Bacteria- and IMD Pathway-Independent Immune Defenses against *Plasmodium falciparum* in *Anopheles gambiae*  

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**Abstract**  
The mosquito *Anopheles gambiae* uses its innate immune system to control bacterial and *Plasmodium* infection of its midgut tissue. The activation of potent IMD pathway-mediated anti-*Plasmodium falciparum* defenses is dependent on the presence of the midgut microbiota, which activate this defense system upon parasite infection through a peptidoglycan recognition protein, PGRPLC. We employed transcriptomic and reverse genetic analyses to compare the *P. falciparum* infection-responsive transcriptomes of septic and aseptic mosquitoes and to determine whether bacteria-independent anti-*Plasmodium* defenses exist. Antibiotic-treated aseptic mosquitoes mounted molecular immune responses representing a variety of immune functions upon *P. falciparum* infection. Among other immune factors, our analysis uncovered a serine protease inhibitor (SRPN7) and Clip-domain serine protease (CLIPC2) that were transcriptionally induced in the midgut upon *P. falciparum* infection, independent of bacteria. We also showed that SRPN7 negatively and CLIPC2 positively regulate the anti-*Plasmodium* defense, independently of the midgut-associated bacteria. Co-silencing assays suggested that these two genes may function together in a signaling cascade. Neither gene was regulated, nor modulated, by infection with the rodent malaria parasite *Plasmodium berghei*, suggesting that SRPN7 and CLIPC2 are components of a defense system with preferential activity towards *P. falciparum*. Further analysis using RNA interference determined that these genes do not regulate the anti-*Plasmodium* defense mediated by the IMD pathway, and both factors act as agonists of the endogenous midgut microbiota, further demonstrating the lack of functional relatedness between these genes and the bacteria-dependent activation of the IMD pathway. This is the first study confirming the existence of a bacteria-independent, anti-*P. falciparum* defense. Further exploration of this anti-*Plasmodium* defense will help clarify determinants of immune specificity in the mosquito, and expose potential gene and/or protein targets for malaria intervention strategies based on targeting the parasite in the mosquito vector.

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**Introduction**  

Human malaria continues to be a scourge of mankind, responsible for approximately over a million deaths annually on average [1]. *Plasmodium falciparum*, the most dangerous malaria parasite, is responsible for the majority of deaths worldwide and is transmitted by the mosquito *Anopheles gambiae* as well as other Anopheline species.

Like other insects, *A. gambiae* relies on its innate immune response to defend against infections with pathogens, including *Plasmodium* [2,3]. Upon activation of pattern recognition receptors that can bind to microbial pathogen associated molecular patterns, two main signaling pathways, the TOLL and immune deficiency (IMD) pathways, launch effective anti-pathogen immune responses through NF-kappaB transcription factors that transcribe effector genes [4]. The link between pattern recognition receptor and immune pathway activation can either be direct, as in the case of the IMD pathway activation by PGRPLC, or can be indirectly mediated through serine protease cascades that are controlled by serpins, as in the case of TOLL pathway activation. The serine protease cascades also play other roles in immune responses, providing signal amplification that leads to the activation of anti-pathogen effector mechanisms such as melanotic encapsulation [4].

