Engineered Resistance to *Plasmodium falciparum* Development in Transgenic *Anopheles stephensi*

**Alison T. Isaacs**, Fengwu Li, Nijole Jasinskiene, Xiaoguang Chen, Xavier Nirmala, Osvaldo Marinotti, Joseph M. Vinetz, Anthony A. James

1 Department of Microbiology and Molecular Genetics, School of Medicine, University of California, Irvine, California, United States of America, 2 Division of Infectious Diseases, Department of Medicine, University of California-San Diego School of Medicine, La Jolla, California, United States of America, 3 Department of Molecular Biology and Biochemistry, University of California, Irvine, California, United States of America, 4 Department of Parasitology, School of Public Health and Tropical Medicine, Southern Medical University, Guang Zhou, GD, China, 5 Department of Entomology and Nematology, University of Florida, Gainesville, Florida, United States of America, 6 USDA/ARS, Center for Medical, Agricultural and Veterinary Entomology, Gainesville, Florida, United States of America

Abstract

Transposon-mediated transformation was used to produce *Anopheles stephensi* that express single-chain antibodies (scFvs) designed to target the human malaria parasite, *Plasmodium falciparum*. The scFvs, m1C3, m4B7, and m2A10, are derived from mouse monoclonal antibodies that inhibit either oocyst invasion of the midgut or sporozoite invasion of salivary glands. The scFvs that target the parasite surface, m4B7 and m2A10, were fused to an *Anopheles gambiae* antimicrobial peptide, Cecropin A. Previously-characterized *Anopheles* cis-acting DNA regulatory elements were included in the transgenes to coordinate scFv production with parasite development. Gene amplification and immunoblot analyses showed promoter-specific increases in transgene expression in blood-fed females. Transgenic mosquito lines expressing each of the scFv genes had significantly lower infection levels than controls when challenged with *P. falciparum*.

Introduction

*Plasmodium falciparum*, a causative agent of human malaria, is a vector-borne parasite that is responsible for more than 500 million clinical disease cases each year [1]. The selection of insecticide-resistant mosquitoes and drug-resistant parasites necessitates an ongoing search for new disease-control methods. A proposed strategy for interrupting transmission is to replace wild, malaria-susceptible mosquito populations with transgenic, *Plasmodium*-resistant mosquitoes [2–4]. Key components of this approach are effector molecules that inhibit parasite development when expressed from a transgene. The mechanisms by which effector molecules function can vary greatly, as the development of the malaria parasites within mosquitoes involves several transitions of environment, physiology and morphology [5].

When mosquitoes feed on infected humans, they ingest parasites in the form of gametocytes. These produce gametocytes that fuse to form diploid zygotes that develop into the motile ookinetes. The ookinetes invade and traverse the mosquito midgut epithelium and then rest beneath the basal lamina of the midgut, forming oocysts. Thousands of sporozoites develop within the oocysts before budding out into the circulatory fluid, the hemolymph, and invading the salivary glands. Several effector molecules have been tested for their ability to target the parasite during either early sporogony in the midgut, or late sporogony in the hemolymph or salivary glands [3–8]. An effector mechanism based on the mosquito signaling protein Akt is the only one to date shown to inhibit completely *P. falciparum* development in a transgenic *Anopheles* mosquito [7].

One effector molecule strategy exploits the finding that monoclonal antibodies (mAbs) that recognize surface-bound or secreted parasite molecules can inhibit pathogen development [9–14]. Two mAbs, 4B7 and 1C3, target parasites early in their development within mosquitoes. 4B7 binds *P. falciparum* surface protein Pf625, a molecule expressed on the surface of ookinetes, and inhibits parasite development completely when fed to *Anopheles* mosquitoes in a gametocytemic bloodmeal [9]. In contrast, 1C3 binds a parasite-secreted enzyme, *P. falciparum* chitinase 1, and inhibits oocyst formation of *P. falciparum* when incorporated into infectious bloodmeals [10]. A third mAb, 2A10, binds *P. falciparum* circumsporozoite protein (CSP), and when pre-incubated with sporozoites, greatly decreases their ability to infect cultured hepatocytes [11,12].

Although the size and complexity of mAbs exclude them from consideration as potential effector molecules, single-chain antibodies (scFvs), which retain the binding specificity of a mAb, are much smaller and can be produced from a single transcription unit [15]. An scFv targeting the *P. gallinaceum* CSP inhibited sporozoite invasion of salivary glands in *Aedes aegypti* in both transient assays and transgenic mosquitoes [13,16]. *Anopheles stephensi* fed *Escherichia coli* expressing transgene...
Malaria eradication will require vector-control strategies that are both self-sustaining and not affected by migration of infected humans and mosquitoes. Replacement of wild malaria-susceptible mosquito populations with transgenic strains refractory to parasite development could interrupt the cycle of disease transmission and support eradication efforts. Production of *P. falciparum*-resistant mosquitoes is a necessary first step towards investigating the population replacement strategy. Here we show that *An. stephensi* engineered to produce *P. falciparum*-targeting effector molecules are resistant to this important human malaria parasite. Two of the three effector molecules represent a novel combination of components derived from the immune systems of mosquitoes and mice. An important feature of these molecules is that they are unlikely to significantly harm the mosquito, as the mosquito component is a *Anopheles* antimicrobial peptide with activity against *Plasmodium*, while the other component is based on a murine antibody selected for its ability to bind specifically to a parasite protein. Transgenes with this design coupled with a gene-drive system could be used alongside vaccines and drugs to provide sustainable local elimination of malaria as part of a long-term strategy for eradication.

