

Effects of Mosquito Genes on *Plasmodium* Development

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Malaria parasites must complete a complex developmental cycle in an *Anopheles* mosquito vector before transmission to a vertebrate host. Sexual development of the parasite in the midgut is initiated in the lumen immediately after the mosquito ingests infected blood, and the resulting ookinetes must traverse the surrounding epithelial layer before transforming into oocysts. The innate immune system of the mosquito is activated during midgut invasion, but to date, no evidence has been published identifying mosquito immune genes that affect parasite development. Here, we show by gene silencing that an *Anopheles gambiae* leucine rich-repeat protein acts as an antagonist and two C-type lectines act as protective agonists on the development of *Plasmodium* ookinetes to oocysts.

Malaria parasites require mosquito vectors for sexual development and transmission to vertebrates. A day after an *Anopheles* mosquito ingests infected blood, *Plasmodium* gametocytes form gametes, zygotes, and ultimately an invasive stage, the ookinete. Ookinetes cross the midgut epithelium and, when they reach the basal lamina, develop into oocysts. However, large losses in parasite numbers occur during invasion (1), sometimes resulting in complete elimination of the parasite (1–3). The mosquito innate immune system is activated during invasion (4–6) and may be implicated in the loss of ookinetes.

From the genome sequence of *A. gambiae* (7), the major African vector of malaria, we have previously identified 242 genes from 18 gene families putatively implicated in innate immunity; many of these genes are markedly diversified from their *Drosophila* homologs, which may reflect adaptation to different pathogens (6). The three genes that we discuss below do not have *Drosophila* orthologs, and their respective families exemplify this diversification. The C-type lectin (CTL) family consists of 22 members, assigned according to sequence features to four different groups: mannose binding (CTLMA), galactose binding, selectins, and other CTLs. In general, CTLs are secreted or membrane-bound Ca²⁺-dependent, sugar-binding proteins. CTLs interact with glycans on cell surfaces, in the extracellular matrix, or on soluble secreted glycoproteins to mediate processes such as cell adhesion, cell-cell interactions, glycoprotein turnover, and pathogen recognition, which lead to innate immune responses (8).

Several mosquito gene families encoding putative pattern recognition receptors (PRRs)

have been identified by bioinformatic and large-scale microarray analyses (5, 6). Some members of these families are up-regulated in *A. gambiae* after challenge with microbial elicitors or infection with bacteria or *Plasmodium* (5, 6). An example is a family named *LRIM* (leucine rich-repeat immune gene) that encodes secreted, membrane-bound or cytoplasmic proteins with numerous leucine-rich repeats (LRRs). In other species, LRR-containing proteins have diverse functions, and some have been assigned key roles in innate immune reactions (9).

Our laboratory is conducting a large-scale functional screen of about 100 mosquito genes, using our recently established gene silencing technique for adult *A. gambiae* (10). Among a small set of genes associated with interesting phenotypes were two *CTL* genes (*CTL4* and *CTLMA2*) and one *LRIM* gene (*LRIM1*), which showed striking effects on

Plasmodium development (11). The vast majority of other silenced genes did not exhibit these phenotypes (12). To achieve gene silencing, double-stranded RNA (dsRNA) was injected in the body cavity of newly emerged female mosquitoes of the G3 strain that are susceptible to the model rodent parasite *P. berghei*. The apparent reduction in transcript levels was determined 4 days later by quantitative reverse transcription polymerase chain reaction (RT-PCR) for *CTLMA2* (96.8% ± 0.1%) and *LRIM1* (86.2% ± 9.9%), or by semiquantitative RT-PCR for *CTL4* (70% ± 4%) (13). The mosquitoes were then infected with a transgenic parasite that expresses the green fluorescent protein (*GFP*) in the ookinete and oocyst stages, which permits convenient and quantitative monitoring of the infection (14). Seven days later, the parasites detected in dsRNA-injected mosquitoes had either developed into fluorescent, living oocysts (which later produced infective sporozoites) or had been killed and appeared as melanized nonfluorescent ookinetes. For each experimental treatment, we counted separately the fluorescent oocysts and the melanized ookinetes and compared these numbers, as well as their sum (total parasites), with the corresponding figures for control mosquitoes injected with dsRNA for the *GFP* gene. Injection of *GFP*-dsRNA did not affect parasite fluorescence, and therefore, *GFP*-dsRNA was used as an internal control for each experiment.