While the TOLL pathway has been shown to suppress infection with the rodent malaria parasite *P. berghei*, the IMD pathway is associated with anti-*P. falciparum* defense and is mediated through the activation of anti-*Plasmodium* effectors such as the fibrinogen-like immunoreceptor 9 (FBN9), leucine-rich repeat domain containing protein 7 (LRRD7), thiostere containing protein 1 (TEP1), and other defense proteins [5–10]. Furthermore, we have shown that the IMD pathway-regulated transcription factor Rel2 is controlling the mosquito midgut microbiota while the Toll pathway and its transcription factor Rel1 does not [8,11]. Finally, while others have shown that the anti-*Plasmodium* IMD pathway is activated by bacteria we have shown that this pathway’s (if activated through
RNAi of IMD pathway negative regulator Caspar anti-Plasmodium activity is independent on the presence of bacteria [12]. The two pathways are also essential to the defense against fungi and bacteria [4]. Recent studies have shown a dependence on bacteria-mediated activation of the IMD pathway to launch an effective anti-Plasmodium immune response in the mosquito gut tissue, which harbors a variety of mostly Gram-negative bacteria [13]. The IMD pathway contributes to microbial homeostasis in this tissue by maintaining a continuous basal level activity, which is stimulated by the midgut microbiota [11]. PGRPLC, a peptidoglycan recognition protein isoform previously identified as a receptor of the IMD pathway in Drosophila, has been shown to play a role in this basal level immune activation by sensing bacteria in the gut tissue [11,13]. Interestingly, PGRPLC has also been shown to be essential for triggering an anti-P. falciparum response through the IMD pathway, but only when bacteria are present in the gut tissue [13]. RNAi-mediated depletion of PGRPLC from antibiotic-treated A. gambiae, which have a greatly reduced microbial flora, does not influence the mosquito’s susceptibility to the parasite, as it does in non-antibiotic-treated septic mosquitoes, suggesting that PGRPLC-mediated activation of the IMD pathway’s anti-Plasmodium defense depends on the presence of midgut bacteria [11,13]. We and others have previously shown that the Plasmodium effector FBN9, LRRD7, and TEP1 are also involved in controlling bacterial proliferation in the midgut tissue, corroborating the intimate relationship between anti-bacterial and anti-Plasmodium defenses [5,11]. Here we wanted to investigate whether P. falciparum ookinete infection of the mosquito midgut activates anti-parasitic immune responses in a bacteria-independent manner. Arrighi et al. previously investigated the role of Plasmodium glycosylphosphatidylinositol (GPI) anchors in the induction of an immune response, and although their study documented the induction of some immune genes, the potential anti-Plasmodium action and possible dependence and relationship of these genes to the microbiota were not investigated [14]. Since the transcriptome of an organism, tissue, or cell type represents a reflection of a physiological state such as immune response, we used whole-genome microarray analysis to investigate the P. falciparum infection-responsive transcriptome in septic and aseptic mosquitoes in order to identify and characterize bacteria-independent immune response signatures and factors. Our analysis revealed a variety of putative immune genes that are regulated upon Plasmodium infection in the absence of midgut microbiota, and we specifically focused on a clip-domain serine protease (CLIPC2) and a serine protease inhibitor (SRPN7), showing that these genes modulate the intensity of the P. falciparum infection in the absence of bacteria. CLIPC2 also controlled systemic bacterial infection and both genes modulated the proliferation of the midgut microflora, indicating their functional versatility. Interestingly, our study suggests that CLIPC2 and SRPN7 may be part of the same protease signaling cascade and that their anti-P. falciparum function is independent of the IMD pathway, since these factors were not regulated by and did not regulate this pathway. Our study points to the existence of bacteria-independent anti-Plasmodium defenses, possibly relating to as-yet unknown immune pathways and mechanisms.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Animal Care and Use Committee of the Johns Hopkins University (ACUC: MO11H184). Commercial anonymous human blood was obtained from Interstate Bloodbank and used for parasite cultures and mosquito feeding and informed consent was therefore not applicable. The Johns Hopkins School of Public Health Ethics Committee has approved this protocol.

Mosquito Rearing, RNA Isolation, and cDNA Synthesis

A. gambiae Keel strain mosquitoes [15] were maintained on a 10% sucrose solution with 12-h light/dark cycles at 27°C and 80% humidity [16]. At specific time points, mosquitoes were anesthetized on ice, and either whole mosquitoes or specific tissues were dissected and collected. RNA was extracted from tissues using the RNeasy kit (Qiagen), and RNA yields were quantified using the Nanodrop 2000 (ThermoFisher) following treatment with DNase (Ambion). cDNA was synthesized from extracted RNA using the Oligo-DT primer and M-MLV Reverse Transcriptase (Promega).

Primer Design and qRT-PCR

The Primer 3 Program (http://frodo.wi.mit.edu) was used to design all primers, except for the bacterial 16s primers [17]. Real-time quantitative PCR (qRT-PCR) to assess transcript abundance and silencing efficiency was performed as described in [5]. Transcript abundance was quantified with Sybr Green PCR Master Mix (Applied Biosystems) using the ABI StepOnePlus Real-Time PCR System and ABI StepOne Software. PCR reactions were performed in duplicate, and melting curve analysis was used to analyze primer specificity. Transcript abundance of target genes was first normalized to the within sample transcript abundance of the mosquito ribosomal S7 gene, and fold changes between samples were determined using the ΔΔct method.

RNAi Gene Silencing

The HiScribe T7 in vitro Transcription Kit (New England Biolabs) was used to generate double-stranded RNA (dsRNA) from PCR-amplified gene oligos. Gene silencing was performed essentially as described in [5,16]. In brief, 3- to 4-day-old female A. gambiae were cold-anesthetized, and cohorts were injected with control GFP dsRNA or experimental gene-specific dsRNA at a concentration of 3 μg/μL (207 ng dsRNA per mosquito). Pools of 15 mosquitoes were collected 1–4 days post-dsRNA injection, and silencing efficiency was assessed by qRT-PCR.

Quantification of Endogenous Mosquito Midgut Bacteria

Colony forming units (CFU) from mosquito midguts were quantified in control untreated, antibiotic-treated, and gene-silenced mosquitoes as described [11,19]. For the gene silencing experiment, 3–4 day old female A. gambiae mosquitoes were injected with dsRNA as described in the section “RNAi Gene Silencing.” 3 days post-injection of dsRNA, mosquito midguts were dissected and processed as described below. At the indicated time points, female mosquitoes were collected, surface-sterilized in ethanol, and washed with 1x PBS, and their midguts were dissected in sterilized 1x PBS. Collected midguts were homogenized, and serial dilutions of homogenate were plated on LB agar plates. After incubation for 2–3 days at 27°C under aerobic or anaerobic conditions, the CFUs per plate were counted, and a titer of CFU/midgut was calculated.