Results

Transgene assembly, transgenesis, and gene copy-number analyses

The scFv genes were synthesized commercially to incorporate either the *AgCPA* signal sequence or the entire *AgCecA* ORF (Figure 1). Codons corresponding to the amino acids serine, proline, alanine, threonine, and arginine displayed the greatest frequency bias differences between *Mus musculus* and *An. gambiae* (Table S1) [24], and these were replaced in the mouse-derived scFv sequences by those favored by the mosquito. DNA sequence encoding a short polypeptide linker (five amino acids) was used to join the heavy- and light-chain variable fragments of m4B7 and m2A10 scFvs and a longer linker (encoding 15 amino acids) joined the two corresponding moieties of m1C3. Long linkers permit intramolecular pairing of variable fragments, while short linkers favor the intermolecular joining of scFv molecules to form multimers containing multiple antigen recognition sites [25]. The m1C3 and m4B7 scFv genes were joined to *AgCPA* regulatory elements and inserted into a pBac [3xP3-EGFP] plasmid to construct the transformation vectors (Figure 2). Similarly, the m2A10 scFv gene was joined to *AvVG1* regulatory elements and inserted into a pBac [3xP3-dsRed] plasmid.

The three transformation plasmids pBac [3xP3-EGFP]-m1C3, pBac [3xP3-EGFP]-m4B7 and pBac [3xP3-dsRed]-m2A10 were injected into 980, 615 and 765 embryos, respectively. Three transgenic m1C3 mosquito lines (21.1, 39.1 and P4.1) were established from EGFP-positive families derived from 78 surviving adults. Two transgenic m4B7 mosquito lines (25.1 and P6.1) were established from 89 adults, and seven transgenic m2A10 mosquito lines (18.1, 20, 34.1, 39.1, 44.1, 44.1, P5.1 and P7.1) were established from 105 adults.

Southern blot analyses were used to verify transgene insertions and to determine the number of integrated constructs in each line (Figure 2). Hybridization of an m1C3 probe to genomic DNA digested with both SphI and XhoI restriction endonucleases produced a diagnostic fragment of ~1.2 kilobase pairs (kb) in transgenic samples, confirming m1C3 integration. Genomic DNA digested with SphI and hybridized to an EGFP probe produced multiple fragments in each transgenic sample, indicating that there were at least three, nine, and ten copies in lines 21.1, 39.1, and P4.1, respectively. Genomic DNA digested with *ApaI*, *AciI*, and *FdiI*, and hybridized to a probe complementary to the m4B7 gene and the *AgCPA* 3’UTR produced two diagnostic fragments of 940 and 810 base pairs (bp), verifying transgene insertion. A second blot, comprising *Xhol*-digested genomic DNA recovered from transgenic mosquitoes and hybridized with a 3XPS EGFP probe, revealed several fragments in each sample, indicating that at least four copies of the m4B7 transgene were present in each line. Lastly, genomic DNA digested with both *XhoI* and *BanHI* and hybridized to an m2A10 probe produced an ~1 kb diagnostic fragment in each transgenic sample. The same probe hybridized to *HindIII*-digested genomic DNA bound multiple DNA fragments in each m2A10 sample, indicating the presence of six, three, three, four, six, seven and three copies in transgenic lines 18.1, 20, 34.1, 39.1, 44.1, P5.1, P7.1, respectively. Transgenic lines were maintained by intercrossing at each generation. However, selection pressures on individual transgene insertions, small founding colony sizes and independent assortment likely result in loss over time of some of the insertions.

Characterization of transgene expression

Reverse-transcriptase-PCR (RT-PCR) and Real-time quantitative RT-PCR (RT-qPCR) were used to evaluate the presence and
relative abundance of m1C3, m4B7 or m2A10 transcription products in non-blood-fed and blood-fed mosquitoes in all of the established transgenic lines. No significant correlation was seen between transgene copy number and amount of transcription product detected (data not shown). Therefore, the lines m1C3 P4.1, m4B7 25.1 and m2A10 44.1, each of which displayed the highest levels of transcript accumulation in their respective group, were selected for use in all further analyses. Southern blot analyses of the generations of m1C3 P4.1, m4B7 25.1 and m2A10 44.1 used in the challenge assays indicated the presence of eight, four, and four copies of the respective transgenes.