The pronounced effects of silencing these three genes on *Plasmodium* development in the mosquito midgut are summarized in Table 1, A and B, and Fig. 1. Functional knockouts (KO) of either of these *CTLs* resulted in massive melanization of the invading ookinetes. On average, *CTL4* KO mosquitoes

Table 1. Numbers of melanized ookinetes and oocysts in KO mosquitoes, reported as experimental data sets for the genotypes listed on the first (left) column. The data sets summarized in each line of (A), (B), and (C) were obtained from two, six, and two independent replicate experiments, respectively. The data for the direct comparison of *LRIM1* and *GFP* genotypes of (C) is also included in (B). Each replicate experiment used different batches of dsRNA and mosquitoes, and all the genotypes within each experiment were assessed by feeding on the same infected mouse. Replicate experiments gave similar results that were pooled, giving the numbers in columns 2 to 7. The probabilities (*P*) indicating whether the distributions of parasites in the midguts of each genotype and the *GFP* controls are similar were determined by the KS and the Student's *t* test, with consistent results. The probabilities shown are from the KS test, except for the *LRIM1/CTL4* dKO in (C), where it is from the *t* test (the sample size did not permit KS analysis for this sample. Mel, melanized; par, parasites.

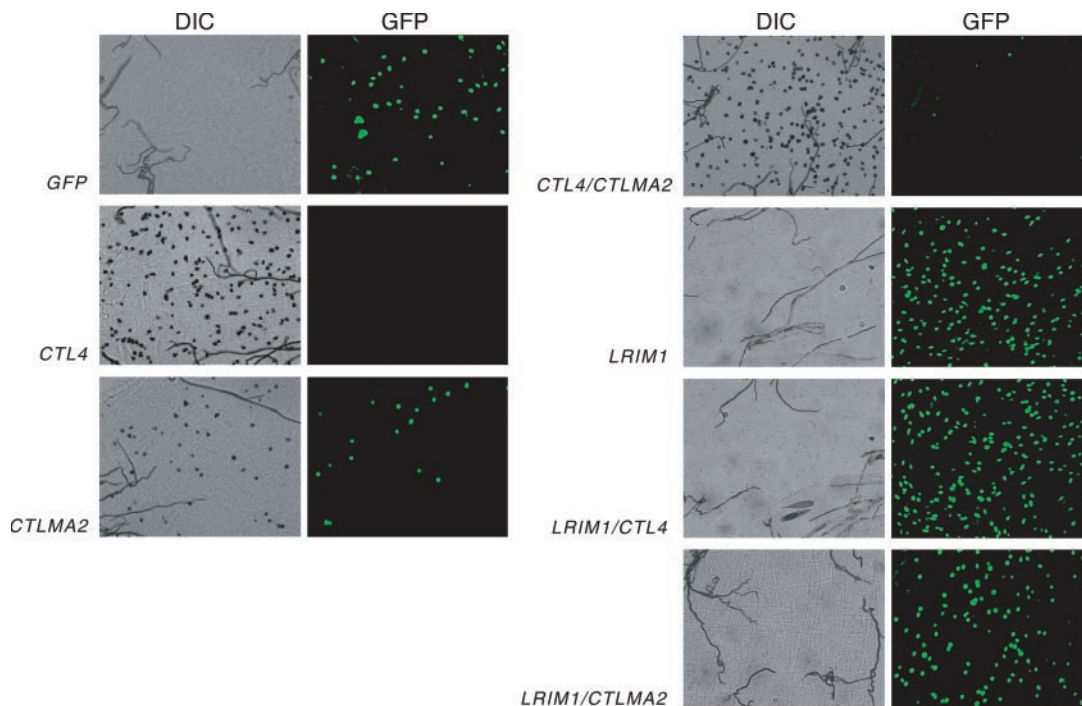
Gene KO	Midguts	Mel par	Oocysts	Total par	Par/midgut [mean (range)]	% Mel	<i>P</i>
(A) <i>CTL4</i>	24	6,447	231	6,678	278(1-664)	96.5	0.043
<i>CTLMA2</i>	34	2,183	1,967	4,150	122(1-377)	52.6	0.060
<i>CTL4/CTLMA2</i>	20	4,422	582	5,004	250(39-648)	88.4	0.338
<i>GFP</i>	25	4	3,955	3,959	158(6-390)	0.1	
(B) <i>LRIM1</i>	61	0	19,398	19,398	318(0-875)	0	0.001
<i>GFP</i>	66	10	5,662	5,672	86(0-301)	0.2	
(C) <i>LRIM1</i>	13	0	6,146	6,146	473(155-875)	0	0.001
<i>LRIM1/CTL4</i>	9	9	3,532	3,541	393(220-574)	0.3	<0.001
<i>LRIM1/CTLMA2</i>	13	3	5,561	5,564	428(60-757)	0.1	0.001
<i>GFP</i>	17	3	1,786	1,792	105(3-252)	0.2	