Antibiotic Treatment

For antibiotic treatment, adult female mosquitoes were collected post-eclosion and given a sterile 10% sucrose solution containing
Microarray Hybridization and Data Analysis

All probe sequences, probe preparation, microarray construction, and microarray hybridizations were performed essentially as described in [5]. Control (Cy3-labeled) and experimental (Cy5-labeled) cRNA probes were generated from 2–3 μg of RNA according to the manufacturer’s instructions (Agilent Technologies Low RNA Input Linear Amplification Kit). Probe hybridization to the microarray slides was accomplished using 2 μg of cRNA, and microarray slides were washed and dried 16 h post-hybridization. Slides were scanned using an Axon GenePix 4200AL scanner at 10-μm pixel size (Axon Instruments, Union City, California, USA); 60% laser power was used, and the photomultiplier tube (PMT) voltage was adjusted to maximize the dynamic range and PMT voltage was adjusted to maximize the dynamic range and.

Bacterial Challenge

Mosquitoes were injected with control dsGFP or experimental dsRNA constructs. Three days post-injection, mosquitoes were cold-anesthetized and injected with 69 μL of either Gram-positive Staphylococcus aureus or Gram-negative Escherichia coli, at the optical density mentioned at the end of this section. Glycerol stocks of both species of bacteria were used to inoculate LB broth cultures, which were grown in a shaker overnight at 37°C for approximately 18 h. The cultures were centrifuged to a pellet that was then washed with 1x PBS three times. A biophotometer (Eppendorf) was used to measure optical density (OD600), and samples were diluted with 1x PBS to the appropriate absorbance prior to injection (OD600; S. aureus = 0.33, E. coli = 3.0).

Results and Discussion

Bacteria-dependent and -independent mosquito transcriptome responses to P. falciparum infection

To examine the impact of P. falciparum infection on the mosquito midgut and carcass transcriptomes in the presence or absence of midgut bacteria, we used A. gambiae whole genome microarrays to compare the mRNA abundance of P. falciparum-infected and naïve mosquitoes of antibiotic- and non-antibiotic treated cohorts. Depletion of the great majority of midgut bacteria was achieved by treating mosquitoes with a broad-spectrum antibiotic cocktail containing 75 μg/ml gentamycin, 100 units/ml penicillin and 100 μg/ml streptomycin for 4 days through their sugar meal, prior to feeding on P. falciparum gametocytes. To assess the efficacy of the antibiotic treatment in the removal of the midgut microbiota, we assayed colony forming unit (CFU) growth on LB agar of both the aerobic and anaerobic bacteria present in sugar-fed and 24-h blood-fed mosquito midguts (Figure 1A, B). Although culturing bacterial isolates exclusively on LB agar may limit the ability to capture the entire spectrum of bacterial species present in the mosquito midgut, we have observed near identical growth of the same bacteria on a variety of mediums (LB, Yeast extract-peptone dextrose, blood agar), (Dimopoulos lab, unpublished data). Our assays showed that no CFU could be detected in antibiotic-treated mosquitoes. Since some midgut bacteria may be unculturable we also determined the relative microbial load of these samples using qRT-PCR with universal primers amplifying the bacterial 16s ribosomal RNA (16s rRNA), (Table S1). The 16s rRNA was amplified 63-fold higher in septic sugar-fed and 272-fold higher in septic blood-fed midguts when normalized to 16s rRNA from aseptic sugar-fed and aseptic blood-fed midguts, respectively (Figure 1C, D). This PCR-amplified bacterial nucleic acid could represent the remains of dead bacteria that existed in the midguts prior to the antibiotic treatment, and the large difference in 16s rRNA abundance between septic and aseptic midguts suggests near complete removal of the microbiota by antibiotic treatment. It is possible that RNA was amplified from a small number of bacteria unaffected by antibiotic treatment. Nevertheless, we have previously demonstrated that the removal of bacteria through antibiotic treatment is efficient to impact on the Plasmodium infection phenotype [11], and for practical purposes we consider our antibiotic-treated mosquitoes aseptic when compared non-antibiotic treated septic mosquitoes.