Transgene-specific transcript accumulation profiles detected by RT-PCR were similar in mRNA samples prepared from the dissected midguts of m1C3 P4.1 and m4B7 25.1 females (Figure 3). Both lines showed constitutive accumulation in midguts from non-blood-fed mosquitoes. In addition, each line showed accumulation of their respective mRNAs at 4 hours post-bloodmeal (hPBM), and signals were evident at 12 and 24 hPBM. m4B7 transcript also could be detected at low levels at 48 hPBM. No amplification products were produced from mRNA prepared from female carcasses or males of each line. As expected, control reactions using mRNA from midguts dissected at 4 hPBM from wild-type, non-transgenic females were negative. RT-qPCR analysis at multiple post-bloodmeal time points was used as an independent measure of m1C3 P4.1 expression. The highest measured level of m1C3 mRNA, 10,000-fold above the control level, was observed in the 16 hPBM midgut sample (paired T-test, one tailed p-value = 0.005), but similar elevated levels also were seen at 24 and 48 hPBM. At this time, we cannot account for the difference in the RT-PCR and RT-qPCR results at 48 hPBM, although this could result from individual females that responded differentially to the feeding regimen. This difference is not expected to have affected

Figure 1. A model of the modified scFv transgenes. A mature mouse immunoglobulin molecule consists of two heavy- and light-chain polypeptides each linked through disulfide (ss) bonds (top image). The single-chain antibodies are composed of the variable regions (Fv) of the heavy (VH) and light (VL) chains (gray and open boxes, respectively) of a mouse monoclonal antibody. The m4B7 and m2A10 scFv transgenes encode a short polypeptide linker of 5 amino acids (Saa) between VH and VL. These transgenes include sequence for a long polypeptide linker of 15 amino acids (15aa) joining the VH to the An. gambiae Cecropin A peptide (CecA), including its signal sequence. The VH region present in the m1C3 transgene is joined to the An. gambiae Carboxypeptidase A gene signal sequence, and joined by a long polypeptide linker to the VL region. Select codons in the variable region genes were codon-optimized to facilitate efficient translation. Anopheles promoter sequences (P) were joined to the scFvs to direct tissue-specific transgene expression.

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the outcome of the challenge experiments because this scFv targets parasites within the first 24 hPBM.

Immunoblot analyses of m1C3 and m4B7 transgenic mosquitoes were unproductive despite repeated attempts. Although high levels of proteinase inhibitors were used during sample preparation, it is possible that the transgene products were degraded quickly in the strong digestive milieu of the post-feeding midgut lumen.

Specific transcript accumulation was evident in m2A10 44.1 females at 12 and 24 hPBM in an expression pattern similar to that of endogenous AsVg1, but was not as abundant at 48 hPBM.

Transgene transcripts detected in whole transgenic m2A10 44.1 females showed sex- and stage-specificity (Figure 3). No signals were seen in samples derived from mRNA prepared from males and non-blood fed transgenic or control wild-type, non-transgenic females.

Figure 2. Southern blot analyses of m1C3, m4B7, and m2A10 transgenic lines. (A) Schematic representations of the single-chain antibody (scFv) transformation constructs. The scFv heavy (VH) and light (VL) variable region genes in the m1C3 construct are joined by sequence encoding a long polypeptide linker (multiple grey boxes). The sequence encoding the VH is joined to the An. gambiae carboxypeptidase A signal sequence (AgCp sig). In the m4B7 and m2A10 constructs, the An. gambiae Cecropin A gene (CecA), including signal sequences (CecA sig), is joined by sequence encoding a long polypeptide linker to the scFv VH and VL genes. The VH and VL genes are joined by a short polypeptide linker (single grey box). All three scFvs are joined to an epitope tag (E tag). The CecA-m2A10 effector gene is flanked by An. stephensi vitellogenin 1 regulatory sequences (AsVg1 5′ UTR, 3′ UTR), while the m1C3 and CecA-m4B7 genes are flanked by AgCP A regulatory sequences (AgCP 5′ UTR, 3′ UTR). A transformation marker (EGFP or DsRed) joined to the Pax3 (3xP3) promoter and the SV40 polyadenylation sequence also is contained between the piggyBac transposase arms (pBacLH, RH). Select restriction endonuclease sites present in the transgene are indicated in the diagram. Probes used to identify the integrated transgenes are indicated by horizontal bars above each schematic representation. (B) Genomic DNA from m1C3 and wild-type control females was digested and hybridized to either an EGFP probe (left) or an m1C3 probe (right). (C) Genomic DNA from m4B7 and wild-type control females was digested and hybridized to an m4B7/Ap1/Asc probe (left) or an m4B7/AgCP A 3′ UTR probe (right). (D) Genomic DNA from m2A10 and wild-type control females was digested and hybridized to an m2A10 probe. The restriction endonucleases used in each experiment are listed above the blot, and the identity of each transgenic line is listed above each lane. The locations of molecular weight markers are indicated in kilobase pairs (kb). doi:10.1371/journal.ppat.1002017.g002
### A

|        | m4B7 | WT |
|--------|------|----|
|        | M    | NBF gut | 4h gut | 12h gut | 24h gut | 48h gut | 4h carc. | 4h gut |
| m4B7   |      | ![Image](image1) | ![Image](image2) | ![Image](image3) | ![Image](image4) | ![Image](image5) | ![Image](image6) | ![Image](image7) |
| AsCP   |      | ![Image](image8) | ![Image](image9) | ![Image](image10) | ![Image](image11) | ![Image](image12) | ![Image](image13) | ![Image](image14) |
| rpS26  |      | ![Image](image15) | ![Image](image16) | ![Image](image17) | ![Image](image18) | ![Image](image19) | ![Image](image20) | ![Image](image21) |

