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Hemocyte differentiation mediates the mosquito late-phase immune response against *Plasmodium* in *Anopheles gambiae*

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Plasmodium **parasites must complete development in the mosquito vector for transmission to occur. The mosquito innate immune response is remarkably efficient in limiting parasite numbers. Previous work has identified a LPS-induced TNFα transcription factor (LITAF)-like transcription factor, LITAF-like 3 (LL3), which significantly influences parasite numbers. Here, we demonstrate that LL3 does not influence invasion of the mosquito midgut epithelium or ookinete-to-oocyst differentiation but mediates a late-phase immune response that decreases oocyst survival. LL3 expression in the midgut and hemocytes is activated by ookinete midgut invasion and is independent of the mosquito microbiota, suggesting that LL3 may be a component of a wound-healing response. LL3 silencing abrogates the ability of mosquito hemocytes to differentiate and respond to parasite infection, implicating hemocytes as critical modulators of the late-phase immune response.**

mosquito | malaria | innate immune response | hemocytes | late-phase immunity

Mosquitoes of the genus *Anopheles* are the obligate vectors for the transmission of the malaria parasite. Despite much effort invested to understand the interactions between the mosquito immune system and the parasite, our knowledge of these processes remains incomplete. The mosquito innate immune response effectively reduces parasite numbers, resulting in a severe bottleneck at the oocyst stage (1). There is evidence to suggest that multiple pathways contribute to parasite killing during two separate stages, or phases, of parasite development (1). An "early phase" occurs ∼18–24 h after blood feeding, as *Plasmodium* ookinetes invade the midgut epithelium and emerge on the basal side facing the hemocoel. During invasion, ookinetes are marked by epithelial nitration in a response regulated by the Jun N-terminal kinase (JNK) pathway, to promote thioester-containing protein 1 (TEP1) binding to the ookinete surface (2, 3). Once bound, TEP1 is believed to initiate a complement-like cascade that ultimately leads to parasite lysis or melanization (4–7).

Parasites that evade this early-phase complement-like recognition and elimination are thought to be subjected to a second, "late-phase" immune response that further decreases parasite numbers. This model is supported by the observation that the number of mature oocysts is significantly less than the number of early oocysts (8). Silencing the transcription factors STAT-A and STAT-B significantly increases oocyst survival, thus implicating the STAT pathway in this response (8). Conversely, constitutive activation of the STAT pathway via silencing the repressor SOCS (suppressor of cytokine signaling) resulted in increased levels of nitric oxide synthase (*NOS*) production and enhanced oocyst killing (8). These observations are in agreement with other studies that implicate NOS and subsequent NO production as important determinants of malaria parasite survival (9–11).

Previously, we have characterized a LITAF-like transcription factor, LPS-induced TNFα transcription factor (LITAF)-like 3 (LL3), in *Anopheles gambiae* that mediates a potent anti-*Plasmodium* immune response against both *Plasmodium berghei* and *Plasmodium falciparum* parasites (12). LL3 expression is strongly up-regulated in response to ookinete invasion and positively regulates the expression of SRPN6, a serine protease inhibitor implicated in anti-*Plasmodium* immunity (13–15), by directly binding to regions of its promoter (12). To date, no other downstream targets of LL3 have been identified.

Here we examine the role of LL3 in the mosquito immune response to *Plasmodium* and find that LL3 is a key determinant of oocyst survival. We demonstrate that LL3 is induced in the midgut and hemocytes of *An. gambiae* in response to ookinete epithelial invasion in a manner independent of the mosquito microbiota and that LL3 expression is critical for hemocyte activation following *Plasmodium* parasite infection. Our analysis provides previously unidentified insights into the mechanisms that operate during the late-phase immune response and provides the first evidence, to our knowledge, highlighting the role of hemocytes in this process.

Results

LL3 Silencing Does Not Influence Ookinete Invasion. Previous experiments have established that LL3 is an important component of the mosquito anti-*Plasmodium* response in *An. gambiae* (12). However, little information exists regarding the timing, expression, and mechanism of LL3 action.

