C-type lectin 4 regulates broad-spectrum melanization-based refractoriness to malaria parasites

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Abstract

Anopheles gambiae melanization-based refractoriness to the human malaria parasite Plasmodium falciparum has rarely been observed in either laboratory or natural conditions, in contrast to the rodent model malaria parasite Plasmodium berghei that can become completely melanized by a TEP1 complement-like system-dependent mechanism. Multiple studies have shown that the rodent parasite evades this defense by recruiting the C-type lectins CTL4 and CTLMA2, while permissiveness to the human malaria parasite was not affected by partial depletion of these factors by RNAi silencing. Using CRISPR/Cas9-based CTL4 knockout, we show that A. gambiae can mount melanization-based refractoriness to the human malaria parasite, which is independent of the TEP1 complement-like system and the major anti-Plasmodium immune pathway Imd. Our study indicates a hierarchical specificity in the control of Plasmodium melanization and proves CTL4 as an essential host factor for P. falciparum transmission and one of the most potent mosquito-encoded malaria transmission-blocking targets.

Introduction

Plasmodium falciparum is the most prevalent malaria parasite in Africa, accounting for 99.7% of the 213 million malaria cases on that continent in 2018 [1]. A comprehensive understanding of the biology and transmission of this human-pathogenic parasite through its main mosquito vector, Anopheles gambiae, is paramount for developing new tools to control malaria. Anophelines are not passive vectors: They possess an effective innate immune system that controls infections with diverse microbes, including Plasmodium parasites, bacteria, and fungi, with some degree of specificity. The susceptibility of mosquitoes to Plasmodium and other pathogens, and, hence, vector competence, is an intricate process determined by a fine balance between antagonistic and agonistic immune mechanisms and factors [2]. Melanization, typically the deposit of a melanin layer on the pathogen surface that results in its encapsulation, is one of the most effective insect defense mechanisms, and extensive studies have shown that A.
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**Abbreviations:** CFU, colony-forming unit; dsRNA, double-stranded RNA; gRNA, guide RNA; hpi, hours postinfection; Imd, immune deficiency; kd, knockdown; LB, Luria broth; PBM, post-blood meal; qRT-PCR, quantitative reverse transcription-PCR; RNAi, RNA interference.

gambiae can melanize and thereby block infection with the rodent malaria parasite *Plasmodium berghei*.

Together with effector molecule-producing immune signaling pathways, the complement-like system acts as a key effector and regulator of *A. gambiae* immunity. The core of this system is an ensemble of hemolymph proteins, including the thioester-containing protein TEP1, which is activated by an unknown protease to form TEP1-cut, which is stabilized by the leucine-rich repeat immune proteins LRIM1 and APL1C, which coordinate its binding to the surface of microbes, leading to their elimination through lysis or melanization [3–9]. A complex cascade of serine proteases appears to regulate the TEP1-mediated pathogen killing. The CLIPA2 serine protease inhibits the TEP1-cut deposition on the microbial surface, while the CLIPA14 serine protease also protects *P. berghei* and other microbes from being melanized, acting more downstream in the process where it appears to regulate phenoloxidase activity [10,11]. Melanization reactions are also key players in *A. gambiae* immunity to bacteria and fungi and have also been shown to kill pathogens through lysis without the formation of a melanotic capsule [9,12–16]. The C-type lectins CTL4 and CTLMA2, which exist mainly as a heterodimer [13,17], have been described as host factors of the rodent parasite *P. berghei*, protecting the ookinete-stage parasites from the TEP1 complement-like system-regulated melanization, thus enabling them to develop into oocysts and ultimately into sporozoites that can infect the vertebrate host [7,18]. With regard to malaria parasites, most studies on the complement-like defense system have employed the rodent parasite *P. berghei* model. Whether the same immune factors and mechanisms are involved in eliminating the clinically relevant human *P. falciparum* has not been clarified and is addressed in the present study.

We have previously used RNA interference (RNAi)-based gene silencing assays to show that partial depletion of *A. gambiae* CTL4 results in the melanization of a very small number of *P. falciparum* ookinetes, but only at unnaturally high infection intensities, and that CTL4 silencing does not affect overall mosquito susceptibility to the human malaria parasite, contrarily to the rodent *P. berghei*. Hence, previous studies by us and others did not prove a significant *P. falciparum* host factor role for *A. gambiae* CTL4, using human malaria laboratory strains and field isolates [19,20].

Here, we used CRISPR/Cas9 genome editing to knockout CTL4 and show that this factor is essential for the protection of the clinically relevant human *P. falciparum* parasite, through a mechanism that does not involve the known complement-like factors, which are important for *P. berghei* melanization [18–20]. Specifically, we show that the immune factors TEP1, LRIM1, and CLIPA2 do not influence melanization of *P. falciparum* NF54 ookinetes in CTL4 null mosquitoes while CLIPA14 does. The CTL4 partner, CTLMA2, plays a protective role for *P. falciparum* even when CTL4 is not present. Intriguingly, the human malaria parasite is not completely blocked by the CTL4-controlled melanization response while the rodent parasite is. Our study points at a significant influence of infection temperature, which is approximately 7 °C higher for *P. falciparum*, on the kinetics of midgut infection and efficiency of parasite melanization, enabling some human malaria parasites to escape this defense system. We also found that the key anti- *P. falciparum* innate immune pathway Imd does not influence parasite melanization, while it is known to mediate lysis-based anti-*P. falciparum* defense [21,22,23]. The CTL4 null mosquitoes are also highly refractory to the fungus *Beauvaria bassiana* through melanization, but more susceptible to bacterial infections, thereby pointing both agonistic and antagonistic roles of the C-type lectin complex for different pathogens [13,24]. We also show that the mosquito midgut microbiota marginally contributes to the CTL4 null- refractoriness to *Plasmodium*. Our study proves a major role for CTL4 in the malaria mosquito biology and pathogen transmission and establishes CTL4 as a potent transmission-blocking target for the development of novel malaria control strategies.
Results

Generation and characterization of CTL4\textsuperscript{null} mutants

We have used our established CRISPR/Cas9 gene editing methodology [25] to generate \textit{A. gambiae} CTL4-knockout mutant mosquitoes (CTL4\textsuperscript{null}). We first created a transgenic \textit{A. gambiae} line expressing 3 guide RNAs (gRNAs), each driven through a U6 snRNA polymerase III promoter, targeting the \textit{CTL4} gene (S1 Fig). Individual gRNAs were first synthesized as short DNA linkers (S1 Table) and separately cloned into plasmid modules carrying the U6 promoter sequence followed by a gRNA expression template, and then assembled by a Golden Gate cloning reaction into the pDSAR transgenesis vector containing the 3xP3-RFP reporter [26] (S1 Fig). This construct with helper plasmid was microinjected into embryos of the \textit{A. gambiae} X1 docking line, and transgenic progeny showing stable red fluorescence in the larvae were selected (S1 Fig). Following amplification of the \textit{CTL4}-gRNA-expressing (CTL4-gRNA) transgenic population for at least 4 generations, a subpopulation enriched with homozygous transgenic mosquitoes was crossed with the \textit{A. gambiae} Vasa-Cas9 strain to generate the CTL4-knockout (CTL4\textsuperscript{null}) mutant mosquitoes (S1 Fig). Because of preadult stage fitness constraints when trying to generate and rear a homozygous population of knockout mosquitoes, we decided to use the CTL4-knockout mutant progeny of the crossing between CTL4-gRNA and Vasa-Cas9 (gRNA/Cas9 transheterozygotes) for our studies, similarly to a previously published study [27]. Transheterozygotes from this cross carrying both transgenes were selected by their red and green fluorescent markers at the larval and adult stages (Fig 1A and 1B). The mutation/deletion in the adult mosquitoes was confirmed by both PCR with flanking primers (Fig 1C, S1 Table), quantitative reverse transcription PCR (qRT-PCR) in the various mosquito tissues (Fig 1D), as well as by sequencing of the PCR products (S1 File). The faint PCR products derived from the CTL4\textsuperscript{null} mosquitoes were also sequenced and shown not to correspond to CTL4 (S4 File). CTL4 protein deletion was also confirmed by western blot analysis, where no truncated CTL4 was observed (Fig 1E) using pools of 10 mosquitoes with a polyclonal serum raised against the mature CTL4. As a result of the CTL4 mutation, the CTL4 protein was truncated from aa 121 (S1 File). These data show that CRISPR/Cas9-mediated disruption of CTL4 is complete in the gRNA/Cas9 transheterozygotes; therefore, the F\textsubscript{0} somatic CTL4 knockout mutants were used as a model for further studies (referred to as CTL4\textsuperscript{null}).

CTL4 is a potent transmission-blocking target for malaria

First, we compared the susceptibility of all 3 control lines (CTL4-gRNA, the Vasa-Cas9, and the \textit{A. gambiae} X1 docking line) to \textit{P. berghei} and \textit{P. falciparum} infections and found no significant differences among the 3 lines (S2 Fig), suggesting that either one of them can serve as a control. We therefore decided to use the X1 line as a control in subsequent experiments (“control” when used hereafter designates the X1 line). To assess the impact of CTL4 knockout on the susceptibility of \textit{A. gambiae} to infection with the rodent \textit{P. berghei} parasite, we fed CTL4\textsuperscript{null} and control (X1) mosquitoes on \textit{P. berghei} (ANKA 2.34)-infected mice. As documented by several studies, \textit{P. berghei} achieves unnaturally high infection intensities in \textit{A. gambiae}, a consequence of \textit{A. gambiae} not being its natural vector. Therefore, to overcome any possible parasite intensity-related dependence on the outcome of infection, we performed assays with \textit{P. berghei} at both high and low infection intensities. Contrary to what we and others previously observed for RNAi-mediated silencing of CTL4 in \textit{A. gambiae} mosquitoes, the CRISPR/Cas9-induced knockout of this gene resulted in a complete (100%) refractoriness to both high and low \textit{P. berghei} infection intensity (Fig 2A–2C) [7,15,18,20,28–30]. A strain-specific dependence to explain our results can be ruled out, since all the \textit{A. gambiae} lines used in our study,
including CTL4null, are derived from the G3 strain, which was also used in most of the studies on CTL4 mentioned above.