### B

|        | m1C3 | WT |
|--------|------|----|
|        | M    | NBF gut | 4h gut | 12h gut | 24h gut | 48h gut | 4h carc. | 4h gut |
| m1C3   |      | ![Image](image22) | ![Image](image23) | ![Image](image24) | ![Image](image25) | ![Image](image26) | ![Image](image27) | ![Image](image28) |
| AsCP   |      | ![Image](image29) | ![Image](image30) | ![Image](image31) | ![Image](image32) | ![Image](image33) | ![Image](image34) | ![Image](image35) |
| rpS26  |      | ![Image](image36) | ![Image](image37) | ![Image](image38) | ![Image](image39) | ![Image](image40) | ![Image](image41) | ![Image](image42) |

### C

|        | WT | m2A10 |
|--------|----|-------|
|        | 24h | M | NBF | 12h | 24h | 48h | 72h |
| m2A10  |      | ![Image](image43) | ![Image](image44) | ![Image](image45) | ![Image](image46) | ![Image](image47) | ![Image](image48) |
| AsVg1  |      | ![Image](image49) | ![Image](image50) | ![Image](image51) | ![Image](image52) | ![Image](image53) | ![Image](image54) |
| rpS26  |      | ![Image](image55) | ![Image](image56) | ![Image](image57) | ![Image](image58) | ![Image](image59) | ![Image](image60) |

### D

**Number of m1C3 transcripts**

| Time post-bloodmeal | 10^0 | 10^1 | 10^2 | 10^3 | 10^4 | 10^5 | 10^6 | 10^7 | 10^8 | 10^9 |
|---------------------|------|------|------|------|------|------|------|------|------|------|
| NBF                 | ![Bar](image61) | ![Bar](image62) | ![Bar](image63) | ![Bar](image64) | ![Bar](image65) | ![Bar](image66) | ![Bar](image67) | ![Bar](image68) | ![Bar](image69) | ![Bar](image70) |
| 4h                  | ![Bar](image71) | ![Bar](image72) | ![Bar](image73) | ![Bar](image74) | ![Bar](image75) | ![Bar](image76) | ![Bar](image77) | ![Bar](image78) | ![Bar](image79) | ![Bar](image80) |
| 8h                  | ![Bar](image81) | ![Bar](image82) | ![Bar](image83) | ![Bar](image84) | ![Bar](image85) | ![Bar](image86) | ![Bar](image87) | ![Bar](image88) | ![Bar](image89) | ![Bar](image90) |
| 16h                 | ![Bar](image91) | ![Bar](image92) | ![Bar](image93) | ![Bar](image94) | ![Bar](image95) | ![Bar](image96) | ![Bar](image97) | ![Bar](image98) | ![Bar](image99) | ![Bar](image100) |
| 24h                 | ![Bar](image101) | ![Bar](image102) | ![Bar](image103) | ![Bar](image104) | ![Bar](image105) | ![Bar](image106) | ![Bar](image107) | ![Bar](image108) | ![Bar](image109) | ![Bar](image110) |
| 48h                 | ![Bar](image111) | ![Bar](image112) | ![Bar](image113) | ![Bar](image114) | ![Bar](image115) | ![Bar](image116) | ![Bar](image117) | ![Bar](image118) | ![Bar](image119) | ![Bar](image120) |
| 72h                 | ![Bar](image121) | ![Bar](image122) | ![Bar](image123) | ![Bar](image124) | ![Bar](image125) | ![Bar](image126) | ![Bar](image127) | ![Bar](image128) | ![Bar](image129) | ![Bar](image130) |
| 1d                  | ![Bar](image131) | ![Bar](image132) | ![Bar](image133) | ![Bar](image134) | ![Bar](image135) | ![Bar](image136) | ![Bar](image137) | ![Bar](image138) | ![Bar](image139) | ![Bar](image140) |
| 15d                 | ![Bar](image141) | ![Bar](image142) | ![Bar](image143) | ![Bar](image144) | ![Bar](image145) | ![Bar](image146) | ![Bar](image147) | ![Bar](image148) | ![Bar](image149) | ![Bar](image150) |
While expression of the m1C3 and m4B7 scFvs is necessary only during the first 24 hours post-bloodmeal, expression of m2A10 must be sustained over several days, as oocysts can mature asynchronously [26]. Denaturing immunoblot analyses were performed on m2A10 44.1 females sampled over the course of four bloodmeals to evaluate whether protein expression could be induced repeatedly (Figure 4; Figure S1). Anti-E tag antibody specifically detected a polypeptide with an approximate Mr of 32 kDa, consistent with the predicted size of m2A10 protein, in transgenic blood-fed females at 24, 48, 72 and 96 hPBM. The continuous presence of m2A10 was detected in females that were given bloodmeals once every five days. Expression of m2A10 also was observed at 12 hPBM in additional females that were given bloodmeals once every five days.