We then compared the genome-wide transcript abundance between infected and non-infected mosquitoes of septic and
aseptic cohorts at 24 h post-ingestion of a *P. falciparum* gametocyte culture or naïve blood; a time period when ookinetes invade the midgut epithelium and the microbial flora has expanded by a 10- to 20-fold in the nutrient-rich blood (Figure 2) [22,23]. Regulation was determined by assessing Log2 ratio values of transcript abundance that were above our cutoff for significance of 0.75, or below $-0.75$. *P. falciparum* infection induced changes in the abundance of as many as 2,183 and 2,429 transcripts in whole mosquitoes belonging to a variety of functional groups in aseptic and septic mosquitoes, respectively, representing approximately 16% and 18% of the *A. gambiae* transcriptome (Figure 2A, B). The abundance of 1,556 transcripts was regulated in the aseptic midguts, as compared to 1,760 in the septic midguts, and 1,154 and 916 transcripts displayed changes in the aseptic and septic carcasses, respectively, upon *P. falciparum* infection (Figure 2C). The abundance of 458 transcripts changed (204 induced and 254 repressed) in the same direction in both aseptic and septic midguts, suggesting that *P. falciparum* infection, and not the presence or absence of the midgut bacteria, was likely to account for this response (Figure 2C). In addition, 46 transcripts with predicted immune functions were uniquely regulated in the septic midgut (11 induced and 35 repressed), suggesting that tripartite interactions between *Plasmodium* parasites, midgut bacteria, and the midgut epithelium affect the expression of this set of genes (Table S2).

When we compared transcript abundance between the midgut and carcass within the aseptic group (Figure 2C), we observed 486 regulated genes (401 induced and 85 repressed) shared between these tissue compartments. This is over double the number of regulated transcripts (66 induced and 133 repressed) that were shared between the midgut and carcass within the septic group. This observation suggests that the presence of midgut bacteria accounts for larger differences between the midgut and carcass transcriptomes, whereas in the absence of midgut bacteria there is an impact on the expression of genes related to the midgut epithelium.
more similarity in the transcriptome between the two tissue compartments.

Transcripts of a suppressor of cytokine signaling (SOCS, AGAP001623, Log2 = 0.97) were upregulated in the P. falciparum infected septic midguts, as was a SOCS negative regulator of the A. gambiae JAK-STAT pathway that had been previously implicated as a host factor in Plasmodium infection [24]. The transcripts of the secreted modular serine protease 22D (SCRASP1, AGAP005625, Log2 = 0.88) were also upregulated in the septic midguts. SCRASP1 has a chitin-binding domain that has been hypothesized to sense chitin in response to injury and to transduce signals via the serine protease domain, even though the signaling pathway to which SCRASP1 belongs has remained elusive [25,26]. Also upregulated in the septic parasite-infected midguts were transcripts of spaetzle-like cytokine 2 (SPZ2, AGAP006403, Log2 = 0.75), which may be involved in TOLL pathway activation [27]. Transcripts of the thioester-containing protein 1 (TEP1, AGAP010815, Log2 = 0.72), an IMD-pathway associated effector molecule with strong anti-Plasmodium activity, was close to being significantly regulated in accordance with previous observations of P. falciparum infection in the septic gut [5,6].

Ultimately, we were interested in identifying the genes involved in bacteria-independent anti-Plasmodium responses, and therefore we focused on transcripts displaying increased abundance in the parasite-infected aseptic midguts, placing a particular emphasis on those with predicted immune functions. Of the 783 transcripts specifically enriched in the aseptic midguts, 17 had predicted functions in immunity, whereas the majority of genes of this group belonged to other diverse or unknown functional groups. Two genes that displayed changes in their transcript abundance in P. falciparum-infected aseptic midguts, LRRD1 (AGAP000360, Log2 = 1.14) and LRRD18 (AGAP000054, Log2 = 1.3), belong to the leucine rich repeat domains (LRRD) gene family, which also contains members with a putative function in pattern recognition and to play key roles in anti-Plasmodium functions [5,28,29]. Fibrinogen-related proteins have been implicated in the pattern recognition processes of human and rodent malaria parasites [30], and two FBN genes (FBN34 AGAP015154, Log2 = 1.04) and a novel gene, XM_001231172 (AGAP010772, Log2 = 1.86) encoding such putative immune factors were upregulated in the aseptic midguts by parasite infection. Another upregulated gene, SCRBU5 (AGAP002738, Log2 = 3.17), belongs to a class of scavenger receptors with diverse roles in pattern recognition, phagocytosis, and Plasmodium infection [31–33]. A non-alternatively spliced region of the AGDSCAM gene (AGAP007092, Log2 = 1.49) was also upregulated. Transcripts of this gene, in theory, can produce over 31,000 splice forms through alternative splicing, and AGDSCAM already has a recognized role in pattern recognition and immunity to Plasmodium infection [19]. Also upregulated in the aseptic midguts were a number of serine proteases and serine protease inhibitors. Studies have previously described roles for these gene families in melanization, immune pathway activation, and anti-parasitic activity [34–36].

Because of the central role of serine protease cascades in regulating insect immune defenses [37,38], we focused the remainder of our analysis on a clip-domain serine protease C2 (CLIPC2, AGAP004317, Log2 = 0.96) and a serine protease inhibitor 7 (SRPN7, AGAP007693, Log2 = 4.16) that were specifically upregulated in the parasite-infected, aseptic mosquito midgut. Their regulation by P. falciparum infection in the absence of the midgut microbiota suggested that they were likely to be involved in regulating bacteria-independent anti-Plasmodium defenses.