To address these questions, we first asked if LL3 plays a role in ookinete invasion and early oocyst formation. Using a fluorescent mCherry *P. berghei* strain, the number of early oocysts was measured at 2 d postinfection. Previous experiments have established that TEP1 is an important component of the mosquito immune response that directly acts on ookinetes to mediate lysis or melanization

Significance

The innate immune response is a major determinant of malaria parasite success in its mosquito host. Previous experiments have implicated LPS-induced TNFα transcription factor (LITAF)-like 3 (LL3) as an integral component of the mosquito immune response to the malaria parasite. This study reports that LL3 influences oocyst survival and demonstrates its role in mosquito blood cell (hemocyte) differentiation in response to parasite infection. Integrating previous data, we provide evidence that hemocytes are critical modulators of the mosquito late-phase immune response. Our findings provide new insight into how parasites are killed in the mosquito host and define major roles for LL3 and the STAT pathways in *Plasmodium* oocyst survival.

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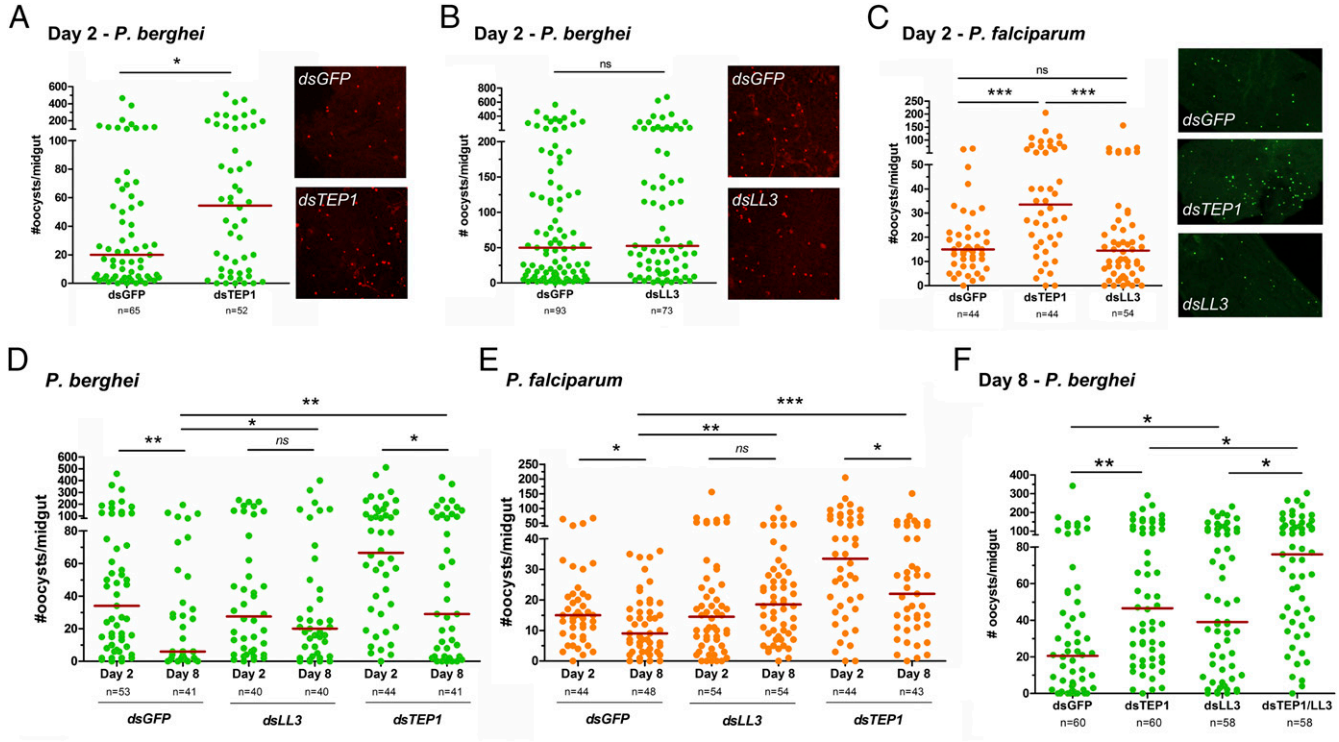