We next addressed the permissiveness of CTL4null for the human malaria parasite *P. falciparum* (NF54). The CTL4-knockout mutants displayed strong *P. falciparum* suppression at low intensity infection, which mimicked the natural infections observed in the field (Fig 2D and 2E). The median oocyst count was significantly (\(p < 0.0001\)) reduced from 1 to 0 parasites/midgut, and the infection prevalence (mosquitoes harboring at least one parasite per total number of mosquitoes) was significantly (\(p < 0.0001\)) reduced from 61.3% to 19.7% in CTL4null when compared to the control at 8 d post-blood meal (PBM) (Fig 2D and S2 Table). Interestingly, CRISPR/Cas9-mediated knockout of CTL4 resulted in the melanization of *P. falciparum* even at the low intensity infection, in contrast to studies based on RNAi-mediated CTL4 silencing, in which *P. falciparum* melanization was shown to be infection intensity-dependent and hence only observed with a high intensity infection [20]. Accordingly, CTL4 knockout also resulted in a profound decrease in sporozoite loads in the salivary glands of mosquitoes at 14 d PBM (Fig 2E and S2 Table). At a high intensity infection, CTL4-knockout mutants yielded a median of 2 oocysts/midgut, as compared to 32 for the control, and the
prevalence of infection also significantly ($p < 0.0001$) decreased 2.2-fold, from 97.3% to 45.0% at 8 d PBM (Fig 2F and S2 Table). Conversely, at the high infection intensity, the total number of $P$. falciparum parasites (live and melanin-coated parasites combined) in the CTL4 null mosquito was significantly ($p < 0.0001$) lower than the number of total parasites in the control mosquitoes (Fig 2F). This discrepancy was not observed in the low $P$. falciparum infection intensity assay (Fig 2D) nor in $P$. berghei infection assays (Fig 2A and 2B), suggesting that CRISPR/Cas9-mediated disruption of CTL4 also results in the killing of $P$. falciparum without the formation of a melanotic capsule at high infection intensity.

In sum, we found that CTL4 null mosquitoes were completely refractory to $P$. berghei as a result of total melanization, and highly refractory to $P$. falciparum NF54, by melanization, or melanization in addition to another killing mechanism. This contrasts to the $A$. gambiae L3-5 genetically selected laboratory strain, which melanizes almost all rodent $P$. berghiei, but is unable to melanize sympatric human malaria $P$. falciparum [31]. The development of $P$. falciparum was not completely blocked in CTL4 null, indicating that the human malaria parasite is capable of partially evading the powerful defense system against which CTL4 protects it. While RNAi-based CTL4 gene silencing resulted in melanization of some parasites but not in a
decrease of infection intensity and prevalence, the more extensive melanization and statistically significant reduction in viable human \textit{P. falciparum} parasites in CTL4\textsuperscript{null} corroborates the assertion that the gene silencing will most often yield a hypomorphic phenotype because of an incomplete depletion of target proteins [20]. The observation that the total number of \textit{P. falciparum} parasites in the CTL4\textsuperscript{null} mosquitoes was significantly lower than the number in the control group suggests that CTL4 protects the human parasite not only from melanization but also from a killing mechanism that is either melanin formation independent or independent of the melanization process and manifests itself at higher levels of infection. Importantly, the level of \textit{P. falciparum} suppression in CTL4\textsuperscript{null} mosquitoes was significantly higher than that achieved through deleting the \textit{FREP1} host factor or by overexpressing the Imd pathway transcription factor \textit{REL2} in Anopheles, indicating CTL4 as a powerful transmission-blocking target [25,21].

**CTL4 knockout-mediated \textit{Plasmodium} killing occurs in the midgut epithelium**

Ookinete-stage \textit{Plasmodium} invades the mosquito midgut epithelium beginning about 18 to 20 h after ingestion of infected blood, depending on parasite species. To address the question of whether the complete refractoriness of CTL4\textsuperscript{null} to \textit{P. berghei} (Fig 2A–2C) occurs before or during midgut invasion, or at both stages, we measured the number of \textit{P. berghei} ookinetes in the blood bolus at 19 h after ingestion, when the majority of parasites have not yet invaded the midgut epithelium. No differences were found in the lumen ookinete loads between CTL4\textsuperscript{null} and control mosquitoes (Fig 3A), indicating that the \textit{P. berghei} decline in CTL4\textsuperscript{null} mosquitoes occurs when the parasite crosses the midgut epithelium. The peak of \textit{P. berghei} ookinete invasion of \textit{A. gambiae} occurs at 24 to 26 h postinfectious blood meal (hpi) [32]. Indeed, we found that all \textit{P. berghei} ookinetes were melanized in the epithelium of CTL4\textsuperscript{null} mosquitoes at 25 hpi, while the epithelium of control mosquitoes displayed live anti-Pbs28-stained fluorescent parasites (Fig 3C), again showing that the CTL4-dependent \textit{Plasmodium} protection takes effect exclusively during the invasion of the midgut epithelium.

We also wanted to investigate at which invasion stage the observed melanotic capsule-independent killing of \textit{P. falciparum} occurs during high-intensity infection in CTL4\textsuperscript{null} mosquitoes (Fig 2F). As was true for the rodent parasite, the number of \textit{P. falciparum} ookinetes in the lumen did not differ between the knockout and control cohorts (Fig 3B), indicating that CTL4 knockout-mediated \textit{P. falciparum} NF54 lysis, together with melanization, takes place in the midgut epithelium. Melanized \textit{P. falciparum} ookinetes were visible in the CTL4\textsuperscript{null} midgut epithelium at 24 to 25 hpi, together with live ookinetes (green) (Figs 2D–2F and 3C). Interestingly, while we observed colocalization of CTL4 with the parasite in control mosquitoes, the staining of CTL4 did not perfectly overlap with the contour of the parasite, suggesting that it might not be engaged in a direct interaction (Fig 3C).

**CTL4 knockout-mediated \textit{Plasmodium} melanization is marginally promoted by the mosquito microbiota**

Studies have shown that the \textit{A. gambiae} melanization response can be triggered by bacteria in the mosquito hemolymph [12,13]. To exclude the possibility that the extreme phenotype of complete \textit{P. berghei} melanization in CTL4\textsuperscript{null} mosquitoes is to some degree triggered by bacteria that can drive enzymatic cascades to reach the threshold needed to melanize ookinetes, we treated mosquitoes with an antibiotic cocktail to eliminate the majority of the bacteria from their midgut [22,33–35]. Interestingly, suppression of the bacteria in CTL4\textsuperscript{null} mosquitoes resulted in a few live \textit{P. berghei} oocysts (median, 0 versus 46.5, respectively; \(p < 0.0001\)
These results demonstrate that the mosquito microbiota weakly promotes *Plasmodium* melanization in CTL4<sup>null</sup> mosquitoes, possibly through the upregulation of immune factors or through bacteria-derived factors that can influence the process.

**CTL4-regulated *Plasmodium* melanization is independent of the Imd pathway**

The immune deficiency (Imd) pathway is one of the major immune signaling pathways that is activated by, and controls, infections with both bacteria and *P. falciparum* in *A. gambiae*. We and others have shown that knockdown of its negative regulator, Caspar, confers a *P. falciparum*-resistant phenotype in *A. gambiae* based on parasite lysis but not melanization [22,23].
We have previously shown that the mosquito microbiota augments the Imd pathway [33], and our experiments with aseptic mosquitoes showed a lesser melanization of *P. berghei*; hence, we hypothesized that perhaps this was due to a modulation of the Imd pathway in the absence of immune-eliciting bacteria in the aseptic mosquitoes [21,33,35,36,37]. In order to investigate whether the Imd pathway could somehow be involved in the CTL4 knockout-induced *Plasmodium* melanization, we activated the Imd pathway by RNAi-mediated silencing of the pathway’s negative regulator *Caspar* (gene knockdown (kd) efficiency of 63.25%) in aseptic control and CTL4 null mosquitoes (Fig 4B) and compared the melanization phenotypes to mosquitoes treated with a control GFP double-stranded RNA (dsRNA). As shown in Fig 4B, Imd pathway augmentation in aseptic CTL4 null mosquitoes did not change the *P. berghei* melanization phenotype since some live oocysts would still form at the aseptic condition. Hence, the formation of some live *P. berghei* oocysts in CTL4 null mosquitoes at aseptic conditions is not due to a lack

Fig 4. Effects of microbiota and Imd pathway on *Plasmodium* suppression in CTL4 null mosquitoes. (A, B) *P. berghei* infection in antibiotics-treated control and CTL4 null *A. gambiae* midguts at 10 dpi, compared to nonantibiotics treated (A) or after injection with dsCaspar or control dsGFP (B). (C, D) *P. falciparum* infection in control and CTL4 null *A. gambiae* females measured at 8 dpi, after RNAi-mediated silencing of IMD (C) and Caspar (D). S5 File displays the underlying data. Dots indicate the number of parasites in an individual midgut (L, live; M, melanized), and horizontal red bars indicate the median, compared by two-tailed p-values by Mann–Whitney test. Bars show the percentage of mosquitoes harboring at least one oocyst, and the Fisher exact test was used to calculate p-values. Significance of parasite numbers: *: p < 0.05, ***: p < 0.0001; horizontal black lines alone: not significant. dpi, days postinfection; RNAi, RNA interference.