Serpins represent a large family of negative regulators of proteolytic cascades that play a critical roles in a variety of processes both vertebrates and invertebrates [39]. In humans, serpins regulate finely tuned processes such as fibrinolytic cascades, clotting, and inflammatory reactions [40]. In arthropods, serpins have been shown to regulate components of the prophenoloxidase (PPO) pathway, which is responsible for the melanization of pathogens, as well as to inhibit processes upstream of the seminal TOLL pathway, which functions in both development and innate immunity [36,38,41–43]. A. gambiae SRPN7 has 1:1 orthologs in both the yellow fever mosquito Aedes aegypti and the Southern house mosquito Culex quinquefasciatus, suggesting that the gene has a conserved, mosquito-specific function. Clip-Domain serine proteases also belong to a large gene family, but unlike the serine protease inhibitors (serpins), they are only found in arthropods [44,45]. Functional studies have demonstrated a role for Clip proteases in the activation of prophenoloxidases (PPOs), which mediate melanization defenses as well as the TOLL pathway [38,46–49]. In mosquitoes, there are five sub-families of Clip proteases (A, B, C, D, and E), and studies on subfamily A and B members have shown that some of these genes regulate the PPO pathway [38,50–52]. Although little is known about the role of subfamily C members in mosquitoes, it is worth noting that in Drosophila subfamily C members include SNAKE and PERSEPHONE, which are involved in TOLL pathway activation in the context of development and immunity, respectively [53,54]. The catalytic triad (His, Asp, Ser) is present in this clip protease indicating likely enzymatic activity similar to what was observed in another clip serine protease [55]. Like SRPN7, CLIPC2 has 1:1 orthologs in both C. quinquefasciatus and A. aegypti, again suggesting a mosquito-specific gene function.

SRPN7 and CLIPC2 infection-responsiveness

Quantitative real-time PCR (qRT-PCR) assays were used to confirm the up-regulation of SRPN7 and CLIPC2 in aseptic P. falciparum-infected mosquitoes (Table S1). The infection-responsive increase in SRPN7 transcript abundance was greatest in the aseptic midgut, although it was modest when compared to that of mosquitoes fed on naive blood (Figure 3A). Since SRPN7 transcripts were previously detected at low levels in adult mosquitoes [56], the increase in transcript abundance upon Plasmodium-infection of the aseptic midgut is intriguing. SRPN7 transcripts have been previously reported to be upregulated in the midguts of mosquitoes fed on a blood meal mixed with Gram-positive and Gram-negative bacteria [11]. Analysis of CLIPC2 has shown nearly a 5-fold increase in transcript abundance after P. falciparum infection of aseptic mosquito guts at 24 h after feeding.
on a gametocyte culture, when compared to mosquitoes fed on naïve blood (Figure 3B). Our earlier studies on the IMD pathway-regulated mosquito transcriptome have suggested that SRPN7 and CLIPC2 are not regulated by the IMD pathway [6]. It is possible that differences in *P. falciparum* infection intensity and/or prevalence between septic and aseptic mosquitoes could have influenced the transcript abundance of these genes, but it is technically challenging to normalize the amount of pre-invasive parasites between antibiotic-treated and untreated cohorts. Nevertheless, our data prove bacteria-independent induction of SRPN7 and CLIPC2 upon *P. falciparum* infection. Overall, the pattern of gene transcript regulation detected by qRT-PCR supported our microarray-based studies, confirming that these genes are not influenced by parasitic infection in septic mosquito guts or carcasses and suggesting the occurrence of a bacteria-independent induction by parasite infection.

Interestingly, the transcript abundance of these genes was not modulated by infection of either septic or aseptic mosquitoes with the rodent malaria parasite *P. berghei*, suggesting a possible functional role in parasite-species-specific immune defenses (Figure 3C, D). *P. falciparum* and *P. berghei* exhibit stark differences in their development within the mosquito that may explain the *P. falciparum*-specific induction observed in this study [37]. The lack of transcript abundance modulation in parasite-infected septic mosquitoes is interesting and may indicate that these genes are also regulated by bacteria in a way that counteracts or masks induction by *P. falciparum* when compared to the non-infected mosquitoes. Interestingly, CLIPC2 was previously reported to be induced in the midguts of mosquitoes fed heat-killed *E. coli* [58], suggesting that CLIPC2 is upregulated in both a bacteria-dependent and bacteria-independent manner. The differential regulation of CLIPC2 by *P. berghei* and *P. falciparum* infection of aseptic mosquitoes does support parasite-mediated regulation rather than an exclusive dependence on bacteria (Figure 3D). Other CLIP C family members, such as the *Drosophila* proteins PERSEPHONE and GRASS, positively regulate two distinct TOLL pathway serine protease cascades [59]. Thus, it is possible that CLIPC2 is involved in an innate immune cascade in *A. gambiae*. Phylogenetically, SRPN7 lies within a mosquito-specific expansion cluster and does not cluster with the serpins that have been shown to regulate melanization [35], and its potential role in innate immunity therefore remains elusive.