Fig. 1. LL3 silencing does not affect *Plasmodium* ookinete invasion and differentiation into oocysts but limits oocyst survival. The number of fluorescent oocysts was determined on day 2 after a *P. berghei*-infected blood meal in *An. gambiae* silenced with dsTEP1 (*A*) or with dsLL3 (*B*). dsGFP served as a control in all experiments. Similar experiments evaluating the effects of dsTEP1 and dsLL3 silencing on *P. falciparum* early oocyst infection were examined by IFA using a *Pfs*28 antibody (*C*). To address oocyst survival, oocyst numbers were examined at 2 and 8 d postinfection with *P. berghei* (*D*) or *P. falciparum* (*E*), following *GFP*, *LL3*, or *TEP1* silencing. Data were collected using the same cohort of mosquitoes for both time points. Oocyst numbers were measured by fluorescence at both time points (*P. berghei*) or by IFA or mercurochrome staining at day 2 or day 8, respectively (*P. falciparum*). Cosilencing experiments were performed to measure the effects of silencing components of both early- (dsTEP1) and late-phase (dsLL3) components on *P. berghei* development (*F*). For all experiments, each dot represents the number of parasites on an individual midgut, with representative images shown on the right for each treatment. Median oocyst numbers are denoted by the horizontal red line. Statistical analysis was performed using the Mann–Whitney test (*A* and *B*) or Kruskal–Wallis with a Dunn's posttest (*C*–*F*) to determine significance. Three or more independent biological replicates were performed for each experimental condition and the data were pooled. Asterisks denote significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). ns, not significant; n, number of midguts assayed.

shortly after emergence from the midgut epithelium (4, 5, 16). As expected, *TEP1* silencing resulted in a significant increase in early oocyst numbers compared with control *GFP*-silenced mosquitoes (Fig. 1*A*). In contrast, *LL3* silencing did not alter early oocyst numbers (Fig. 1*B*). Similar results for TEP1 and LL3 were also found when early *P. falciparum* oocyst numbers were examined (Fig. 1*C*). These results establish that *LL3* silencing does not influence the success of ookinete invasion and early oocyst formation, indicating that LL3 does not have a role in TEP1 and complement-like immune function.

LL3 Limits *Plasmodium* Oocyst Survival. Whereas LL3 does not influence ookinete invasion (Fig. 1 *A*–*C*), previous experiments suggested that mature oocyst numbers increase following *LL3* silencing (12). To address these differences, we compared oocyst numbers at early (day 2) and late (day 8) time points (Fig. 1 *D* and *E*). In control mosquitoes oocyst numbers significantly decrease between day 2 and day 8 postinfection for both *P. berghei* (Fig. 1*D*) and *P. falciparum* (Fig. 1*E*). In contrast, when *LL3* is silenced, oocyst numbers remain constant between day 2 and day 8 for both *Plasmodium* species (Fig. 1 *D* and *E*). This apparent protection from oocyst degradation most likely accounts for the increase in oocyst numbers following *LL3* silencing that we previously observed (12). Additional experiments in which *TEP1* was silenced demonstrate that oocyst numbers also decrease between day 2 and day 8, even though the initial infection intensity is higher compared with control mosquitoes (Fig. 1 *D* and *E*). However, we did not measure the duration of *TEP1* silencing and cannot exclude the possibility, although unlikely, that TEP1 levels return to normal to have an active role during the late-phase response.

These results imply that the early and late responses to *Plasmodium* are distinct processes. To support this hypothesis, we examined oocyst numbers in mosquitoes lacking both early- and late-phase immune components by respectively cosilencing mosquitoes with TEP1 and LL3. Importantly, when *TEP1* and *LL3* are cosilenced, we observed a significant additive increase in oocyst numbers compared with when either *TEP1* or *LL3* is silenced alone (Fig. 1*F*), suggesting that these mosquitoes are deficient in their ability to prevent ookinete invasion and the removal of developing oocysts. Together, these observations suggest that specific components of both early- and late-phase immune responses shape vector competence.