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of a bacterially mediated modulation of the Imd pathway (Fig 4A and 4B). These data also suggest that the Imd pathway is not involved in CTL4 knockout-mediated melanization of Plasmodium. In A. gambiae, the Imd pathway has emerged as a key defense system against the human malaria parasite P. falciparum through a lytic killing mechanism (not melanization) [22,23]. To further explore a possible implication of the Imd pathway in the CTL4 knockout-induced melanization of P. falciparum, we independently silenced the IMD receptor protein (gene kd efficiency of 68.10%) and the negative regulator Caspar to inactivate and activate the pathway, respectively, prior to P. falciparum infection. Silencing IMD did not influence P. falciparum melanization in CTL4null mosquitoes, whereas it did influence their susceptibility to parasite infection with live parasites in both the CTL4null and control groups (Fig 4C), in agreement with our previous studies [22,23]. Silencing Caspar did also not influence the number of melanized P. falciparum ookinetes in the CTL4null mosquitoes, corroborating the independence of the CTL4-regulated melanization from the Imd pathway (Fig 4D).

### CTL4-regulated P. falciparum melanization is temperature dependent

As described above, P. falciparum infection in CTL4null mosquitoes resulted in a powerful but somewhat leaky phenotype where the infection was not completely abrogated (Fig 1). Optimal infections of A. gambiae with P. berghei and P. falciparum occur at 19 to 20 °C and 27 °C, respectively, and this rather large temperature difference results in a slower rate of development/infection for the rodent parasite than for the human parasite within its vector [38,39]. We hypothesized that it might also influence immune response kinetics and exposure of the parasites to these immune responses, that could explain the complete versus incomplete melanization of P. berghei and P. falciparum, respectively, in CTL4null mosquitoes. To determine whether the leaky P. falciparum infection phenotype of the CTL4null mosquitoes could be attributed to the higher infection temperature, we performed P. falciparum infection experiments at both 19 °C and 27 °C with control and CTL4null mosquitoes fed on the same P. falciparum NF54 gametocyte culture. First, we determined the effect of temperature on P. falciparum development and infection kinetics of the midgut lumen and epithelium in control mosquitoes. Reducing the temperature to 19 °C resulted in a dramatic reduction of ookinetes in both the midgut lumen and epithelium, as well as a significantly reduced number of formed oocysts (Fig 5A and 5B). In addition to the overall reduction of the various parasite stages at 19 °C, we also observed the expected slower midgut invasion and oocyst development kinetics (Fig 5A and 5B). Next, we assessed the effect of the lower temperature on P. falciparum melanization in CTL4null mosquitoes. Interestingly, reducing the temperature to 19 °C did not affect the intensity and prevalence of parasite melanization compared to infection at 27 °C, but it completely abolished the formation of live oocysts in the CTL4null mosquitoes (Fig 5C). These data show that melanization of P. falciparum at 19 °C is more efficient and extensive than that at 27 °C, since the proportion of melanized parasites in relation to the total number of ookinetes in the lumen and midgut epithelium, and live oocysts, is much larger at the lower temperature. These findings show that the observed differences in the proportions of melanized P. berghei and P. falciparum in CTL4null mosquitoes is to a significant extent due to a difference in the temperature required for the sexual development of either parasite, as the more efficient melanization at the lower temperature is likely attributed to a slower invasion kinetics that prolongs the exposure of ookinetes to the mosquito’s melanization-mediated defense.

### CTL4 is a selective antagonist of bacterial infection

Mosquito immune defense mechanisms have most likely principally evolved to combat infections with pathogens that are more prevalent and virulent than Plasmodium [40], such as
Fig 5. Influence of temperature on *Plasmodium* suppression in CTL4null mosquitoes. (A) *P. falciparum* ookinetes in the lumen of control *A. gambiae* females at 24 and 40 hpi at 19 °C and 24 hpi at 27 °C, and oocysts at 13 dpi at 19 °C and 8 dpi at 27 °C. (B) *P. falciparum* ookinetes in the midgut epithelium of control *A. gambiae* females at 24 and 40 hpi at 19 °C and 27 °C, and oocysts at 13 dpi at 19 °C and 8 dpi at 27 °C. (C) *P. falciparum* infection in control and CTL4null *A. gambiae* females measured at 8 dpi at 19 °C or 27 °C. S5 File displays the underlying data. Dots indicate the number of parasites in an individual midgut (L, live; M, melanized), and horizontal red bars indicate the median.
CTL4 controls melanization of human malaria parasites

bacteria and fungi, which are mainly present in the mosquito’s external environment and intestine. Hence, many of the immune genes and immune signaling pathways that mediate antibacterial and antifungal defenses are also involved in anti-

Plasmodium immunity [2]. Knowing that CTL4 is essential for mosquito immunity/tolerance against bacterial systemic infections, we first investigated whether it also contributes to controlling the mosquito’s midgut microbiota. We first compared the number of Luria broth (LB)-culturable bacteria found in the midgut of either sugar-fed control and CTL4null mosquitoes, and the results showed a marginally nonsignificant ($p = 0.0612$) increased bacterial load in CTL4-deficient mosquitoes (Fig 6A). To also capture contributions of nonculturable bacteria, we compared the total microbiota by qRT-PCR of bacterial 16s ribosomal RNA in both sugar-fed and blood-fed control and CTL4null mosquitoes (Fig 6B). Results again showed that bacterial proliferation in the A. gambiae sugar-fed midgut is not significantly controlled by CTL4 but that CTL4 deletion affects the proliferation of bacteria following a blood meal, which was reduced in the CTL4null mosquitoes, thereby suggesting that CTL4 has an agonistic effect on the midgut microbiota upon blood feeding through an unknown mechanism.

Next, we investigated the role of CTL4 in controlling systemic bacterial infections by monitoring the survival of CTL4null and control mosquitoes after injection of either control PBS,

compared by two-tailed $p$-values by Mann–Whitney test. Bars show the percentage of mosquitoes harboring at least one oocyst, and the Fisher exact test was used to calculate $p$-values. Significance of parasite numbers: $^\ast: p < 0.05, ^\ast\ast: p < 0.01; ^\ast\ast\ast: p < 0.001; ^\ast\ast\ast\ast: p < 0.0001$; horizontal black lines alone: not significant. dpi, days postinfection; hpi, hours postinfection.

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Fig 6. Influences of CTL4 on mosquito interactions with bacteria and fungi. (A, B) Midgut microbial flora of control and CTL4null A. gambiae females was compared using two-tailed $p$-values by Mann–Whitney (A) and ANOVA followed by a Tukey test (B). (C) Control and CTL4null A. gambiae females were injected with either E. coli (350,000 CFU), S. aureus (420,000 CFU), and B. subtilis (62,000 CFU), or PBS as a control, and longevity was analyzed up to 8 dpi. Kaplan–Meier survival analysis with a log-rank test; SE of replicates are indicated. (D) Abdomens were dissected to check bacterial melanization 3 d following E. coli and S. aureus challenge by injection, and the Fisher exact test was used to determine the difference between control and CTL4null A. gambiae females. (E) Fungi spores were efficiently melanized (white arrows) in the CTL4null abdomens 12 h and 48 h following injection of B. bassiana (2.15 x 10^6 spores/ml), and the number of live spores (green arrows) was visibly higher in the control abdomens. (F) Melanized and live fungi were measured in midguts tissues from 24 h to 7 d after mosquitoes fed on a sucrose solution containing 2.15 x 10^6 B. bassiana spores/ml, and the Fisher exact test was used to calculate the difference between control and CTL4null A. gambiae females. (G) Melanized fungi were detected on the midgut tissue of CTL4null mosquitoes after dipping (surface exposure) in B. bassiana spores (1 x 10^8 spores/ml), compared by the Kaplan–Meier survival analysis with a log-rank test; SE of replicates are indicated. Significance: $^\ast\ast\ast\ast: p < 0.0001$. S5 File displays the underlying data. CFU, colony-forming unit; dpi, days postinfection; SE, standard error.

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gram-negative *Escherichia coli*, or gram-positive *Staphylococcus aureus* and *Bacillus subtilis* into the hemolymph. This assay showed a decreased survival of CTL4<sup>null</sup> mosquitoes upon challenge with gram-negative *E. coli*, but not with the gram-positive bacteria (Fig 6C), in agreement with previous studies [13,24,32]. To investigate whether the increased CTL4<sup>null</sup> mortality following *E. coli* challenge was related to bacteria-induced melanization, which could potentially produce toxic byproducts that are detrimental to the mosquito, we measured the mosquitoes’ abdominal melanization, which is visible in the cuticle 3 d after challenge with *E. coli* and *S. aureus* (Fig 6D). No differences were observed in the percentage of melanization between the CTL4<sup>null</sup> and control cohorts, indicating that the decreased survival of *E. coli*-challenged CTL4<sup>null</sup> mosquitoes was not due to a CTL4 knockout-induced increase in bacterial melanization (Fig 6D). Similarly, Schnitger and colleagues [13] did not observe an increase in phenol oxidase enzymatic activity in bacteria-challenged CTL4-silenced mosquitoes. In conclusion, our bacteria challenge assays suggest that CTL4 acts as an antagonist of systemic bacterial infections in a melanization-independent manner. It could be argued that the melanization observed in both CTL4<sup>null</sup> and control mosquitoes in Fig 6C is a response to wounding provoked by the mechanical injection of bacteria, which equally affected both the CTL4<sup>null</sup> and control cohorts. However, in contrast to another study in which thoracic melanization of the injection site was observed in CTL4-silenced mosquitoes but not in the dsLacZ control [15], we did not observe any increased abdominal melanization in our CTL4<sup>null</sup> cohort (Fig 6D).