Overall, our data suggest that CLIPC2 and SRPN7 are being induced by *P. falciparum* infection through a bacteria-independent mechanism.

### SRPN7 influences mosquito susceptibility to Plasmodium falciparum infection

We have shown that SRPN7 and CLIPC2 transcript abundance changes upon *P. falciparum* infection in a bacteria-independent fashion. To investigate whether SRPN7 or CLIPC2 could modulate mosquito permissiveness to *Plasmodium* infection, we used RNAi (RNA interference)-mediated gene silencing in conjunction with *Plasmodium* infection assays under aseptic conditions. Depletion of SRPN7 resulted in a significant decrease (P<0.001) in the intensity of *P. falciparum* infection in comparison to GFP dsRNA-injected controls (Figure 4A). However, there was no significant difference in the prevalence of *P. falciparum* infection between SRPN7-depleted mosquitoes when compared to the GFP dsRNA-injected controls (Table S3).

Dipteran serpins function in diverse processes ranging from inhibition of signaling cascades, such as the TOLL pathway and the prophenoloxidase activation system, to developmental processes such as morphogenesis [59–61]. An earlier study has shown that the Anopheles *SRPN6* has an anti-*Plasmodium* function that is dependent on the mosquito strain/species [34]. The significant reduction in *P. falciparum* infection intensity that we observed upon SRPN7 depletion suggests that this serpin functions as an inhibitor.
of an anti-*Plasmodium* defense that involves a serine protease cascade. The fact that, phylogenetically, *SRPN7* does not cluster with the serpins known to be involved in melanization cascades, and the knowledge that the Keele strain mosquitoes used in our study have a weak melanization response and do not melanize *P. falciparum* together suggests that *SRPN7* may be regulating a previously undescribed anti-*Plasmodium* mechanism. Alternatively, the role of *SRPN7* in the Keele strain melanization response could be involved in parasite clearance as opposed to direct melanization [34].

Although *CLIPC2* was upregulated nearly 5-fold in response to *P. falciparum* infection in aseptic midguts, RNAi-mediated depletion of its transcript resulted in no statistical difference in the intensity of *P. falciparum* infection, although there was a slight increase in the overall infection intensity (Table S3). This result may suggest a predominant role for *CLIPC2* in some non-defense-related process that occurs during *Plasmodium* infection, such as tissue repair or the stress response. Alternatively, an anti-*Plasmodium* defense mediated by *CLIPC2* might regulate a single component within a plethora of defenses normally elicited by the endogenous microflora, which we have previously shown can have a significant effect on the intensity of *Plasmodium* infection [11].

We and others have previously shown that different mosquito immune responses are involved in the defense against infection with the two malaria parasite species *P. falciparum* and *P. berghei*. The IMD pathway has been associated with defense against *P. falciparum*, whereas the TOLL pathway is associated with defense against *P. berghei* [4]. We have also shown that *SRPN7* and *CLIPC2* transcripts are induced in aseptic mosquito midguts upon infection with *P. falciparum* but not *P. berghei*. To investigate whether *SRPN7* and *CLIPC2* are regulating a general anti-*Plasmodium* defense or alternatively *Plasmodium*-species-specific defenses, we performed RNAi-mediated gene silencing upon infection with *P. berghei*. Interestingly, independent depletion of either *SRPN7* or *CLIPC2* resulted in no statistical difference in the intensity of *P. berghei* infection when compared to control GFP dsRNA-injected control mosquitoes (Figure 4B, Table S3). This result supports the disparity between the mosquito immune response to either *P. falciparum* or *P. berghei* infection and underscores the importance of utilizing the human malaria parasites in mosquito infection studies in order for the results to be of relevance to human disease transmission.

**Figure 4. *Plasmodium* infection intensity in aseptic mosquitoes after depleting *SRPN7* or *CLIPC2* through RNAi gene silencing.** (A) *P. falciparum* infection intensity following RNAi-mediated depletion of *SRPN7* (Dunn’s post test, p<0.05) and *CLIPC2* (Dunn’s post test, p>0.05). (B) *P. falciparum* infection intensity following double RNAi-mediated depletion of *SRPN7* and *CLIPC2* (Kruskal-Wallis test p = 0.42). Circles represent the number of oocysts from a single midgut; horizontal black bars represent the median oocysts in each RNAi treatment. Three independent biological replicates were pooled, and significance was determined by a Kruskal-Wallis test followed by Dunn’s post-test in the case of multiple comparisons. Statistical analysis of the double RNAi knockdown was performed using a Mann-Whitney test. RNAi treatments were compared to dsGFP-injected control mosquitoes. doi:10.1371/journal.pone.0072130.g004