LL3 Expression Is Strongly Induced in Mosquito Hemocytes. In our initial characterization of LL3, we focused primarily on its activation in the mosquito midgut following ookinete invasion (12). To determine if LL3 has a role in other mosquito tissues, we examined *LL3* gene expression in the midgut, in hemocytes, and in the carcass of naïve sugar-fed mosquitoes and following infection with *P. berghei* (Fig. 2*A*). As previously reported (12), *LL3* is significantly up-regulated in the midgut at 24 h post-*P. berghei* infection (Fig. 2*A*). In contrast, membrane-attack ookinete protein (MAOP) *P. berghei* mutant parasites do not induce *LL3* expression.

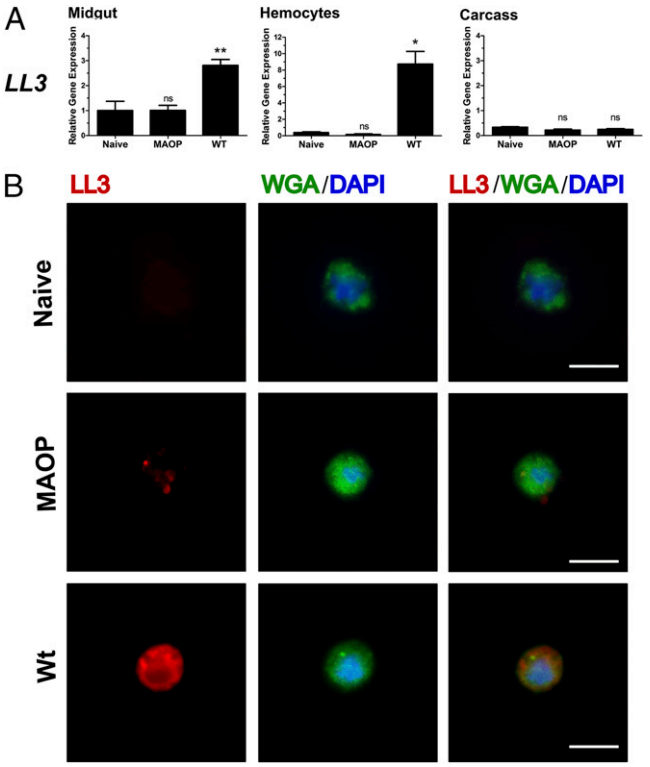

Fig. 2. Activation of LL3 in the midgut and in hemocytes requires ookinete invasion. (*A*) Mosquito tissues (midgut, hemocytes, carcass) were obtained from naïve sugar-fed mosquitoes or from noninvasive MAOP (control) or wild-type (WT) *P. berghei* parasites 24 h after infection. LL3 expression determined by qRT-PCR is displayed for each tissue and experimental condition. Note differences of the *y* axis scales. Statistical analysis was performed using the Mann–Whitney test to determine significance and is denoted by asterisks (*$P < 0.05$, **$P < 0.01$). The data represent a pool of three or more independent biological replicates for each experimental condition. ns, not significant. (*B*) Hemocytes were perfused from naïve sugar-fed mosquitoes or 4 d after infection with noninvasive MAOP (control) or wild-type (WT) *P. berghei* parasites, as indicated to the left of the panels. Following fixation, hemocytes were stained with a LL3 peptide antibody (red), FITC-conjugated WGA, and DAPI. Representative images are displayed for each experimental condition. (Scale bar, 10 μm.) Carcass, mosquito tissues after removal of the midgut.

MAOP mutant parasites form ookinetes that attach to the midgut epithelium but do not invade (17), thus serving as a control for parasite infection in the absence of midgut invasion. In addition, further analysis demonstrated that *LL3* is highly induced in circulating hemocytes in response to wild-type parasites (Fig. 2*A*). By comparison, *LL3* expression is low in carcass and does not change in response to *Plasmodium* infection (Fig. 2*A*), further suggesting that the fat body and sessile hemocytes attached to the body wall are likely not involved in *LL3* regulation. These results indicate that ookinete invasion activates *LL3* expression in both the midgut and circulating hemocytes, thus implicating both immune tissues in LL3-mediated parasite killing (Fig. 1).