### CTL4 protects fungi from melanization

Mosquitoes are extensively exposed to fungi in their natural environments. To investigate whether CTL4 plays a role in mosquito immunity against fungi, we used the entomopathogenic fungus *Beauvaria bassiana*, which is virulent and has been shown to trigger the melanization response in *A. gambiae* [9,14,30]. After challenging mosquitoes with an intrathoracic injection of a *B. bassiana* spore solution, the number of live fungus spores was visibly higher in the abdomens of control mosquitoes as compared to the CTL4<sup>null</sup> mosquitoes, in which the majority of the spores were efficiently melanized (Fig 6E). The CTL4<sup>null</sup> mosquitoes also showed an increased melanization of *B. bassiana* associated with the midgut tissues, compared to the control cohort, at all assayed time points up to 7 d after feeding on a sterile sucrose solution containing *B. bassiana* spores (Fig 6F and 6G). Taken together, these assays demonstrate that CTL4 prevents melanization of fungi in both the *A. gambiae* abdomen and midgut tissues, suggesting that it plays a similar agonistic role for *B. bassiana* as it does for *Plasmodium*.

Although these experiments were helpful in elucidating the role of CTL4 in the *A. gambiae* immune response to fungi, the natural route of fungal infection in the mosquito does not require ingestion by the host (as for *Plasmodium* and bacteria) but rather consists of a direct penetration through the mosquito cuticle into the hemocoel after surface exposure. Therefore, we also monitored survival of CTL4<sup>null</sup> and control mosquitoes after surface exposure by dipping mosquitoes in a *B. bassiana* spore solution. Interestingly, CTL4<sup>null</sup> mosquitoes exhibited enhanced tolerance to *B. bassiana* infection, as reflected in their higher survival rate when compared to the control group (Fig 6H). The RNAi-mediated silencing of 2 key factors of the TEP1 complement-like system, TEP1 and CLIPA8, has been shown to increase the susceptibility of mosquitoes to *B. bassiana* infection by reducing fungal melanization, revealing these 2 genes as positive regulators of fungal melanization [9]. Interestingly, both these immune proteins are also antagonists of *Plasmodium* infection, promoting parasite lysis and/or melanization [3,29]. The fact that CTL4 is an agonist/host factor and TEP1 and CLIPA8 are both antagonists/restriction factors of both *Plasmodium* and fungi indicates the existence of at least a partial overlap in the genetic modules that regulate the melanization response of *A. gambiae*.
to both microorganisms. It is therefore conceivable that CTL4 plays a broader role in protecting eukaryotic unicellular organisms. The enhanced tolerance of CTL4 null mosquitoes to B. bassiana infection is a particularly attractive feature that provides CRISPR/Cas9-mediated CTL4 knockout transgenic mosquitoes with a survival advantage over wild-type populations in the context of a fungal-based mosquito biocontrol strategy.

**CTL4 regulation of Plasmodium falciparum is independent of the TEP1 complement-like system**

We were interested in exploring the implication of other factors of the complement-like defense system in CTL4 knockout-dependent Plasmodium melanization. For this purpose, we used standard RNAi-based gene-silencing assays in conjunction with parasite infections. It is important to note that the RNAi-based approach used to silence genes achieves incomplete depletion of the target proteins and that a complete depletion would likely result in stronger phenotypes, similarly to what we have observed between our previous [20] and the present study. First, we addressed the C-type lectin CTLMA2, which forms a dimer with CTL4 and yields a similar phenotype when silenced by RNAi in wild-type mosquitoes [13,18–20]. While no effect on P. falciparum infection was observed between the CTLMA2-silenced (gene kd efficiency of 87.02%) and the GFP dsRNA-treated control mosquitoes (Fig 7A), silencing this gene in CTL4 null mosquitoes resulted in a marginal reduction of live oocysts when compared to GFP-treated mosquitoes, but no change in ookinete melanization, suggesting that CTLMA2 also protects against melanotic capsule-independent parasite killing (Fig 7A). Previous studies have suggested that CTL4 and CTLMA2 are secreted into the hemolymph in the form of a disulfide-linked heterodimer [13,17], thus explaining their similar roles in the mosquito defense against bacteria and Plasmodium. However, our results (Fig 7A) reveal a minor agonistic role for CTLMA2 that is independent of CTL4. These results suggest that in the context of the A. gambiae immune response to the human malaria parasite, CTLMA2 may still be moderately active as a monomer or homodimer [13] that functions differently from the CTL4-CTLMA2 heterodimer.

The LRIM1/APL1C complex is a Plasmodium antagonist required for the binding of TEP1 to ookinetes [4–7] and hence acts upstream of CTL4. Several studies using RNAi gene silencing have demonstrated that the leucine-rich repeat protein LRIM1 is necessary for P. berghei melanization in A. gambiae [6,18]. To investigate the implication of LRIM1 in P. falciparum melanization, we silenced LRIM1 (gene kd efficiency of 80.96%) and observed no variation in the number of live parasites in either the control or the CTL4 null cohorts, or melanized parasites in the CTL4 null mosquitoes (Fig 7B), thereby showing that LRIM1 is not necessary for the CTL4 knockout-mediated melanization of P. falciparum NF54.

TEP1 is one of the most studied factors in the A. gambiae anti-Plasmodium defense as a Plasmodium antagonist that provides protection against both human and rodent malaria parasites, triggering immune responses that include lysis and melanization [3,7,22,32,41]. TEP1 is acting upstream of a cascade of clip-domain serine proteases and leucine-rich domain proteins that control lysis and melanization of P. berghei and P. falciparum, and melanization of P. berghei in absence of CTL4 [7]. It has been proposed that some P. falciparum strains have partly adapted to geographically sympatric vectors through evasion of complement-like immune responses [41]. Nevertheless, the P. falciparum NF54 strain does not evade TEP1-mediated lysis completely, as shown by us and others in TEP1-silencing experiments [32,22,42]. Interestingly, TEP1 knockdown (gene kd efficiency of 79.32%) did not abolish melanization-based resistance to P. falciparum in CTL4 null mosquitoes, but it did reverse the lysis-mediated killing of this parasite in the control mosquitoes (Fig 7C). This result is in sharp contrast to the P.
Fig 7. Interaction of CTL4 with complement-like immune-related genes in Plasmodium suppression. (A, B) *P. falciparum* infection intensity and prevalence in control and CTL4null *A. gambiae* females measured at 8 dpi, after RNAi-mediated silencing of CTLMA2 (A) and LRIM1 (B). (C, D) *P. falciparum* infection intensity and prevalence (C) measured at 8 dpi were compared to *P. berghei* (D) infection intensity and prevalence measured at 10 dpi, following RNAi-mediated silencing of TEP1 in control and CTL4null *A. gambiae* females. (E, F) *P. falciparum* infection intensity and prevalence in control and CTL4null *A. gambiae* females measured at 8 dpi, after RNAi-mediated silencing of
Here, we reveal the role of CTL4 in immunity against the human malaria parasite and against fungi and bacteria using CRISPR/Cas9-generated CTL4null mice. When TEP1, APL1C, and LRIM1 are silenced, the parasites are protected from being melanized by the CTL4/CTLMA2 complex and CLIPA2 and CLIPA14 in wild-type mosquitoes. When CLIPA2 and CLIPA14 are silenced, the P. berghei ookinetes are lyzed by the action of TEP1 and the Imd pathway and TEP1 and APL1C, and LRIM1, and they are protected from being lysed by CLIPA2 and CLIPA14 and are protected from being melanized by the CTL4/CTLMA2 complex, CLIPA2 and CLIPA14 in wild-type mosquitoes. When TEP1, APL1C, and LRIM1 are silenced, the P. berghei ookinetes develop into oocysts. When CLIPA2 and CLIPA14 are silenced, the P. berghei ookinetes are lyzed. In CTL4null mosquitoes, the Imd pathway is promoting lysis of the parasites, while silencing of CLIPA2 will enhance melanization of the parasites. TEP1, APL1C, and LRIM1 are promoting melanization of the ookinetes in CTL4null mosquitoes, and silencing of CLIPA2 and CLIPA14 will also promote melanization of the ookinetes in CTL4null mosquitoes.

**Discussion**

Here, we reveal the role of CTL4 in immunity against the human malaria parasite and against fungi and bacteria using CRISPR/Cas9-generated CTL4null A. gambiae mosquitoes. We show that CTL4's protective role is exclusively exerted in the mosquito midgut epithelium on invading ookinetes, and, unlike the murine parasite P. berghei, the human malaria parasite P. falciparum is not completely melanized in CTL4null mosquitoes. This partial refactoriness is to a significant extent related to the higher temperature of P. falciparum infections, which allows this parasite to develop and traverse the midgut epithelium quicker than the rodent parasite at the lower temperature (Fig 5). It is possible that other differences in the biology of P. berghei and P. falciparum infections in A. gambiae also relate to the 7°C difference in infection temperature. While the complete removal of CTL4 in CTL4null mosquitoes resulted in strong melanization phenotypes and significant suppression of both P. berghei and P. falciparum, its...
partial depletion through RNAi-based silencing only yielded strong melanization and suppression of *P. berghei* in *A. gambiae* [20]. This suggests that the rodent parasite has a higher dependence on the quantity of available CTL4, most likely because of the slower rate of invasion of the midgut epithelium, while the faster migrating *P. falciparum* may not enable the melanization-mediated defense system to target it as effectively.