**SRPN7 and CLIPC2 may function in the same serine protease cascade**

Since serpins and Clip-domain serine proteases function together as signal transducers and inhibitors in proteolytic signaling cascades, we performed a double knockdown assay of *SRPN7* and *CLIPC2* in aseptic *P. falciparum*-infected mosquitoes to provide a baseline indication as to whether these factors could be functioning in the same cascade, and thereby reciprocally influence their knockdown infection phenotypes. Interestingly, co-silencing of the two genes abolished the effects on *P. falciparum* infection that was observed when each gene was silenced independently (Figure 4C, Table S3). Although the potential for a direct interaction between a serpin and serine protease should be examined by a rigorous biochemical analysis, this experiment, taken together with the bacteria-independent opposite effects of *SRPN7* and *CLIPC2* depletion on susceptibility to *P. falciparum* infection, suggest that *SRPN7* and *CLIPC2* may be operating in the same cascade that regulates anti-*Plasmodium* defense. Alternatively, *SRPN7* and *CLIPC2* could be negative and positive regulators, respectively, of separate processes and thus the result could merely be explained by a canceling effect of silencing both transcripts. Without a biochemical analysis addressing interaction between the two proteins, it may be more accurate to assume that these genes are negative and positive regulators, possibly of the same cascade or independent cascades.

**CLIPC2 and SRPN7 influence systemic bacterial infection and the midgut microbiota**

We have previously shown that anti-*Plasmodium* factors also play versatile functions in antibacterial defense and wanted to investigate whether *SRPN7* or *CLIPC2* could play a role in the mosquito’s ability to fight systemic bacterial infection, or in the control of its midgut microbiota. While RNAi-mediated depletion of *SRPN7* or *CLIPC2* did not affect the mosquito’s survival upon experimental infection with *S. aureus*, mosquitoes depleted of *CLIPC2* showed increased survival when infected with *E. coli*, suggesting that *CLIPC2* may be a host factor for this bacterium (Figure 5A, B). The mosquito’s midgut microbiota needs to be under continuous immune control to avoid an over-proliferation that could be detrimental to the insect. We have previously shown that factors of the IMD immune pathway play a crucial role in controlling the midgut microbiota, and conversely, that the
microbiota is responsible for priming basal immune activity [10,11]. Surprisingly, independent silencing of SRPN7 and CLIP2 resulted in a significant decrease of the mosquito’s midgut microbiota, suggesting that these putative immune factors act as agonists of the mosquito’s natural midgut bacteria, through an unknown mechanism (Figure 3C). The midgut microbiota are predominately Gram-negative [62], and the decrease in the midgut bacteria in response to the silencing of CLIP2 corroborates the increase in survival that was observed in E. coli-challenged mosquitoes depleted of CLIP2. Whereas silencing of SRPN7 resulted in a decrease in the mosquito’s midgut microbiota, silencing of this gene had no effect on survival after systemic bacterial challenge, suggesting that the function of SRPN7 may be associated with the midgut as opposed to the fat body. Although we have hypothesized that SRPN7 and CLIP2 operate in the same or different serine protease cascades to activate defense mechanisms, the effects of SRPN7 and CLIP2 depletion on resistance to bacterial infection and the microbiota, respectively, cannot be fully explained by this model and suggest that these two factors may be associated with multiple and functionally diverse serine protease cascades. Nevertheless, our results further corroborate the functional unrelatedness of SRPN7 and CLIP2 with the bacteria-dependent IMD pathway-mediated anti-Plasmodium defense system and highlight the functional versatility and complexity of mosquito immune factors.

SRPN7 and CLIP2 do not regulate the IMD immune pathway

Although serpins and clip serine proteases have been identified as regulators of the TOLL pathway, it is unclear whether similar cascades are involved in regulating the activation of the IMD pathway. The TOLL pathway is primarily responsible for immunity against the rodent parasite P. berghei, whereas the IMD pathway is associated with immunity against the human parasite P. falciparum [4]. Since SRPN7, and to some degree CLIP2, appear to modulate P. falciparum infection intensity, we hypothesized that these genes could be involved in regulating the IMD pathway. In order to determine whether SRPN7 or CLIP2 is involved in activating the IMD pathway, we used qRT-PCR to measure the abundance of three IMD-pathway-regulated, anti-Plasmodium gene transcripts (TEP1, FBN9, LRRD7) at 24, 48, 72, and 96 hours following RNAi-mediated gene silencing of SRPN7 or CLIP2 in aseptic mosquitoes (Table S1). We chose to monitor the expression of these genes over 4 days because of the previously reported temporal regulation of IMD pathway-driven gene transcript abundance [63]. The silencing efficiency over 4 days averaged 62% for SRPN7 and 59% for CLIP2, respectively (Figure 6A, B). Depletion of either SRPN7 or CLIP2 did not influence the transcript abundance of TEP1, FBN9, or LRRD7 over the course of the experiment when compared to GFP dsRNA-injected control mosquitoes (Figure 6C, D). These three potent anti-Plasmodium genes were previously found to be upregulated by the IMD pathway upon silencing of the negative regulator CASPAR and upon overexpression of the IMD pathway transcription factor REL2 [6]. In our earlier studies we have also shown that the abundance of SRPN7 and CLIP2 transcripts is not regulated by the IMD pathway [6]. FBN9 is induced by the native microbiota in the mosquito midgut, and all three genes are involved in controlling its microbiota as well as in systemic bacterial challenge [5,11]. Furthermore, none of these genes were upregulated in the P. falciparum-infected aseptic midguts. In the absence of bacteria, SRPN7 and CLIP2 were upregulated in the P. falciparum-infected aseptic midguts, and depletion of SRPN7, and to some degree CLIP2, modulated the intensity of P. falciparum infection, yet these genes do not appear to regulate the expression of anti-Plasmodium factors through the IMD pathway. These findings suggest that bacteria- and IMD pathway-independent anti-P. falciparum defenses exist, and they underscore the complexity of the mosquito’s anti-Plasmodium immune mechanisms.