Discussion

Previous evaluations of mosquitoes engineered genetically to express anti-Plasmodium effector genes featured analyses of transgene copy numbers, transgene transcription levels, detection of transgene effector proteins, binding of effector molecules to the target parasite stage and a phenotype of reduced parasite mean intensities of infection and prevalence [7,8,16,22,27–29]. Remarkably, no single study includes all of these data and the emphasis has been on the impact of transgene presence on parasite numbers. Expression of the two midgut-directed scFvs, m1C3 and m4B7, was detected by RT-PCR, but not by immunoblots. The rapid degradation of these scFvs in the midgut environment may have inhibited immunoblot detection. However, the observation that m4B7 25.1 and m1C3 P4.1 transgenic mosquitoes have reduced parasite loads supports the conclusion that these scFvs are expressed in the midgut. Both transgene transcription and translation products were detected in m2A10 44.1 mosquitoes. The finding that the immunoblot analyses of non-denatured m2A10 44.1 samples detected the presence of scFv multimers is consistent with the expectation that the short polypeptide linker joining the VH and VL regions promotes intermolecular scFv interactions. The size of these multimers was similar to the predicted sizes of m2A10 multimers comprising four, seven, nine, and fifteen scFv molecules. Such scFv multimers are reported to have high affinity to target epitopes [23].

Both m1C3 and m4B7 expressed in transgenic lines P4.1 and 25.1, respectively, inhibited parasite development during early...
sporogony, resulting in significantly reduced mean intensities of oocyst infection in eight of ten challenge experiments. The results of two of the m4B7 25.1 challenge experiments are consistent with the interpretation that there is a threshold level of initial parasite density above which this scFv, at the levels expressed in these transgenic lines, cannot efficiently inhibit ookinete development. The finding that m2A10 44.1 and wild-type control mosquitoes did not differ in midgut infection supports the conclusion that transgene integration alone does not necessarily impair parasite development.

When expression of m2A10 in line 44.1 was induced repeatedly by blood feeding, a highly significant decrease in sporozoite load was observed in transgenic mosquito salivary glands. For this transgenic line, the greatest reduction in prevalence was found in an experiment in which the mean intensity of oocyst infection was low. It is likely that these scFvs would effectively impair \textit{P. falciparum} transmission in field conditions, as infected wild-caught \textit{An. gambiae} carry few oocysts. Studies of \textit{An. gambiae} by Billingsley \textit{et al}. [30] and Taylor [31] found mean numbers of oocysts per infected mosquito of 1.55 and 3.38, respectively.

Incorporation of multiple transgenes is typical for piggyBac-mediated insertions into \textit{An. stephensi} [22,23,32]. Although it is reasonable to expect that higher transgene copy numbers should yield higher expression levels, no statistically-significant correlations have been reported. We hypothesize that many of the multiple copies have little or no expression as a result of position effects, and that the majority of transgene expression comes from single or small numbers of the transgenes. To mitigate copy-number issues, we have used piggy-Bac-mediated transposition to integrate target sites for \textit{Qc31} site-specific recombination into multiple locations in the \textit{An. stephensi} genome and are now testing individual lines for permissiveness for optimum transgene expression [33]. These lines have the added benefit of having been evaluated for the impact on fitness of the introduced exogenous DNA at the specific insertion site, and therefore the effects of anti-pathogen transgene product expression can be

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**Figure 4. Bloodmeal-induced expression of m2A10 scFv.** (A) Immunoblot analyses of transgenic m2A10 44.1 \textit{An. stephensi}. An anti-E tag antibody was used to detect m2A10 protein in whole body homogenates of transgenic females. Purified m2A10 scFv protein, which contained an E tag but did not contain Cecropin A, was used as a positive control (+). Female mosquitoes were given bloodmeals (bm) once every five days, and examined at 24, 48, 72, and 96 hours after each meal. (B) An immunoblot of both whole females (W) and a hemolymph preparation of females (H) from transgenic m2A10 44.1 and wild-type control mosquitoes (WT) 48 hours post-bloodmeal. Anti-E tag antibody was used for detection of m2A10. (C) An immunoblot of m2A10 and wild-type control female hemolymph samples, prepared in non-denaturing conditions (left panel) and probed with anti-E tag antibody. Non-bloodfed (NBF) and 24 hours post-bloodmeal (24hPBM) females were compared. NativeMark protein standard (Invitrogen) was used for molecular weight estimation. Control images of the Coomassie-stained gels are provided in Figure S1.

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Two anti-*Plasmodium* effector molecule strategies have yielded transgenic mosquitoes with zero prevalence: expression of the signaling molecule Akt, and expression of a combination of Cecropin A and Defensin A [7,29]. The latter study was conducted with the *P. gallinaceum/Ae. aegypti/G. gallus* model system. The study of Akt demonstrated the feasibility of producing an *Anopheles* mosquito that is completely resistant to *P. falciparum*, however this effector molecule may not be an optimal component of a population replacement strategy as these mosquitoes have a significantly reduced lifespan [7]. A synthetic peptide designed to interact with *P. yoelii* reduced midgut infections of this parasite by 67–87% in *An. gambiae* but was considerably less efficacious against *P. falciparum* [8]. Quantitative comparisons of the efficacy of alternative effector molecules are hindered currently by differences in expression that result from variations in transgene location and copy number. Site-specific recombination approaches will allow such evaluations in well-characterized ‘locking-site’ mosquito strains [8,33].