Importantly, our study demonstrates that the functions of key anti-*Plasmodium* effectors in the defense against the human malaria parasite differ from those acting against the rodent counterpart. The TEP1 and LRIM1 effectors, and the CLIPA2 agonist, which regulate the melanization of *P. berghei*, do not influence *P. falciparum* NF54 melanization. TEP1 does also not bind to the surface of the *P. falciparum* oocokine, in contrast to the mechanism regulating *P. berghei* melanization (Fig 7G). However, the serine protease CLIPA14, which is further downstream in the molecular cascade leading to melanization, influences *P. falciparum* NF54 melanization similarly to *P. berghei* melanization. Taking our findings together with previous studies, it becomes apparent that the parasite species specificity of this molecular cascade is higher upstream, where *A. gambiae* TEP1, LRIM1, and CLIPA2 are specific for the regulation of *P. berghei* killing and melanization, while the species specificity decreases downstream, as CLIPA14, CTL4, and CTLMA2 regulate melanization of both parasite species (Fig 7H) [10,11,20]. It is quite likely that other unknown members of the TEP, LRIM, and CLIP families may be implicated in regulating *P. falciparum* melanization.

We also show that CTL4 knockout-mediated killing of *P. falciparum* is not exclusively the result of capsule-dependent melanization, since more than 50% of the oocokines appeared to be killed without the formation of a melanotic coating of the parasites in CTL4<sup>null</sup> mosquitoes at a high intensity of parasite infection. This killing mechanism is likely the result of an incomplete melanization reaction that does not result in the formation of a melanotic capsule, as shown for *Drosophila* [16]. Interestingly, silencing of CTLMA2, which resulted in a decreased number of live *P. falciparum* oocysts in the CTL4<sup>null</sup> mosquitoes, was not accompanied by an increase in oocokine melanization, suggesting that CTLMA2 may protect the parasite from additional nonmelanization-related killing mechanisms and is not exclusively exerting its function as an interacting partner with CTL4. While the Imd pathway is a key anti-*P. falciparum* lysis-based defense system, it does not influence melanization of *Plasmodium* in CTL4<sup>null</sup> mosquitoes. It will be interesting to address the effects of CTL4 knockout on different malaria parasites strains in future studies.

Mosquitoes are continuously exposed to a variety of bacteria and fungi, and our infection assays with CTL4<sup>null</sup> mosquitoes show that CTL4 is also an agonist of fungal infections through a melanization-based defense similarly to that mounted against *Plasmodium*, but an antagonist of systemic bacterial infections. The opposite roles of CTL4 in fungal and bacterial systemic infections are particularly interesting: like fungi, *Plasmodium* is also unicellular eukaryote, in contrast to bacteria being prokaryotes with a cell wall. Our results suggest that both *Plasmodium* and *B. bassiana* recruit CTL4 as a protective factor against melanization-based killing. The mosquito’s natural midgut microbiota is weakly promoting *Plasmodium* melanization, likely though priming of additional immune factors, but not the antibacterial and anti-*Plasmodium* Imd pathway.

The CTL4 agonist system described in this study is particularly interesting as a malaria transmission-blocking target because it achieves a level of refractoriness to the human malaria parasite that we have not observed with other genetically modified *Anopheles* lines, without compromising the life span of adult mosquitoes (S3 Fig). Modeling studies have shown that total transmission blocking, or refractoriness, is not necessary to achieve an epidemiologically significant impact on disease prevalence and that even a 35% transmission-blocking effect would result in malaria elimination from a hypoendemic area [43,44]. Furthermore, studies
using Anopheles stephensi and Plasmodium yoelii infection models, whose sporozoite infectivity is similar to that observed for P. falciparum, have shown that the likelihood of transmission is significantly decreased at low sporozoite loads (<10,000 sporozoites) in the salivary glands [45]. Our CTL4null mosquitoes exceeded a 35% reduction in P. falciparum infection prevalence, and the salivary gland sporozoite loads were strongly suppressed, predicting a significant impact on malaria transmission as a result of a population replacement of wild-type with CTL4-deficient mosquitoes. However, the adverse effects of a CTL4 germline-based knockout on embryonic development call for alternative approaches to inactivate CTL4 function at the life stage and in the tissues that are relevant for Plasmodium development. B. bassiana is also an entomopathogenic fungus that has been developed into an ecologically friendly biopesticide for mosquito control [14,46–50]. The increased resistance of CTL4-deficient mosquitoes to both Plasmodium and B. bassiana could thereby be exploited to provide a competitive/selective advantage to the malaria-refractory CTL4null mosquitoes against their wild-type counterparts through B. bassiana exposure.

Materials and methods

Ethics statement

All animal work was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH), USA. The protocols and procedures used in this study were approved by the Animal Care and Use Committee of the Johns Hopkins University (Permit Number: MO18H82) and the Johns Hopkins School of Public Health Ethics Committee. Commercial anonymous human blood was used for parasite cultures and mosquito feeding; thus, informed consent was not required.

Generation of U6-gRNA constructs

Putative gRNA sequences targeting the A. gambiae CTL4 (AGAP005335) gene were identified using ZiFiT (zifit.partners.org). Sequences with no off-target sites, 20 nucleotides long, starting with a guanine (G) to facilitate transcription by the U6 promoter, and followed by a protospacer adjacent motif (PAM; NGG, where N is any nucleotide), were chosen (S1 Table). The final selected gRNA target sequences are within the region encompassing the CTL4 RNAi primers. For each of the 3 gRNA targets selected for the CTL4 gene, we designed a forward and a reverse primer, complementary to each other (S1 Table). To generate each of the three pKSB-gRNA vectors (pKSB-CTL4-gRNA-1, -2, -3), each of the 3 double-stranded DNA fragments was individually cloned into a pBluescript vector backbone that contained the U6 snRNA polymerase III promoter (AGAP013557), CRISPR RNA invariable sequences, and the RNA polIII TTTTT terminator (as in [25]), followed by sequencing confirmation. The pKSB-CTL4-gRNA vectors were used to assemble 3 gRNA copies of CTL4 into a destination pDSAR vector via the Golden Gate assembly system (New England Biolabs, E1600S) for embryo microinjection of the A. gambiae docking line X1 (S1 Fig) [26]. The final plasmid, pDSAR-CTL4-gRNA3 (with 3xP3-RFP), allowed screening of gRNA-positive larvae or adult mosquitoes by using the red fluorescent eye marker (Fig 1, S1 Fig).

Mosquito rearing and embryo microinjection

All the A. gambiae s.s. mosquito lines used in this study were derived from the G3 strain, including the docking line X1 as well as the Vas2-Cas9 [51] and CTL4-gRNA3 lines described above, here referred to as Cas9 and CTL4-gRNA, respectively. Mosquitoes were reared and maintained under laboratory conditions according to Simões and colleagues [52]. Embryo
microinjection solution was prepared by combining maxi-prep purifications (Invitrogen) of 2 plasmids: the pDSAR-CTL4-gRNA3 construct (160 ng/μl) and a helper plasmid, pENTR-R4R3-Vasa2-integrase (200 ng/μl), expressing the phiC31 integrase under the Vasa promoter [26,53], in 1/10 of 10× injection buffer solution (0.1 mM NaHPO4 buffer and 5 mM KCl (pH 6.8)).

Approximately 700 A. gambiae X1 docking line embryos were injected [26,54] and maintained on wet filter paper for 2 d before hatching [25]. The hatched G0 larvae were screened for transient red fluorescence expression at the second instar larval stage under a fluorescence stereomicroscope. Approximately 29.5% of the hatched larvae showed RFP fluorescence, and the RFP+ G0 were sexed at pupal stage and emerged adults were crossed with X1 adults from the opposite sex (virgin females and males were prepared in the same way as the gRNA line). The G1 progeny were examined for RFP fluorescence at both the larval and adult stages. Positive G1 mosquitoes were outcrossed with X1 for 2 generations, followed by 2 generations of self-crossing to enrich the homozygous CTL4-gRNA mosquitoes by the screening of RFP in the larvae.

**Generation of CRISPR/Cas9 CTL4-knockout mutants**

Nearly fully homozygous CTL4-gRNA mosquitoes starting from generation 5 were crossed with the Vasa-Cas9 strain to generate CTL4-knockout (CTL4*null*) mutants (S1 Fig). Next, we sought to obtain a line of homozygous CTL4*null* mosquitoes by crossing the CTL4 knockout mutants with X1 mosquitoes, but the progeny of these crossings resulted in either nonhatching eggs or only wild-type descendants, as described in Yang and colleagues [27]. Given this challenge, we decided to use the CTL4*null* mosquitoes, which are the transheterozygous progeny of the crossing of the CTL4-gRNA virgin females with the Vasa-Cas9 males (S1 Fig), for all our assays. All generations of CTL4*null* mosquitoes were screened for both red and green fluorescence (Fig 1). The mutation/deletion in CTL4*null* females was assessed by PCR with flanking primers and confirmed by qRT-PCR in the different mosquito tissues (Fig 1). The faint PCR products derived from CTL4*null* were also sequenced by Sanger sequencing, after gel excision and purification, showing that they do not contain CTL4 sequences (S4 File). The mutation of the CTL4 gene was determined by alignment of the mutant sequence with the wild-type sequence (S1 File). All primers sequences used are listed in S1 Table.

