar larvae, as shown with the bars indicating the mean values and SE. Each dot represents the hatch rate of the eggs laid by an individual female after blood meal. (**G**) Life spans of male mosquitoes of the three transgenic lines and AsWT controls maintained on 10% sucrose solution. (**H** and **I**) Comparison of life spans of the female hybrid transgenic line mosquitoes (MultiEffScFv) to the parental single transgenic lines (MultiEff and ScorpScFv) and the AsWT controls maintained on 10% sucrose solution (H) or after one naïve blood meal on mice (I).

DISCUSSION

We have explored the use of versatile anti-*Plasmodium* transgenes, expressed in the mosquito midgut and fatbody compartments after blood feeding, to achieve refractoriness to the human malaria parasite *P. falciparum* in the Asian malaria vector *A. stephensi*.

The selection of effector molecules for parasite blocking has been facilitated by the existence of a variety of both mosquito-encoded endogenous and nonmosquito exogenous candidates. We have previously shown that transgenic midgut-specific, blood meal–inducible expression of the IMD pathway transcription factor gene *Rel2* results in a multi-effector attack on the malaria parasite in the midgut lumen and epithelium, thereby achieving near-complete refractoriness (*10*). Transgenic midgut expression of *Rel2* also provides a reproductive fitness advantage in a cage population because of a modification in the mosquito's microbiota that, in turn, influences mate choice (*11*). We have now transgenically expressed multiple antiparasitic factors in the midgut of *Anopheles* mosquitoes from a single transgene through a construct that produces polycistronic mRNAs after blood feeding (*28*). While it is possible that this strategy results in a lower quantity of each of the effectors than would have been achieved through single-effector constructs (*24*), targeting the parasite with five different effectors surely impedes the development of resistance and may also potentiate blocking through synergistic effects (*28*).

Most studies on mosquito anti-*Plasmodium* immunity have focused on the pre-oocyst stages, and only relatively little is known about mosquito-encoded defense molecules that target the sporozoite-stage parasite. Inspired by earlier studies on transgenic mosquitoes expressing a CSP protein–targeting m2A10 single-chain antibody fused with Cecropin A, we developed transgenic mosquitoes expressing similar single-chain antibodies but fused to one of the three anti-*Plasmodium* effectors PLA2, CecC, or Scorpine. Both *ScorpScFv* and *CecScFv* transgenic mosquitoes showed increased resistance to *Plasmodium*. As predicted, the most effective suppression of sporozoites was achieved when the mosquitoes were provided additional naïve blood meals at 5 and 9 days after the infectious blood meal; this supplemental feeding resulted in the enrichment in recombinant antiparasitic effectors when the oocysts ruptured and released sporozoites (*19*). While the use of an adult-specific constitutive promoter would ensure the presence of antiparasitic recombinant proteins for *Plasmodium* targeting regardless of infection stage, such an approach would pose a high risk of adverse fitness effects. We therefore used two well-characterized and widely used blood meal–inducible promoters for driving our transgenes in the midgut or fatbody compartments after ingestion of the parasites. Provision of multiple blood meals also turns out to mimic a real field scenario because *Anopheles* mosquitoes acquire blood meals every 3 to 4 days throughout their life span (*32*).

Transgenic mosquitoes expressing the midgut-specific *Rel2* or *MultiEff* in combination with the fatbody-specific *ScorpScFv* showed the most potent *P. falciparum* suppression. While expression of the midgut-specific effector transgenes *Rel2* or *MultiEff* resulted in a sporozoite infection intensity and prevalence that were similar to those of the dual-transgenic mosquitoes, the expression of *ScorpScFv* or *CecScFv* alone in the fatbody tissue did not, suggesting that the most effective targeting of the *Plasmodium* parasite occurs in the midgut tissue, which is also considered the most important bottleneck in the malaria infection cycle (*33*). Nevertheless, coexpression of multiple effector transgenes that can target the parasite at multiple stages limits the probability of the emergence of parasite resistance and the loss of refractoriness should one of the transgenes become inactive. The emergence of parasite resistance to the transgenic blocking mechanisms is possible, but is very difficult to address experimentally, because no efficient animal models exists for studying transmission of the human malaria parasites. Using strategies such as those explored in our study can mitigate and delay the emergence of resistance, thereby providing a deployment time window that would have an epidemiologically significant impact on disease transmission.