The finding that m2A10 44.1 mosquitoes display up to 97% decreases in the mean intensity of *P. falciparum* infection, as well as decreased prevalence of infection, supports the argument that this scFv may be an effective component of a malaria resistance transgene. The m1C3 and m4B7 scFv genes conferred significant reductions in mean intensities of infection, and if expressed in a rodent malaria parasite, *P. berghei*, in which the effector molecules SM1, PLA2 and CEL-III show a significant inhibition of parasite development (81.6%, 87% and 84.8%, respectively) [22,27,28]. Reductions of mean intensities of infection in salivary glands below 400 were sufficient to block transmission. In contrast, experimental infections of humans with *P. vivax* showed that 10 sporozoites were sufficient to cause malaria [36]. We have opted to take the conservative approach and are attempting to achieve zero prevalence of human parasites in mosquito salivary glands [16].

![Table 1. Oocyst prevalence and mean intensity of infection in *P. falciparum*-infected mosquitoes.](https://doi.org/10.1371/journal.ppat.1002017.t001)

| Exp. | Strain | Oocyst prevalence* (n) | Mean intensity of oocyst infection* +/- SD (n) | P* |
|------|--------|-------------------------|---------------------------------------------|----|
| 1    | Control| 84%(45)                 | 15.3+/−12.3 (38)                             | <0.0001 |
|      | m4B7   | 48% (50)                | 2.9+/−2.4 (24)                               |    |
| 2    | Control| 70% (67)                | 4.6+/−4.4 (47)                               | <0.0001 |
|      | m4B7   | 41% (93)                | 2.9+/−2.9 (38)                               |    |
| 3    | Control| 77% (64)                | 6.3+/−4.5 (49)                               | <0.0001 |
|      | m4B7   | 40% (92)                | 2.4+/−1.9 (37)                               |    |
| 4    | Control| 88% (48)                | 19.8+/−21.4 (42)                             | 0.3736 |
|      | m4B7   | 88% (43)                | 14.1+/−10.5 (38)                             |    |
| 5    | Control| 83% (46)                | 17.4+/−17.8 (38)                             | 0.2883 |
|      | m4B7   | 88% (41)                | 11.2+/−9.5 (36)                              |    |
| 6    | Control| 83% (23)                | 22.5+/−13.5 (19)                             | 0.0068 |
|      | m1C3   | 64% (22)                | 12.0+/−11.6 (14)                             |    |
| 7    | Control| 86% (22)                | 38.8+/−36.8 (19)                             | 0.0007 |
|      | m1C3   | 46% (24)                | 14.3+/−18.1 (11)                             |    |
| 8    | Control| 48% (23)                | 7.1+/−5.8 (11)                               | 0.0004 |
|      | m1C3   | 9% (33)                 | 3.0+/−2.0 (3)                                |    |
| 9    | Control| 67% (12)                | 10.5+/−4.4 (8)                               | 0.0043 |
|      | m1C3   | 39% (23)                | 3.9+/−3.6 (9)                                |    |
| 10   | Control| 46% (24)                | 3.7+/−2.1 (11)                               | 0.0065 |
|      | m1C3   | 15% (20)                | 1.0+/−0.0 (3)                                |    |

*Prevalence reflects the percent of mosquitoes infected; mean intensity of infection reflects the mean number of parasites found in infected mosquitoes.

A Mann-Whitney U test was used to evaluate statistical significance of oocyst mean intensity infection data. A one-tailed P value is listed for each experiment.

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The discovery and characterization of several effector molecules that completely inhibit *P. falciparum* development will support the engineering of mosquitoes that express multiple effector molecules. Such mosquitoes may have a reduced likelihood of selecting for resistant parasites. Along with vaccines, drugs, and insecticide-treated nets, parasite-resistant transgenic mosquitoes would be a useful component in a malaria-control strategy, especially in regions where existing interventions have been unable to eliminate disease transmission.

**Materials and Methods**

**Mosquito rearing and maintenance**

A colony of *Anopheles stephensi* (gift of M. Jacobs-Lorena, Johns Hopkins University) bred in our insectary for >5 years was used in the experiments. The mosquitoes were maintained in conditions that maximize larval nutrition, and adult size and fitness [37]. These conditions include maintenance of cultures at 27°C with 77% humidity and 12 hr day/night, 30 min dusk/dawn lighting cycle. Larvae were fed a diet of powdered fish food (Tetramin) mixed with yeast. Adults were provided water and raisins *ad libitum*. Anesthetized chickens, mice, or rabbits were used for blood feeding. Transgenic and wild-type control mosquitoes used in parasite challenge experiments were reared in parallel using standardized insectary procedures.

**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. The protocol was approved by the Intuitional Animal Care and Use Committee of the University of California, Irvine (NIH Animal Welfare Assurance number: A3416.01 (approved February 20, 2008), Protocol Number: 1998-1411 (approved May 21, 2010). The vertebrates used as bloodmeal donors for mosquitoes were anesthetized on a regimen that avoids the build-up of drug tolerance, and all efforts were made to minimize suffering.