To validate these results at the level of protein expression, circulating hemocytes were perfused from age-matched naïve sugar-fed mosquitoes or from mosquitoes 4 d after infection with either MAOP or wild-type *P. berghei* parasites and examined for LL3 expression by immunofluorescence (IFA) (Fig. 2*B* and Fig. S1). Staining with FITC-conjugated wheat germ agglutinin (WGA) was used to identify hemocytes (18, 19). Minimal levels of LL3 were detected in hemocytes from naïve or MAOP-infected mosquitoes. In contrast, ∼90% of hemocytes perfused from mosquitoes following wild-type *P. berghei* infection displayed strong LL3 fluorescence (Fig. 2*B* and Fig. S1), confirming the quantitative RT-PCR (qRT-PCR) results (Fig. 2*A*) and indicating that LL3 protein and transcript abundance are directly correlated. Although LL3 is localized primarily in the cytoplasm of the fixed granulocyte population examined (Fig. 2*B* and Fig. S1), fixation heavily enriches (∼95%) for the more adherent granulocyte population (18), excluding other hemocyte subtypes. Thus, it is unclear if this protein localization pattern also applies to other hemocyte subtypes.

LL3 Expression Is Independent of the Mosquito Microbiota. The mosquito midgut microbiota is believed to be an integral component of the anti-*Plasmodium* immune response (20) and has been implicated in the priming of the mosquito immune system (21). In addition, Rodrigues et al. (21) suggested that disruption of the midgut epithelial barrier by invading ookinetes may allow bacteria

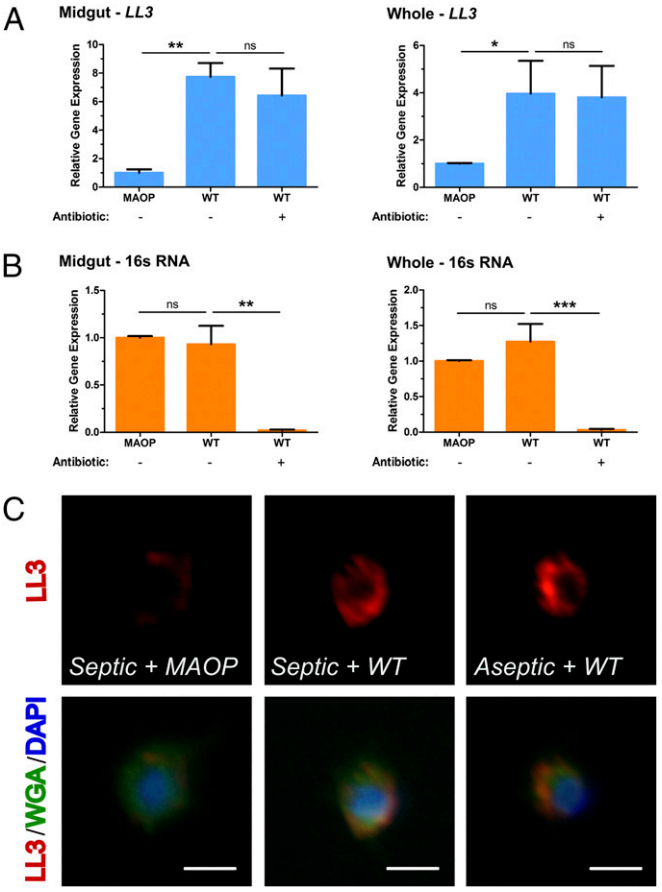

Fig. 3. LL3 expression in response to ookinete invasion is independent of the mosquito microbiota. (*A*) LL3 expression was analyzed by qRT-PCR in dissected midguts or in whole mosquitoes 24 h postinfection with noninvading MAOP or with wild-type (WT) *P. berghei* parasites. Before blood feeding, mosquitoes were maintained in the presence (+) or absence (–) of antibiotics to remove the mosquito microbiota. (*B*) The reduction of the bacterial abundance following antibiotic treatment was verified by qRT-PCR using universal 16S bacterial primers. In the experiments illustrated in *A* and *B*, expression is relative to the levels of gene expression in mosquitoes infected with noninvasive MAOP parasites and without antibiotic treatment. For *A* and *B*, statistical analysis was performed using the Mann–Whitney test to determine significance denoted by asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Four independent biological replicates were performed for each experimental condition. (*C*) LL3 protein expression was examined by IFA in hemocytes obtained from mosquitoes 4 d postinfection with MAOP or with wild-type *P. berghei* parasites. Following fixation, hemocytes were stained with a LL3 peptide antibody (red), FITC-conjugated WGA (a hemocyte marker), and DAPI. Representative images are displayed for each experimental condition. (Scale bar, 20 μm.)