While several of our tested transgenes and their combinations resulted in high-level resistance, total refractoriness was not attained in our assays. This may be a result of the high efficiency of our laboratory infection model or may indicate that the parasite targeting was not spatiotemporally perfect. It is also possible that some ookinete-stage parasites circumvented cells in which the effector transgenes were expressed or that some early or late events in midgut invasion or sporozoite translocation to the salivary glands occurred outside the peak concentration periods of the transgenic effectors. Nevertheless, several modeling studies have shown that total transmission blocking, or refractoriness, is not necessary to achieve an epidemiologically significant impact on disease prevalence and even a 35% transmission-blocking effect would result in malaria elimination from a hypoendemic area (*34*, *35*). Here, we have shown that anti-*Plasmodium* effector-gene combinations targeting multiple infection stages can exceed a 35% reduction in *P. falciparum* infection prevalence assuming an effective population replacement of WT with transgenic mosquitoes.

While our *Rel2*-expressing mosquitoes did not display any measurable fitness cost under laboratory conditions as measured by longevity, size, and fecundity, the expression of the exogenous effector transgenes *ScorpScFv* and *MultiEff* was associated with a slightly decreased fecundity and longevity when mosquitoes were provided a blood meal. The transient upregulation of the transgenes is unlikely to pose an energetic cost that would affect these fitness parameters; also, we saw a lack of fitness costs when the endogenous *Rel2* was transgenically expressed (*11*, *12*). Hence, the decreased fecundity and longevity could be a result of the exogenous nature of the recombinant effectors that may adversely interfere with some of the mosquito's biological processes. However, overexpression of an essential biological process could also adversely impact mosquito fitness as in the case when Akt, a single activated protein kinase that is essential to insulin signaling, was activated in the midguts (*36*). More realistic life-history studies must be performed under conditions that better mimic the natural environment of the mosquitoes. A slight transgene-mediated fitness cost may also not preclude its use in a transgenic mosquito–based malaria control strategy, because an effective gene-drive system could mitigate such effects (*2*, *4*). Future modeling studies would have to consider multiple parameters, such as transgene spread and prevalence in the population, fitness costs, loss of transgene activity because of mutations or emergence of parasite resistance, and *Plasmodium* transmission-blocking efficacy.

MATERIALS AND METHODS

Ethics statement

This study was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (NIH). The Johns Hopkins University Animal Care and Use Committee has approved this protocol, with

permit number MO18H82. Commercial anonymous human blood was used for parasite cultures and mosquito feeding, and informed consent was therefore not applicable. The Johns Hopkins School of Public Health Ethics Committee has approved this protocol.

Mosquito rearing

A. stephensi Liston strain mosquitoes were maintained on sugar solution at 27°C and 80% humidity with a 12-hour light/dark cycle according to standard procedures (*10*). Larvae were reared on cat food pellets and ground Tetramin fish food powder. Adult mosquitoes were maintained on 10% sucrose and fed on mouse blood (from mice anesthetized with ketamine) for egg production.

Generation of aseptic mosquitoes through antibiotic treatment

On the basis of modifications of previously established methodology, adult female mosquitoes were provided a 10% filter-sterilized sugar solution containing 50 μg/ml of gentamicin sulfate (Sigma) and penicillin (100 U/ml)–streptomycin (100 μg/ml) (Thermo Fisher Scientific) immediately after eclosion (*10*, *30*). The antibiotic treatment efficacy was confirmed by real-time qPCR using the bacterial ribosomal gene *16S* primers as previously described in (*11*). To minimize possible effects of the antibiotics on *Plasmodium*, the antibiotic-supplemented sugar was replaced with regular filter-sterilized sugar 1 day before the *P. falciparum*–infected blood meal.