**scFv sequence modifications**

The sequences of the 4B7 and 2A10 variable heavy- and light-chain regions (V_H and V_L, respectively) were derived from cDNA synthesized from 2A10 and 4B7 hybridoma cell lines (obtained from E. Nardin [New York University], and the Malaria Research and Reference Reagent Resource Center, respectively). 2A10 cDNA was synthesized from total RNA isolated from the hybridoma cell line using primers designed from the known V_H and V_L sequence [38]. V_H and V_L cDNA from the 4B7 hybridoma cell line was amplified from its total RNA using the heavy and light primer mixes respectively, provided in the Mouse scFv Module/Recombinant Phage Antibody System (Amersham Biosciences). The modified scFv genes, including either AgCP4

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Figure 5. *P. falciparum* oocyst or sporozoite burden in transgenic and wild-type control mosquitoes. Box plots display the observed distribution of oocyst per midgut or sporozoite per salivary gland pair values for wild-type control mosquitoes and m1C3 P4.1, m4B7 25.1, or m2A10 44.1 transgenic mosquitoes. The boxes in each panel represent from bottom to top the 25th to 75th percentiles, while the vertical lines and small horizontal bars delimit the minimum and maximum values. Horizontal bars within the boxes indicate the median value of each group. (A) Plots of data from all challenge experiments with m4B7 25.1 and m1C3 P4.1 mosquitoes. (B) The plot represents data from m2A10 44.1 experiments 1–4 and 5–7 separately, as indicated.

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signal sequence or the entire AgCecA ORF, were synthesized commercially (Epoch Biolabs) to allow for incorporation of novel features. The variable regions of 4B7, 2A10, and 1C3 [17] were optimized by replacing the codons corresponding to the amino acids serine, proline, alanine, threonine, and arginine in the mouse-derived sequences with those favored in 

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digested of the pBac [3xP3-EGFP]-m4B7 plasmid with both NaeI and PstI. The m2A10 probe was generated through a restriction digest of the pBSK-m2A10 plasmid with both BamHI and BssBI. Probes were labeled with 32P using the Megaprime DNA labeling system (Amersham).

RT-PCR
Total RNA was isolated from whole or dissected mosquitoes using Trizol (Invitrogen). For m2A10 RT-PCR analyses, 10 males or 2–3 whole females were used for each RNA preparation. One microgram of RNA was treated with DNase I (Promega) for each 50 µl RT-PCR reaction. For m4B7 and m1C3 RT-PCR analyses, 6 males, 4–15 female midguts, or 4 female carcasses were used for each RNA preparation. Two hundred fifty nanograms of RNA were treated with DNase I for each 12.5 µl RT-PCR reaction. Gene-specific primers and a OneStep RT-PCR Kit (Qiagen) were used for amplification of diagnostic products from m2A10, m4B7, m1C3, AsCPA [32], AsVg1 [23], or An. stephensi ribosomal protein S26 gene [23] transcripts (Table S2). For m2A10 RT-PCR analyses, amplification of diagnostic products from AsVg1 and ribosomal protein S26 gene-specific primers was performed in a single reaction. Diagnostic amplification reactions for the m1B7 25.1 and m1C3 Pri.1 lines were initiated with one cycle at 50°C for 30 s, one cycle at 95°C for 15 s, and 32 cycles denaturation at 94°C for 30 s, annealing at a reaction-specific temperature (Table S2) for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. Diagnostic amplification reactions for the m2A10 44.1 line were performed as described, except that 30 cycles of amplification were completed. For each sample, an additional control RT-PCR reaction tested for the presence of genomic DNA contamination using ribosomal protein S26 gene primers but omitting the reverse transcription step. Multiple biological replicates (≥2) were performed for selected time points for each of the RT-PCR series of experiments.

Real-time quantitative RT-PCR analysis
Female mosquito midguts and carcasses and male midguts were dissected in phosphate-buffered saline (PBS), homogenized in Trizol Reagent (Invitrogen), and total RNA extracted. Midguts were dissected at different time points (4 h, 8 h, 16 h, 24 h, 48 h, 72 h, 7 d, and 15 d) after a bloodmeal. RNA was treated with DNase I (Invitrogen) at 1 U/µg RNA to remove potential genomic DNA contamination. Further purification was performed using a DNA-free kit (Ambion). A total of 0.4 µg of RNA was used for reverse transcription in a reaction volume of 20 µl using ThermoScript RT-PCR System (Invitrogen); Real-time quantitative PCR was performed on an Opticon 2 Real-Time PCR Detection System using the Opticon Monitor software version 3.1 (Bio-Rad laboratories). m1C3 expression was quantified with Platinum SYBR Green qPCR SuperMix UDG with ROX (Invitrogen) using gene-specific primers (Table S2) to amplify a diagnostic fragment 211 bp in length. A series of quantitative standards were generated from serial 10-fold dilutions (a range of 1010–1 molecules) of TOPO-m1C3cxFv, in which full-length m1C3 was cloned. Each assay was run in triplicate wells in a 25 µl final reaction volume containing 2.5 µl of Platinum SYBR Green, 400 nM each forward and reverse primer, and 2.5 µl cDNA sample. Each run included negative controls consisting of wild-type control cDNA and water instead of cDNA. The amplification protocol consisted of 2 min at 95°C, 2 min at 95°C, followed by 40 cycles of amplification (94°C for 15 s, 60°C for 45 s, plate read of SYBR Green I fluorescence), after which a melting-curve reaction was conducted from 42°C to 95°C with plate readings every 1°C. GraphPad Prism software was used to calculate statistical significance using paired T-tests.