**CTL4 and Tep1 antibodies and western blotting**

Polyclonal CTL4 and TEP1 antibodies were generated by Boster Biological Technology in rabbit targeting antigens corresponding to peptide sequences of the 2 genes (cDNA sequences used are available in S2 and S3 Files). Control and CTL4*null* 3 pools of 10 mosquitoes were homogenized in PBS with protease inhibitor cocktail (cOmplete, EDTA-free Protease Inhibitor Cocktail, Roche), and samples were stored overnight at 4 °C. Protein concentrations were measured with a Micro BCA Protein Assay Kit (Thermo Fisher Scientific). Western analysis was done following a previously established protocol with modifications [55]. About 20 μg of total protein were mixed with NuPAGE LDS Sample Buffer (4X) (Thermo Fisher Scientific) and incubated at 95 °C for 10 min before they were separated on a 4% to 20% SDS-PAGE gradient gel (Novex WedgeWell 4% to 20%, Tris-glycine, 1.0-mm Mini Protein Gel, 10-well, Thermo Fisher Scientific). Proteins were transferred to a nitrocellulose membrane through a Trans-Blot Turbo Transfer System (BioRad), following incubation in blocking buffer (1X PBS with 0.05% Tween-20, 5% nonfat milk powder) overnight at 4 °C. Anti-A. gambiae CTL4 rabbit polyclonal antibody was diluted 1:500 in blocking solution and incubated for 1 h. The blot was washed 3 times for 15 min each with 1xPBS (Tweem-200.05%), incubated with HRP-linked
anti-rabbit IgG secondary antibody (Cell Signaling Technology, 7074) at 1:15,000 dilution for 1 h, washed again, and developed using ECL Prime Western Blotting Detection Reagent (GE Healthcare). In addition to sample normalization with the BCA protein assay, the membrane was stripped for 20 min with Restore Plus Western Blot Stripping Buffer (Thermo Scientific), blocked and incubated with anti-beta actin primary antibody-loading control (Abcam, 8224) at 1:1,000 dilution, followed by HRP-linked anti-mouse IgG secondary antibody (Cell Signaling Technology, 7076) at 1:15,000 dilution.

Plasmodium infection and longevity assessment

To assess anti-Plasmodium activity, female mosquitoes were fed on an anesthetized P. berghei (ANKA 2.34 strain)-infected mouse or through artificial membrane feeders on an NF54 P. falciparum gametocyte culture in human blood. For high-infection P. berghei experiments, female mosquitoes were allowed to feed for a longer period on mice with higher parasitemia. High (approximately 0.1%) or low (approximately 0.01%) gametocytemia was used for P. falciparum NF54 infection experiments. Unfed females were removed, P. berghei- and P. falciparum-infected mosquitoes were kept at 19 °C or at 27 °C, respectively, except for some P. falciparum-infected cohorts that were kept at 19 °C for temperature-dependent experiments. At 19 h postinfection (hpi), infected midguts were dissected in PBS, the blood bolus was removed, and the midguts were homogenized and transferred to a glass slide on which they were fixed in methanol. Midguts were then stained with Giemsa, and ookinetes in the midgut lumen were counted using a Leica DM 2500 microscope. For temperature-dependent experiments with the control mosquitoes, the lumen ookinetes were counted using the blood bolus at both 24 hpi and 40 hpi for 19 °C and 24 hpi for 27 °C treatments through Giemsa staining. The epithelium ookinetes at 24 hpi and 40 hpi for 19 °C treatment, and 24 hpi ookinetes and 40 hpi early oocysts for 27 °C were measured through immunohistochemical staining with anti-Pfs25 antibodies as previously described [33,56]. Oocysts and sporozoites were counted as described previously [20,57]. To evaluate the impact of knocking out CTL4 in A. gambiae adult female mosquitoes, females were divided into 3 cohorts: either maintained on filtered-sterile 10% sucrose solution alone, provided with a single naïve mouse blood meal, or infected with P. berghei, as detailed in [52]. The life span of these 3 groups (35 females per group) was monitored for 40 d.

Immunohistochemical staining and confocal microscopy

IFA and confocal microscopy were done essentially according to Dong and colleagues [56] with modifications. P. falciparum- and P. berghei-infected mosquito midguts were dissected at approximately 24 hpi in 1% paraformaldehyde, the blood bolus was removed, and the midguts were washed with PBS and fixed in 4% paraformaldehyde for at least overnight. The midguts were then incubated for 1 h with a blocking solution of 10% goat serum (Sigma) and then further incubated overnight with primary antibodies: anti-A. gambiae CTL4 rabbit polyclonal or anti-A. gambiae TEP1 rabbit, at 1:400 dilution; anti-P. falciparum Pfs25 mouse monoclonal (BEI resources, MRA-28), or anti-P. berghei Pbs28 mouse monoclonal (kindly provided by Professor Sinden, Imperial College London), at 1:500 dilution. After 3 PBS washes, the midguts were incubated for 2 h with secondary antibody: AlexaFluor568 goat anti-rabbit (Life Technologies, A11011) for CTL4 and TEP1, and AlexaFluor 488 goat anti-mouse (Life Technologies, A11029), at 1:500 dilution, for the parasites. Following another 3 washes, the midguts were stained with DAPI, mounted with ProLong Gold antifade reagent, and examined with a Zeiss LSM 710 confocal microscope, collecting 0.2 to 1 mm optical sections. The confocal microscopy settings were kept at similar level across different slides for the comparison purpose, and
DAPI staining intensity from each treatment was used for standardization. Stacks of thin optical slices of the samples were projected with Zeiss Zen software, and overlays of different channels were prepared through ImageJ (Fiji). The fluorescent intensities from the colocalization of total TEP1 protein with \textit{P. berghei} or \textit{P. falciparum} ookinetes in either control or \textit{CTL4} null midguts were measured using the Image J (Fiji) software. A total of 30 midguts (with at least 3 ookinetes in each gut) were assayed for each treatment. The intensities of the green fluorescence of either \textit{P. berghei} or \textit{P. falciparum} ookinetes were used as internal references for normalization. Mean ratio of intensities of either control or mutant samples was first obtained by dividing TEP1 intensities (red channel) with intensities of Pbs28 (for \textit{P. berghei}, green channel) or Pfs25 (for \textit{P. falciparum}, green channel). Fold change of TEP1 mean intensities in \textit{CTL4} null compared to the controls were calculated by dividing the mean ratio of \textit{CTL4} null with that of control samples (S3 Table).

**Antibiotic treatment and RNAi-mediated gene silencing**

Newly emerged adult female mosquitoes were provided a fresh filtered-sterile 10% sucrose solution containing 75 μg/ml gentamicin sulfate (Sigma) and 100 units-μg/ml of penicillin–streptomycin (Thermo Fisher Scientific) for 3 d, as described in [57], which was replaced 1 d before \textit{P. berghei} infection. PCR products were generated from cDNA using gene-specific primers that included a T7 promoter sequence (S1 Table). dsRNA was synthesized, at least 50 females per group were injected with the dsRNA, and the efficiency of the gene silencing for each gene of interest was assessed by qRT-PCR (S1 Table), following the protocol as described in [20].

**Bacterial challenge**

For the enumeration of endogenous bacteria from mosquito midguts, non-blood-fed mosquitoes were surface sterilized with ethanol and rinsed with PBS, and their midguts were dissected and homogenized in PBS. Dilutions of this homogenate were plated into LB agar plates and incubated at room temperature for 3 d, after which the bacterial colonies were counted and the number of colony-forming units (CFUs) of culturable bacteria was calculated. Total bacterial load was assessed by qRT-PCR to measure 16s RNA abundance, as in [57]. Mosquito longevity assays were performed as in [52], by intrathoracic injection of 350,000 CFU for the \textit{E. coli} suspension, 420,000 CFU for the \textit{S. aureus} suspension, or 62,000 CFU for \textit{B. subtilis} suspension. Three days after the bacterial injection, the abdomens of the mosquitoes infected with \textit{E. coli}, \textit{S. aureus}, and the control PBS-injected (20 females per group) were dissected to score for the presence of melanized bacterial clumps.

**Fungal challenge**

\textit{Beauvaria bassiana} (\textit{B. bassiana} strain 80.2, kindly provided by Professor Silverman, University of Massachusetts Medical School) was cultured, and spores (conidia) used for mosquito challenges were collected as in [9,58]. Freshly prepared spores were used for all experiments. A solution (69 nl) containing \(2.15 \times 10^6\) \textit{B. bassiana} spores/ml was injected into adult females (50 females per group) using a nanoinjector. At 12 and 48 h after fungal injection, abdomens were dissected and scored for the presence of live or melanized \textit{B. bassiana} spores, and images were captured using a Leica DM 2500 microscope. For the midgut fungal infection, a solution containing \(2.15 \times 10^8\) \textit{B. bassiana} spores/ml was added to filtered-sterile 10% sucrose, and newly emerged female adult mosquitoes were allowed to feed on this mixture for 48 h, after which the fungus-supplemented sucrose solution was replaced with regular filtered-sterile 10% sucrose solution. Mosquito midguts were washed with PBS and dissected at 24, 48, and 72 h.
and 5 and 7 d after fungal challenge, and the presence of live or melanized fungus was evaluated. To measure the longevity following challenge with *B. bassiana*, 3- to 4-day-old female mosquitoes were dipped (i.e., surface exposed) for 10 min in a 1-ml solution containing $1 \times 10^9 B. bassiana$ spores/ml and 0.05% Tween-20 [59]. Dipped females from each group were transferred to cups and kept on filtered-sterile 10% sucrose solution, and dead mosquitoes were counted and removed from cups daily until all females were dead.

**Statistical analysis**

All experiments were repeated at least 3 times. Each biological replicate corresponds to a different mosquito population cage, and each population corresponds to a different generation. All graphs were generated using GraphPad Prism8 software, and the statistical methods used for each experiment are indicated in the respective figure legends. Detailed values and statistics are presented in S2 Table. The statistical analysis for *Plasmodium* infection experiments was also performed excluding zeros (noninfected females) (S4 Fig).