Generation of mosquito transformation constructs: *pAsVg-CecScFv*, *pAsVg-ScorpScFv*, *pAsVg-PLAScFv*, and *pAgCp-MultiEff*

A transgene cassette with *AsVg-CecC-ScFv-3′UTR* (hereafter denoted *CecScFv*) was paper-cloned and synthesized through GenScript Inc., including the *AsVg* promoter and its native terminator *AsVg 3′-UTR*. The transgene sequences are listed in the Supplementary Materials. The gene cassettes were first cloned into the cloning vector *pUC57-Kan*, followed by Asc I digestion and insertion of the transgene into the same restriction site on *pBac[3xP3-DsRed]*. For the *AsVg-Scorp-ScFv-3′UTR* (denoted *ScorpScFv*) and *AsVg-PLA2-ScFv-3′UTR* (denoted *PLAScFv)* constructs, the *Scorpine* or Phospholipase (*PLA2*) sequences were synthesized (IDTDNA gBlock) and followed by cloning into the *pUC57Kan-AsVg-CecScFv* plasmid by replacing the *CecC* with the *Scorpine* or *PLA2* gene sequences at Pme I and Spe I RE sites. The *Scorpine* and *PLA2* sequences are described in the Supplementary Materials. To generate the construct with an array of genes encoding five anti-*Plasmodium* effectors, the codons were optimized, and the whole transgene cassette, including the P2A viral sequences, was synthesized through GenScript Inc. The sequence information is listed in the Supplementary Materials. The gene cassette was first cloned into the *pUC57-Kan* plasmid and confirmed by sequencing. The *pUC57-Kan-MultiEff* plasmid was then digested with Bam HI and Xho I, and this gene fragment was inserted into the *pENTR-carboxypeptidase P-antryp1T* (provided by S. Yoshida) on the same RE sites (*12*). The cassette with the *AgCp* promoter, *MultiEff* transgene, and trypsin terminator (*AgCp-MultiEff-TrypT*) was then amplified through PCR using primers (AgCp-Pro-F and Tryp-Ter-R) in table S1 and cloned into the Fse I site of *pBac[3xP3-eGFPafm]*.

Generation of transgenic mosquitoes

For the germline transformation, the mosquito transformation constructs generated above and the helper plasmid *phsp-pBac* were prepared using the Qiagen EndoFree Maxi Prep Kit and resuspended in 1× microinjection buffer according to an established protocol (*10*, *37*). A mixture of 0.25 μg/μl of the plasmid containing the respective gene with helper plasmid DNA (0.20 μg/μl) was injected into *A. stephensi* embryos using the Eppendorf FemtoJet Microinjector and quartz needles as described in (*37*). In general, to generate transgenic mosquitoes, about 700 to 800 *A. stephensi* eggs were injected, and the larval survivors that hatched (~20%) were screened for transient expression of the *3xP3-GFP* marker (green eyes) or *3xP3-dsRed* marker (red eyes). Transient expression of *GFP* or *dsRed* was screened, and all the surviving adult mosquitoes were crossed to WT mosquitoes, giving rise to several independent transgenic lines (described in each section). Among all these lines, the most potent anti-*Plasmodium* lines were selected for further study. The AsWT control colony mosquitoes were reared in parallel to the transgenic mosquitoes for inclusion in all the infection assays.

gDNA and RNA isolation, PCR, and qRT-PCR

gDNA of the transgenic larvae or adult mosquitoes was prepared by using a Qiagen mini DNA kit; a pool of five larvae and five adult mosquitoes was used in each sample preparation. The PCR primers used to validate transgene insertion on the chromosome are listed in table S1.

To measure the activation of the transgene after a blood meal, midgut samples were collected from six midguts from *AgCp* transgenic mosquitoes at 3 hours after a blood meal and compared with unfed G8 homozygote mosquitoes. For *AsVg* transgenic mosquitoes, fatbody samples were collected 12 hours after blood feeding. RNA was extracted using TRIzol reagent (Thermo Fisher Scientific). Reverse transcription was carried out at 37°C for 2 hours using an MMLV kit (Promega), with 20 μl of reaction mixtures containing oligo(dT) primers and 1 μg of total RNA. The qRT-PCR assays were performed according to a standard protocol (*38*) by using SYBR Green PCR Master Mix (Thermo Fisher Scientific) and an ABI StepOne Plus real-time PCR system. The relative fold induction or repression of gene expression in the experimental samples was determined by comparing these values to their respective controls after normalizing the transcript levels with the *A. stephensi* ribosomal *S7* gene. The primers used for qRT-PCR are listed in table S1.