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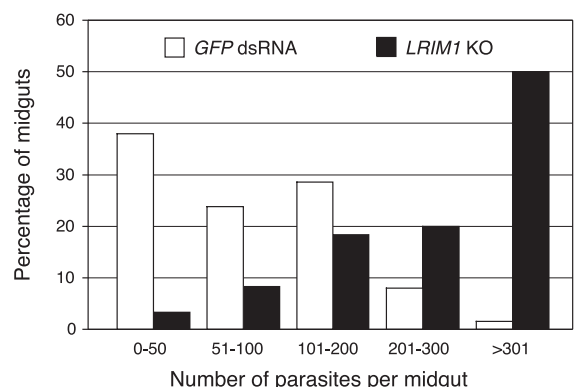
Fig. 1. *Plasmodium* oocysts (green, right) and melanized ookinetes (black spots, left) in midgut of mosquitoes are shown, from controls (*GFP*) or from samples with the labeled genes silenced; midgut dissection and visualization were performed 7 days after infection (13). Melanized ookinetes were detected in bright field (by differential interference contrast, left), and oocysts were visualized with the fluorescein isothiocyanate filter (*GFP*, right).



melanized 97% of the parasites and *CTLMA2* KO mosquitoes 53%. Furthermore, in a substantial fraction of the midguts (33% of *CTL4* KO and 8% of *CTLMA2* KO), every parasite was melanized. In strong contrast, the background melanization in the *GFP* KO controls was sporadic and negligible (0.1%). The *CTL4/CTLMA2* double-knockout (dKO) phenotype was reasonably similar to that of *CTL4* alone (88% of the parasites were melanized and 20% of *CTL4/CTLMA2* dKO midguts showed complete melanization). These results suggested that the two lectins have similar functions that normally protect parasites against melanization. The *CTL4* KO appeared to have a secondary phenotype increasing the mean (melanized) parasite numbers by nearly twofold. However, comparisons of the distributions of parasite numbers in the midguts of all four genotypes by the Kolmogorov-Smirnov (KS) (Table 1) and *t* tests indicated that this secondary effect is not highly significant.

The *LRIM1* KO phenotype was quite different and indicated that this gene has a strong antagonistic rather than protective function vis à vis the parasite. Rather than resulting in killing and melanization of parasites, inactivation of this gene led to a substantial increase in oocyst numbers, averaging 3.6-fold in these experiments: The mean number of ookinetes per gut was 323 for *LRIM1* KO and 90 for the corresponding *GFP* KO controls (Fig. 1 and Table 1B). KS analysis of the distribution of oocyst numbers in the midguts of *GFP* and *LRIM1* KO mosquitoes (Fig. 2) indicated that their difference is highly significant ($P = 0.001$).

Fig. 2. Distribution of parasite numbers per midgut of *GFP* and *LRIM1* KO mosquitoes. Data five independent experiments, all showing similar distributions, were pooled (Table 1B). Midguts were grouped in five successive classes according to their number of oocysts. There is a pronounced shift toward higher oocyst numbers in the *LRIM1* KO midguts. Half of these midguts were superinfected, with more than 300 oocysts each, and the other two well-represented classes showed 101 to 300 oocysts. In contrast, the most-represented class of *GFP* KO midguts had 0 to 50 oocysts; midguts showing more than 200 oocysts were rare, and those showing more than 300 oocysts were extremely rare. According to the KS test, the difference of these distributions is highly significant ($P < 0.001$).



Genetic epistasis analysis using *LRIM1/CTL* dKOs exhibited negligible melanization but a fourfold increase in oocyst numbers relative to the *GFP* controls. The results were comparable to those observed with the *LRIM1* KO alone (Fig. 1 and Table 1C). Thus, the antagonistic function of *LRIM1* against the parasite appears to be epistatic and does not require the CTL function. In contrast, the melanization triggered in the absence of *CTL4* or *CTLMA2* requires the function of *LRIM1*; unlike the massive melanization that characterized the KOs of *CTLs* alone, the *LRIM1/CTL* dKOs showed only negligible and sporadic melanization at the background level (0.1 to 0.2%). Coinjection of dsRNAs against *GFP* and any of these three mosquito genes resulted in the expected single KO phenotype.