**Supporting information**

**S1 Fig.** Generation of the CTL4-gRNA-expressing transgenic line. The generation of the CTL4-gRNA-expressing transgenic line is outlined. (DOCX)

**S2 Fig.** *Plasmodium* infection intensities do not differ between X1, Cas9, and CTL4-gRNA mosquitoes. *P. falciparum* (a) and *P. berghei* (b) infection intensity in X1, Cas9, and CTL4-gRNA *A. gambiae* females at 8 or 10 dpi, respectively. No significant differences between the medians (horizontal red lines) of the 3 groups are represented by the horizontal black lines above the graphs. (DOCX)

**S3 Fig.** Longevity of adult female CTL4null *A. gambiae*. Life spans of non-blood-fed (sugar-fed) (a), naïve blood-fed (b), and *P. berghei*-infected blood-fed (c) did not differ between control and CTL4null *A. gambiae* females (Kaplan–Meier survival analysis with a log-rank test; SE of replicates are indicated). (DOCX)

**S4 Fig.** *Plasmodium* infection results excluding uninfected individuals. (DOCX)

**S1 Table.** List of gRNA target sequences and primers used in the study. (DOCX)

**S2 Table.** Statistical analysis of *Plasmodium* infections. (DOCX)

**S3 Table.** Quantification of fluorescent intensity from antibody staining of ookinetes and TEP1 in the midgut at 24 hpi in Fig 7G. N indicates total gut samples assayed, with only the values of 20 representatives shown. Mean ratio and standard errors (SE) were calculated from the total parasites. (DOCX)

**S1 File.** Sequences: Control vs. CTL4null. (DOCX)
S2 File. CTL4 gene and protein sequences used for antibody production and qPCR. (DOCX)

S3 File. Sequences used for polyclonal antibodies production. (DOCX)

S4 File. Sequences from the lower gel from qPCR amplification of CTL4null in Fig 1D. (DOCX)

S5 File. The underlying data for all graphs. (XLSX)

S1 Raw Images. The original PCR gels of Fig 1C and 1D and western blots of Fig 1E. (PDF)

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References
1. WHO. World Malaria Report. 2018:2019.
2. Simões ML, Caragata EP, Dimopoulos G. Diverse host and restriction factors regulate mosquito pathogen interactions. Trends Parasitol. 2018; 34:603–16. https://doi.org/10.1016/j.pt.2018.04.011 PMID: 29793806
3. Blandin S, Shiao SH, Moita LF, Janse CJ, Waters AP, Kafatos FC, et al. Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector Anopheles gambiae. Cell. 2004; 116:661–70. https://doi.org/10.1016/s0092-8674(04)00173-4 PMID: 15006349
4. Fraiture M, Baxter RHG, Steinert S, Chelliah Y, Frolet C, Quispe-Tintaya W, et al. Two mosquito LRR proteins function as complement control factors in the TEP1-mediated killing of Plasmodium. Cell Host Microbe. 2009; 5:273–84. https://doi.org/10.1016/j.chom.2009.01.005 PMID: 19286136
5. Baxter RHG, Steinert S, Chelliah Y, Volohonsy G, Levashina EA, Deisenhofer J, et al. A heterodimeric complex of the LRR proteins LRIM1 and APL1C regulates complement-like immunity in Anopheles gambiae. Proc Natl Acad Sci U S A. 2010; 107:16817–22. https://doi.org/10.1073/pnas.100575107 PMID: 20826443
6. Povelones M, Waterhouse RM, Kafatos FC, Christophides GK. Leucine-rich repeat protein complex activates mosquito complement in defense against *Plasmodium* parasites. Science. 2009; 324:258–61. https://doi.org/10.1126/science.1171400 PMID: 19264986

7. Povelones M, Upton LM, Sala KA, Christophides GK. Structure-function analysis of the *Anopheles gambiae* LRAM1/APL1C complex and its interaction with complement C3-like protein TEP1. PLoS Pathog. 2011; 7:e1002023. https://doi.org/10.1371/journal.ppat.1002023 PMID: 21533217

8. Povelones M, Bhagavatula L, Yassine H, Tan LA, Upton LM, Osta MA, et al. The CLIP-domain serine protease homolog SPCPL1 regulates complement recruitment to microbial surfaces in the malaria mosquito *Anopheles gambiae*. PLoS Pathog. 2013; 9:e1003623. https://doi.org/10.1371/journal.ppat.1003623 PMID: 24039584

9. Yassine H, Kamareddine L, Osta MA. The Mosquito Melanization Response Is Implicated in Defense against the Entomopathogenic Fungus *Beauveria bassiana*. PLoS Pathog 2012; 8:e1003029. https://doi.org/10.1371/journal.ppat.1003029 PMID: 23166497

10. Yassine H, Kamareddine L, Chamat S, Christophides GK, Osta MA. A Serine Protease Homolog Negatively Regulates TEP1Consumption in Systemic Infections of the Malaria Vector *Anopheles gambiae*. J Innate Immun. 2014; 6:806–18. https://doi.org/10.1159/000363296 PMID: 25012124

11. Nakhtleh J, Christophides GK, Osta MA. The serine protease homolog CLIPA14 modulates the intensity of the immune response in the mosquito *Anopheles gambiae*. J Biol Chem. 2017; 292:18217–26. https://doi.org/10.1074/jbc.M117.797787 PMID: 28928218

12. Schnitzer AKD, Kafatos FC, Osta MA. The Melanization Reaction Is Not Required for Survival of *Anopheles gambiae* Mosquitoes after Bacterial Infections. J Biol Chem. 2007; 282:21884–8. https://doi.org/10.1074/jbc.M701635200 PMID: 17537726

13. Schnitzer AKD, Yassine H, Kafatos FC, Osta MA. Two c-type lectins cooperate to defend *Anopheles gambiae* against Gram-negative bacteria. J Biol Chem. 2009; 284:17616–24. https://doi.org/10.1074/jbc.M808298200 PMID: 19380589

14. Rhodes VL, Thomas MB, Michel K. The interplay between dose and immune system activation determines fungal infection outcome in the African malaria mosquito, *Anopheles gambiae*. Dev Comp Immunol. 2018; 85:125–33. https://doi.org/10.1016/j.dci.2018.04.008 PMID: 29469553

15. Sousa GL, Bishnoi R, Baxter RHG, Povelones M. The CLIP-domain serine protease CLIPC9 regulates melanization downstream of SPCPL1, CLIPA8, and CLIPA28 in the malaria vector *Anopheles gambiae*. PLoS Pathog. 2010; 6:e1000985. https://doi.org/10.1371/journal.ppat.1000985 PMID: 20345027

16. Dudzic JP, Hanson MA, Iatsenko I, Kondo S, Lemaitre B. More Than Black or White: Melanization and Toll Share Regulatory Serine Proteases in *Drosophila*. Cell Rep. 2019; 27:1050–61. https://doi.org/10.1016/j.celrep.2019.03.101 PMID: 31018123

17. Bishnoi R, Sousa GL, Contet A, Day CJ, Hou C-FD, Profitt LA, et al. Solution structure, glycan specificity and of phenol oxidase inhibitory activity of *Anopheles* C-type lectins CTL4 and CTLMA2. Sci Rep. 2019; 9:15191. https://doi.org/10.1038/s41598-019-5155-2 PMID: 31645596

18. Osta MA, Christophides GK, Kafatos FC. Effects of mosquito genes on *Plasmodium* development. Science. 2004; 30:2030–2. https://doi.org/10.1126/science.1091789 PMID: 15044804

19. Cohuet A, Osta MA, Morlais I, Awono-Ambene PH, Michel K, Simard F, et al. *Anopheles and Plasmodium*: from laboratory models to natural systems in the field. EMBO Rep. 2006; 7:1285–9. https://doi.org/10.1038/sj.embor.7400631 PMID: 17099691

20. Simões ML, Mlambo G, Tripathi A, Dong Y, Dimopoulos G. Immune Regulation of *Plasmodium* Is *Anopheles* Species Specific and Infection Intensity Dependent. mBio. 2017; 8:01631–17.

21. Dong Y, Das S, Cirimotich C, Souza-Neto JA, McLean KJ, Dimopoulos G. Engineered *Anopheles* Immunity to *Plasmodium* Infection. PLoS Pathog. 2011; 7:e1002458. https://doi.org/10.1371/journal.ppat.1002458 PMID: 22216006

22. Garver LS, Bahia AC, Das S, Souza-Neto JA, Shiao J, Dong Y, et al. *Anopheles* lmd pathway factors and effectors in infection intensity- dependent anti-*Plasmodium* action. PLoS Pathog. 2012; 8; e1002737. https://doi.org/10.1371/journal.ppat.1002737 PMID: 22685401

23. Garver LS, Dong Y, Dimopoulos G. Caspar controls resistance to *Plasmodium falciparum* in diverse anopheline species. PLoS Pathog. 2009; 5:e1000335. https://doi.org/10.1371/journal.ppat.1000335 PMID: 19282971

24. Dekmak AS, Yang X, Zu Dohna H, Buchon N, Osta MA. The Route of Infection Influences the Contribution of Key Immunity Genes to Antibacterial Defense in *Anopheles gambiae*. J Innate Immun. 2020; 13:107–26. https://doi.org/10.1159/000511401 PMID: 33207342

25. Dong Y, Simões ML, Marois E, Dimopoulos G. CRISPR/Cas9-mediated gene knockout of *Anopheles gambiae* FREP1 suppresses malaria parasite infection. PLoS Pathog. 2018; 14:e1006898. https://doi.org/10.1371/journal.ppat.1006898 PMID: 29518156
26. Volohonsky G, Terenzi O, Soichot J, Naujoks DA, Nolan T, Windbichler N, et al. Tools for Anopheles gambiae Transgenesis. G3 (Bethesda). 2015; 5:1151–63. https://doi.org/10.1534/g3.115.016808 PMID: 25869647

27. Yang J, Schleicher TR, Dong Y, Park HB, Lan J, Cresswell P, et al. Disruption of mosGILT in Anopheles gambiae impairs ovarian development and Plasmodium infection. J Exp Med. 2019; 217:e20190682.