Conclusions

The Anopheles mosquito’s anti-Plasmodium defense system is actively engaged in limiting parasite infection of the midgut epithelium by mounting immune responses against the ookinetes...
in the midgut lumen and epithelium [8]. While these immune responses have been shown to be regulated to some extent by midgut microbiota-mediated activation of the IMD pathway, we show here for the first time that other, as yet uncharacterized, microbiota- and IMD pathway-independent immune responses also participate in limiting *P. falciparum* infection. The potential affiliation of *SRPN7* and *CLIPC2* with a serine protease activation cascade suggests that these genes are controlling the activation of an effect mechanism, rather than representing effectors themselves. The regulation and parasite killing mechanism of these defenses appear to be quite different from those previously characterized since (a) *SRPN7* and *CLIPC2* are not regulated by, nor do they regulate, the IMD pathway and (b) they act against *Plasmodium* independently of the midgut microbiota. The observation that *SRPN7* and *CLIPC2* were only regulated in the *P. falciparum*-infected aseptic midguts, strongly suggests that an upstream pattern recognition molecule is sensing *P. falciparum* and culminating in the activation of an undescribed pathway. Alternatively, a molecule upstream of *SRPN7* and *CLIPC2* could be sensing damage to the midgut epithelium mediated by *P. falciparum* invasion. *SRPN7* and *CLIPC2* were neither induced by nor involved in anti-*P. berghei* defense, suggesting an association with defense against *P. falciparum* and demonstrating the ability of the mosquito immune system to discriminate between infections of closely related pathogens. A *P. falciparum*-specific defense pathway could be exploited in a translational approach to control *Plasmodium* in the mosquito, as opposed to the human host. A biochemical analysis of their interacting partners will be necessary to confirm that these molecules are true partners and that they regulate the same effector mechanism. In summary, we have discovered *SRPN7* and *CLIPC2* in the bacteria-independent, *Plasmodium* infection-responsive transcriptome and demonstrated the existence of IMD pathway-independent defenses against *P. falciparum*.

**Supporting Information**

**Table S1** Primers used in this study. PCR primer sequences used in this study. “Gene Target” displays the target gene of the corresponding primer. “AGAPID” lists the Vectorbase identifier if applicable such as in the case of a primer designed to produce double-stranded RNA for targeted gene silencing. “Primer name” is an arbitrary identifier for a particular primer sequence. “Primer sequence” displays the forward and reverse primers designed for a specific gene. “Primer use” shows the use of a given primer (i.e. RNAi is for generating double stranded RNA whereas qRT-PCR indicates a primer was used in real-time PCR analyses).

**Table S2** Microarray data analyzed in this study. Master tab; Microarray assayed Log2 transformed transcript abundance ratio of corresponding genes using a cutoff of 0.75 for upregulation and
0.75 for downregulation. The “Gene Description” column provides a gene name for the corresponding transcript if one is available. The “Functional Group” indicates the assignment of a transcript to a particular functional group dependent on the predicted function of the gene (i.e., putative immune and apoptotic; R/S/M: endogenous T cell-related and mitochon-
drial; C/S: cytoskeletal, structural; MET: metabolism; R/T/T: replication, transcription, translation; P/D: proteolysis, digestion; TRP: transport; DIV: diverse; UKN: unknown functions). The “AGAP-ID” column lists the Vectorbase identifier for a particular gene (http://www.vectorbase.org). The following four columns display Log2 ratio values from either the midgut or carcass experiments: BB ST SD. Analyzed the data: BB ST SD GD. Contributed reagents/materials/analysis tools: GD. Wrote the paper: BB GD.

Table S3 Supplementary data from Figure 4. Supplementary data from Plasmodium infection assays displayed in Figure 4.

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