At 24 hours post infection, when the ookinetes invade the midgut epithelium, expression levels of the three genes were assessed by quantitative RT-PCR (Fig. 3) in dissected mosquito midguts and in carcasses (mosquito remnants after midgut isolation). Mosquitoes fed on uninfected blood were used as controls. Both *CTLs* and *LRIM1* were specifically up-regulated in the carcasses of infected as compared with uninfected mosquitoes; they were also expressed at a substantially higher level in the carcass compared with the midgut. However, the levels of *LRIM1* transcripts were greatly and transiently increased in the midgut (Fig. 3D) 24 to 28 hours after infection, the known period of ookinete invasion of the midgut epithelium (15). In the absence of specific antibody reagents, we

do not know as yet whether *LRIMI* up-regulation occurs in the midgut epithelium itself or in hemocytes, many of which attach to the midgut during infection (16). The specific up-regulation of these genes in the carcass at 24 hours, when the parasites are still confined in the midgut, may reflect an uncharacterized signaling process across organs and may entail either released parasite products or mosquito factors. Both epithelial and systemic immune responses (within or outside the midgut, respectively) have been noted previously with immune markers such as defensin and Gram-negative-binding protein (4, 17).

Our data identify *CTL4* and *CTLMA2* as genes whose products protect the ookinetes against a potent mosquito innate immune response, melanization. The susceptible G3 mosquito strain that we have used permits survival of an average of more than a hundred unmelanized parasites per midgut, of which only a negligible fraction (0.1%) are melanized. We ascribe the protection against melanization to the two lectins, because their functional knockouts trigger massive melanization of the parasites (highest in the case of the *CTL4*). Thus, these vector proteins appear to have been subverted to protect the parasite. Preliminary experiments suggest that *CTL4* is a secreted lectin present in the hemolymph (18). Secreted CTLs have been implicated previously in elimination of unrelated pathogens in vertebrates (19) and invertebrates (20). *CTL4* may also have a secondary mildly antagonistic effect on parasite numbers; although our data indicate that this effect is not highly significant, it merits further study. Unquestionably, however, the predominant effect of the *CTL4* gene, like *CTLMA2*, is to promote susceptibility by inhibiting parasite melanization.

The protective action of CTLs against parasite melanization does not negate the distinct, antagonistic, and epistatic action

that is mediated by the *LRIMI* gene. The last-mentioned action was inferred by the substantial (fourfold on average; range 3.6- to 4.5-fold) increase in oocysts in all the *LRIMI* KO experiments summarized in Table 1. Apparently, *LRIMI* (at least in part) causes a substantial majority of the ookinetes to be killed while crossing the G3 midgut, before oocyst formation. These parasites, which appear when *LRIMI* is silenced, are "missing" in wild-type mosquitoes but have not been visualized (in contrast to melanized parasites); this suggests the possibility that their massive death may occur by lysis. In a different vector-parasite combination, *A. gambiae*-*P. gallinaceum*, ookinete lysis inside the midgut epithelial cells has been reported (21). No ortholog of *LRIMI* has been detected in any other organism. However, many proteins with LRR domains are linked to the initiation of innate immune reactions, including the Toll-like receptors (TLRs) of insects and mammals (9), the NOD proteins in mammals (22), and the R proteins in plants (23). Although *Drosophila* Toll is not itself a PRR, cumulative evidence supports the idea that mammalian TLRs and NODs are true PRRs, responsible for direct recognition of pathogens (24, 25). Repetitive LRR motifs appear to provide a versatile structural framework for the formation of protein-protein interactions and are thought to form amphipathic structures with a hydrophobic surface that interacts with membranes (26).

In summary, about three-quarters of the invading *P. berghei* ookinetes normally disappear in crossing the midgut epithelium, at least in part through the major (direct or indirect) antagonistic action of *LRIMI* (fig. S1). The remaining parasites survive and form oocysts, provided that they are protected against melanization by *CTL4* and *CTLMA2* (and potentially other protective mosquito molecules). However,

melanization also requires the presence of *LRIMI*: Absence of CTLs does not result in melanization if *LRIMI* is functionally inactivated. It remains to be determined whether *LRIMI* triggers melanization directly or kills the remaining CTL-unprotected parasites, the remnants of which, in turn, trigger local melanization. Identification of both parasite antagonists and protectors in the vector sets the stage for dissecting the molecular interactions between *Anopheles* and *Plasmodium* and for potentially guiding the development of novel malaria control strategies based on those interactions.

References and Notes

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Supporting Online Material

www.sciencemag.org/cgi/content/full/303/5666/2030/DC1
 Materials and Methods
 Fig. S1
 References and Notes

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Fig. 3. Transcript levels of *CTL4* (A), *CTLMA2* (B), and *LRIMI* (C) genes in the midgut (M) and carcass (C) of mosquitoes 24 hours after blood feeding on mice infected with *P. berghei* (black bars) or uninfected (gray bars). (D) Temporal expression profile of *LRIMI* in midguts between 18 and 38 hours after feeding on infected blood.