28. Abraham EG, Pinto SB, Ghosh A, Vanlandingham DL, Budd A, Higgs S, et al. An immune-responsive serpin, SRPN6, mediates mosquito defense against malaria parasites. Proc Natl Acad Sci U S A. 2005; 102:16327–32. https://doi.org/10.1073/pnas.0508335102 PMID: 16260729

29. Volz J, Müller H-M, Zdanowicz A, Kafatos FC, Osta MA. A genetic module 505 regulates the melanization response of Anopheles to Plasmodium. Cell Microbiol. 2006; 8:1392–405. https://doi.org/10.1111/j.1462-5822.2006.00718.x PMID: 16922859

30. El Moussawi L, Nakhle N, Kamaraddine L, Osta MA. The mosquito melanization response requires hierarchical activation of non-catalytic clip domain serine protease homologs. PLoS Pathog. 2009; 1: e1008194.

31. Collins FH, Sakai RK, Vernick KD, Paskewitz S, Seeley DC, Miller LH, et al. Genetic selection of a Plasmodium-refractory strain of the malaria vector Anopheles gambiae. Science. 1986; 234:607–10. https://doi.org/10.1126/science.3532325 PMID: 3532325

32. Dong Y, Aguilar R, Xi Z, Warr E, Mongin E, Dimopoulos G. Anopheles gambiae immune responses to human and rodent Plasmodium parasite species. PLoS Pathog 2006; 2:e52. https://doi.org/10.1371/ journal.ppat.0020052 PMID: 16789837

33. Dong Y, Manfredini F, Dimopoulos G. Implication of the mosquito midgut microbiota in the defense against malaria parasites. PLoS Pathog 2009; 5:e1000423. https://doi.org/10.1371/journal.ppat.1000423 PMID: 19424427

34. Cirimotich CM, Dong Y, Clayton AM, Sandiford SL, Souza-Neto JA, Mulenga M, et al. Natural microbe-mediated refractoriness to Plasmodium infection in Anopheles gambiae. Science. 2011; 2011 (332):855–8. https://doi.org/10.1126/science.1201618 PMID: 21566196

35. Bahia AC, Dong Y, Blumberg BJ, Miambgo G, Tripathi A, Ben Marzouk-Hidalgo OJ, et al. Exploring Anopheles gut bacteria for Plasmodium blocking activity. Environ Microbiol. 2014; 16:2980–94. https://doi.org/10.1111/1462-2920.12381 PMID: 24428613

36. Clayton AM, Cirimotich CM, Dong Y, Dimopoulos G. Caudalis a negative regulator of the Anopheles IMD Pathway that controls resistance to P. falciparum infection. Dev Comp Immunol. 2012; 39:323–32. https://doi.org/10.1016/j.devimm.2012.10.009 PMID: 23178401

37. Meister S, Agianian B, Turlure F, Relogio A, Morlais I, Kafatos FC, et al. Anopheles gambiae PGRPLC-mediated defense against bacteria modulates infections with malaria parasites. PLoS Pathog. 2009; 5: e1000542. https://doi.org/10.1371/journal.ppat.1000542 PMID: 19662170

38. Margos G, Navarette S, Butler G, Davies A, Willers C, Sinden RE, et al. Interaction between Host Complement and Mosquito-Midgut-Stage Plasmodium berghei. Infect Immun. 2001; 69:5064–71. https://doi.org/10.1128/IAI.69.5.5064-5071.2001 PMID: 11447187

39. Murdock CC, Sternberg ED, Thomas MB. Malaria transmission potential could be reduced with current and future climate change. Sci Rep. 2016; 6:27771. https://doi.org/10.1038/srep27771 PMID: 27324146

40. Simões ML, Dimopoulos G. A mosquito mediator of parasite-induced immune priming. Trends Parasitol. 2015; 31:402–404.41. https://doi.org/10.1016/j.pt.2015.07.004 PMID: 26254960

41. Molina-Cruz A, DeJong RJ, Ortiga C, Hayle A, Abban E, Rodrigues J, et al. Some strains of Plasmodium falciparum, a human malaria parasite, evade the complement-like system of Anopheles gambiae mosquitoes. Proc Natl Acad Sci U S A. 2012; 109:e1957–62. https://doi.org/10.1073/pnas.1121183109 PMID: 22623529

42. Eldering M, Morlais I, van Gemert G-J, van de Vege-Bolmer M, Graumans W, Siebelink-Stoter R, et al. Variation in susceptibility of African Plasmodium falciparum malaria parasites to TEP1-mediated killing in Anopheles gambiae mosquitoes. Sci Rep. 2016; 6. https://doi.org/10.1038/srep26861587

43. Blagborough AM, Churcher TS, Upton LM, Ghani AC, Gething PW, Sinden RE. Transmission-blocking interventions eliminate malaria from laboratory populations. Nat Commun. 2013; 4:1812. https://doi.org/10.1038/ncomms2840 PMID: 23652000

44. Blagborough AM, Delves MJ, Ramakrishnan C, Lal K, Butcher G, Sinden RE. Assessing transmission blockade in Plasmodium spp. Methods Mol Biol. 2013; 923:577–600. https://doi.org/10.1007/978-1-62703-026-7_40 PMID: 22990806
45. Aleshnick M, Ganusov VV, Nasir G, Yenokyan G, Sinnis P. Experimental determination of the force of malaria infection reveals a non-linear relationship to mosquito sporozoite loads. PLoS Pathog. 2020; 16:e1008181. https://doi.org/10.1371/journal.ppat.1008181 PMID: 32453765

46. Blanford S, Chan BHK, Jenkins N, Sim N, Turner RJ, Read AF, et al. Fungal pathogen reduces potential for malaria transmission. Science. 2005; 308:1638–41. https://doi.org/10.1126/science.1108423 PMID: 15947189

47. Blanford S, Shi W, Christian R, Marden JH, Koekemoer LL, Brooke BD, et al. Lethal and pre-lethal effects of a fungal biopesticide contribute to substantial and rapid control of malaria vectors. PLoS ONE. 2011; 6:e23591. https://doi.org/10.1371/journal.pone.0023591 PMID: 21897846

48. Kikankie CK, Brooke BD, Knols BGJ, Koekemoer LL, Farenhorst M, Hunt RH, et al. The infectivity of the entomopathogenic fungus Beauveria bassiana to insecticide-resistant and susceptible Anopheles arabiensis mosquitoes at two different temperatures. Malar J. 2010; 9. https://doi.org/10.1186/1475-2875-9-71 PMID: 20210990

49. George J, Blanford S, Domingue MJ, Thomas MB, Read AF, Baker TC. Reduction in host-finding behaviour in fungus-infected mosquitoes is correlated with reduction in olfactory receptor neuron responsiveness. Malar J. 2011; 10. https://doi.org/10.1186/1475-2875-10-219 PMID: 21812944

50. Ragavendran C, Dubey NK, Natarajan D. Beauveria bassiana (Clavicipitaceae): a potent fungal agent for controlling mosquito vectors of Anopheles stephensi, Culex quinquefasciatus and Aedes aegypti (Diptera: Culicidae). RSC Adv. 2017; 7:3838–51.

51. Hammond A, Galizi R, Kyrou K, Simoni A, Siniscalchi C, Katsanos D, et al. A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector Anopheles gambiae. Nat Biotechnol. 2016; 34:78–83. https://doi.org/10.1038/nbt.3439 PMID: 26641531

52. Simões ML, Dong Y, Hammond A, Hall A, Crisanti A, Nolan T, et al. The Anopheles FBN9 immune factor mediates Plasmodium species-specific defense through transgenic fat body expression. Dev Comp Immunol. 2017; 67:257–65. https://doi.org/10.1016/j.dci.2016.09.012 PMID: 27667688

53. Papathanos PA, Windbichler N, Menichelli M, Burt A, Crisanti A. The vasal regulatory region mediates germline expression and maternal transmission of proteins in the malaria mosquito Anopheles gambiae: a versatile tool for genetic control strategies. BMC Mol Biol. 2009; 10. https://doi.org/10.1186/1471-2199-10-65 PMID: 19573226

54. Meredith JM, Underhill A, McArthur CC, Eggleston P. Next-generation site-directed transgenesis in the malaria vector mosquito Anopheles gambiae: self-docking strains expressing germline-specific phiC31 integrase. PLoS ONE. 2013; 8:e55264. https://doi.org/10.1371/journal.pone.0055264 PMID: 23516619

55. Sandford SL, Dong Y, Pike A, Blumberg BJ, Bahia AC, Dimopoulos G. Cytoplasmic Actin Is an Extracellular Insect Immune Factor which Is Secreted upon Immune Challenge and Mediates Phagocytosis and Direct Killing of Bacteria, and Is a Plasmodium Antagonist. PLoS Pathog. 2015; 11:e1004631. https://doi.org/10.1371/journal.ppat.1004631 PMID: 25668622

56. Dong Y, Dimopoulos G. Anopheles Fibrinogen-related Proteins Provide Expanded Pattern Recognition Capacity against Bacteria and Malaria Parases. J Biol Chem. 2009; 284:9835–44. https://doi.org/10.1074/jbc.M807084200 PMID: 19193639

57. Dong Y, Simões ML, Dimopoulos G. Versatile transgenic multistage effector-gene combinations for Plasmodium falciparum suppression in Anopheles. Sci Adv. 2020; 6:eaaay5898. https://doi.org/10.1126/sciadv.aay5898 PMID: 32426491

58. Dong Y, Morton JC Jr, Ramirez JL, Souza-Neto JA, Blumberg BJ, Pike A. The entomopathogenic fungus Beauveria bassiana activate toll and JAK-STAT pathway-controlled effector genes and anti-dengue activity in Aedes aegypti. Infect Biochem Mol Biol. 2012; 42:126–132.59. https://doi.org/10.1016/j.ibmb.2011.11.005 PMID: 22198333

59. Angleró-Rodríguez YI, Blumberg BJ, Dong Y, Sandiford SL, Pike A, Clayton AM, et al. A natural Anopheles-associated Penicillium chrysogenum enhances mosquito susceptibility to Plasmodium infection. Sci Rep. 2016; 6:34084. https://doi.org/10.1038/srep34084 PMID: 27678168