Immunoblot analysis
Mosquitoes were blood-fed on chickens and homogenized in a protease inhibitor solution made from complete mini (Roche) and Pefabloc SC (Roche). An equal volume of Laemmli sample buffer (Bio-Rad) with 0.1 M diithiothreitol was added. Homogenates were separated on a 12% Tris-HCl polyacrylamide gel in 1×Tris/Glycine/SDS buffer (Bio-Rad), transferred to Immun-Blot PVDF membrane (Bio-Rad), and incubated with goat anti-E tag polyclonal antibody conjugated to horse radish peroxidase (Abcam). ECL Plus Western Blotting Detection Reagents (GE Healthcare) were used to detect bound antibody. Ten females were used for each hemolymph sample preparation. Legs were removed with forceps and the proboscis was cut with a scissors. Individuals were inserted into a pipette tip plugged with glass wool and threaded through a 0.3 ml tube placed in a 1.5 ml collection tube. Centrifugation at 530 g for 10 min at 4°C extracted hemolymph. Each hemolymph sample was mixed with 25 µl 0.15 M NaCl and centrifuged at 2040 g for 5 min at 4°C. Fifteen microliters of the middle fraction of the sample was transferred to a new 1.5 ml tube, to which 10 µl of the protease inhibitor solution was added. For immunoblots with non-denatured samples, native sample buffer (Bio-Rad), 4–15% Tris-HCl polyacrylamide gels (Bio-Rad), 1×Tris/Glycine electrophoresis buffer (Bio-Rad), and native transfer buffer (25 mM Tris, 25 mM Glycine, pH 9.2) were used.

Parasite challenge experiments
Four to six day-old transgenic and wild type female mosquitoes were fed with P. falciparum NF 54 gametocytes using a membrane feeding apparatus. After 15 min of feeding, un-engorged mosquitoes were removed and engorged mosquitoes were maintained in the insectary under standard conditions [37] with daily access to a 10% sucrose solution or water and raisins. Midguts were dissected 9 days after the infectious bloodmeal, stained with 0.1% mercurochrome and the number of oocysts in each preparation counted. Uninfected bloodmeals were provided to transgenic and wild-type control mosquitoes following the membrane feeding. For the m4B7 25.1 experiments, mosquitoes were allowed to feed on the first and second days post-infection. Mosquitoes in the m2A10 experiments were allowed to feed on the 4th, 8th, and 12th days post-infection. Engorged and un-engorged females were not separated after the uninfected bloodmeals in m2A10 experiments 1–4, while un-engorged females were discarded in experiments 5–7. Samples of wild-type control and m2A10 females were dissected for oocyst counts on the 10th day post-infection. The salivary glands of all remaining m2A10 and wild-type control females were dissected 17–19 days post-infection. A hemacytometer was used to count salivary gland sporozoites [13]. The samples in experiments 5–7 were dried on 6 mm well slides and stored at −20°C. Sporozoites were stained using SlowFade Gold antifade reagent with DAPI (Invitrogen) and counted with a Zeiss Axioskop using the Axiovision camera and software. Sporozoites were counted using one of three methods, depending on parasite density. Method 1: If the number of sporozoites in each of five fields was counted, and a total of 3 or more sporozoites was found, an average sporozoite/mm2 measurement was calculated. When a field contained greater than 50 parasites, Improvement Velocity software was used to count the number of sporozoite nuclei in the DAPI image (Measurement protocol: 1. Find 2D nuclei: separate touching nuclei with a separation guide of 0.4 µm, reject nuclei with an area of less than 0.2 µm2. 2.
Exclude objects by size: exclude objects (>10 μm²). Method 2: If 3 sporozoite requirement of method 1 was not met, fields were examined until 3 sporozoites were counted, and average sporozoite/mm² measurement was calculated. Sporozoite/mm² values were used to calculate the total number of sporozoites present in the 6 mm² slide well area. Method 3: If a total of 3 sporozoites were not found in up to 25 fields, the entire 6 mm² slide well area was examined for an exact count. GraphPad Prism software was used to calculate statistical significance using Mann-Whitney U tests.

Accession numbers

The GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) accession numbers for the m1C3, m4B7, and m2A10 genes are HQ315886, HQ315365, and HQ315604, respectively.

Supporting Information

Figure S1 Immunoblot loading controls. Cooommassie-stained polyacrylamide gels used in immunoblot analyses of m2A10 expression. (A) Polyacrylamide gels used in m2A10 immunoblot presented in Figure 4A. (B) Polyacrylamide gel used in m2A10 immunoblot presented in Figure 4B. (C) A Cooommassie-stained polyacrylamide gel loaded with an equal volume of each sample used in the immunoblot of non-denatured hemolymph samples (Figure 3C) displayed protein migration. All labeling as in Figure 4. (TIF)

Table S1 Comparison of Anopheles gambiae (256,340 codons) and Mus musculus (18,786,705 codons) codon usage for selected amino acids [23]. (DOC)

Table S2 Transgene-specific primers used in expression analyses. (DOC)

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Author Contributions

Conceived and designed the experiments: ATI FL NJ XC XN OM JMV. Performed the experiments: ATI FL NJ. Analyzed the data: ATI FL NJ. Wrote the paper: ATI FL OM JMV AJ.

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