To measure the possible influence of transgene expression on the expression of endogenous AMP and anti-*Plasmodium* effector genes, and the microbiota in the transgenic mosquitoes midguts, we measured mRNA abundance in midgut samples at 0-, 24-, 48-, and 72-hour PBM. Midgut total RNA was used for complementary DNA (cDNA) synthesis and subjected to ABI StepOne Plus real-time PCR using the primers listed in table S1.

P. falciparum infection assays and statistical analysis

To determine anti-*Plasmodium* activity, the transgenic and WT mosquitoes were fed on NF54 *P. falciparum* gametocyte cultures (provided by the Johns Hopkins Malaria Research Institute Core Facility) (*39*) through membranes at 37°C (*38*, *40*). The adult mosquitoes were starved for 3 to 6 hours before feeding to ensure engorgement. Unfed mosquitoes were sorted, and the fed mosquitoes were further incubated for 7 days at 27°C for the counting of *P. falciparum* oocyst loads. Midguts were dissected out in phosphate-buffered saline (PBS), followed by staining in 0.1% PBS–buffered mercurochrome (MilliporeSigma) and examination under a light-contrast microscope (Olympus). At least three biological replicates were performed for

each experiment, and equal numbers of samples from different replicates were pooled to produce dot plots using GraphPad Prism 8 software.

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 30 μl of PBS and then homogenized on ice. The homogenate was centrifuged at 2000 rpm for 10 min to pellet tissue debris. Then, 10 μl of the suspension was placed in a Neubauer counting chamber and at least 5 min were allowed to elapse (to ensure that the sporozoites had sedimented at the bottom of the chamber) for accurately counting the sporozoites. The sporozoites were counted with a Leica phase-contrast microscope at ×400 magnification.

The dot plots of the oocyst and sporozoite numbers in the gut epithelium and salivary glands, respectively, for each treatment were generated using GraphPad Prism8 software, along with the median value. *P* values of infection intensities were calculated through the Mann-Whitney test and used to determine the significance of parasite numbers as described in (*30*). The significance of the infection prevalence was determined through the χ^2 test or the Fisher's exact test using GraphPad Prism 8.

Wing length, life span, fecundity, and egg hatching rate measurements

Adult wing length (in both males and females) was used as a surrogate measurement for mosquito size. Mosquitoes were anesthetized on ice and kept on a cold plate for wing length measurement. Wing length was measured manually from the distal end of the alula to the tip of the wing (without the hairy fringe), through a microscope objective containing a scale bar calibrated to a 1-mm stage micrometer, without taking pictures or using software (*11*).

To measure the life span of the various mosquito lines, adult mosquitoes were sexed into male and female cohorts and placed into cups within 12 hours of emergence, with a cotton pad constantly impregnated with a 10% sucrose solution. They were held there until all the mosquitoes in that cup had died, and the number of dead mosquitoes in the cup was recorded and the dead mosquitoes were removed daily (*10*, *11*). For the life span assays of female mosquitoes receiving a single blood meal (blood-fed on mice), the mosquitoes were offered a blood meal 5 days after emergence, and only mosquitoes taking a blood meal on day 5 were retained for the rest of the study. The survival percentage represents the mean survival percentage for all three biological replicates of 35 mosquitoes each. Statistical significance was determined by Kaplan-Meier survival analysis with pooled data from three replicates by using GraphPad Prism8 software, and *P* values were determined by Wilcoxon test as described in (*10*, *36*).

For the fecundity assay, approximately fifty 7-day-old adult female AsWT, CecScFv, ScorpScFv, and MultiEff mosquitoes were allowed to feed on mice. Mosquitoes were anesthetized on ice immediately following the blood meal, and all non-engorged mosquitoes were discarded. At 3 days after blood feeding, female mosquitoes were separated into individual vials (50-ml Falcon tubes) containing moist filter paper with 1 ml of water on the bottom and allowed to oviposit, and the number of eggs laid by each female was recorded 4 to 5 days after the blood feeding using light microscopy. The females that died before laying eggs were excluded from the assays. After each count, the eggs were submerged in larval rearing water in the same individual tubes to allow the eggs to hatch. First- and second-instar larvae were counted under a light microscope. Statistical significance was determined using the Mann-Whitney test with GraphPad Prism6 software. The control mosquitoes were reared under the same conditions as the transgenic mosquitoes.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/20/eaay5898/DC1

View/request a protocol for this paper from *Bio-protocol*.

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