present in the midgut microbiota to come into direct contact with injured epithelial cells to initiate an immune response. To address whether the presence of the mosquito microbiota shapes the late-phase immune response, we examined oocyst numbers in septic and aseptic backgrounds (Fig. S2). In agreement with previous studies (20), we observed increased oocyst numbers in aseptic mosquitoes, either when examined at day 2 or at day 8 (Fig. S2). However, the presence or absence of the microbiota did not influence the effects of the late-phase immune response on oocyst survival as significant losses in oocyst numbers were observed between day 2 and day 8 for both septic and antibiotic-treated mosquitoes (Fig. S2). These data support the hypothesis that the late-phase immune response is independent of the presence of commensal bacteria and may be triggered by ookinete invasion alone.

To determine if the midgut microbiota plays a role in the induction of LL3 expression after ookinete invasion, we examined *LL3* transcript abundance in septic and antibiotic-treated aseptic mosquitoes. *LL3* expression is dramatically increased in both the midgut as well as whole mosquitoes following ookinete invasion (Fig. 3*A*). When treated with antibiotics, bacterial titers were effectively reduced by greater than 100-fold (Fig. 3*B*), yet *LL3* expression after ookinete invasion is comparable to that detected in septic mosquitoes (Fig. 3*A*). These results indicate that *LL3* transcriptional activation is independent of the mosquito microbiota.

Consistent with this finding, LL3 protein expression in circulating hemocytes was unaffected by antibiotic treatment when examined by IFA (Fig. 3*C* and Fig. S3). Weak fluorescence was detected in mosquito hemocytes when infected with invasion-deficient MAOP mutant parasites (Fig. 3*C* and Fig. S3). However, when mosquitoes were infected with wild-type *P. berghei* parasites, LL3 was strongly induced in circulating hemocytes of both septic and aseptic mosquitoes (Fig. 3*C* and Fig. S3). Together, these results suggest that *LL3* expression in the midgut and circulating hemocytes is likely a component of a stress or epithelial damage response as a result of ookinete invasion.

LL3 Is an Important Regulator of Hemocyte Differentiation. Previous studies have shown that the composition of the mosquito hemocyte population is dynamic and is influenced by blood feeding and infection status (21–26). In response to *Plasmodium* infection, prohemocyte precursors undergo differentiation into oenocytoid and granulocyte cell populations (21, 26). However, the mechanisms that regulate these responses have yet to be investigated.

The requirement for ookinete invasion to promote *LL3* expression in the midgut epithelium and circulating hemocytes (Fig. 3) suggests that LL3 may play an important role in hemocyte function. In control dsGFP mosquitoes, significant shifts in the composition of hemocyte subpopulations were detected following ookinete invasion (MAOP vs. WT parasites) without altering total hemocyte numbers (Fig. S4*A*), in agreement with previous reports (21, 26). Mosquitoes infected with wild-type *P. berghei* parasites had a reduced proportion of prohemocytes and significantly increased representation of circulating oenocytoids and granulocytes, suggesting that ookinete invasion triggers hemocyte differentiation (Fig. 4*A*). In contrast, in *LL3*-silenced mosquitoes, the composition of hemocyte subpopulations remained unchanged following *Plasmodium* infection (Fig. 4*A*). To further address this question, we investigated whether hemocyte differentiation correlates with cell division using incorporation of the DNA synthesis precursor EdU (5-ethynyl-2′-deoxyuridine) (Fig. 4*B*) to label newly divided hemocytes (Fig. 4*C*). In *GFP*-silenced mosquitoes fed with the noninvasive MAOP parasite, ∼25% of the perfused hemocytes incorporated the EdU label, similar to previous reports that demonstrated that blood feeding promotes hemocyte proliferation (19, 23). When *GFP*-silenced mosquitoes were fed with wild-type parasites, ookinete traversal triggered a significant increase in EdU-labeled hemocytes, but this increase was abrogated by *LL3* silencing (Fig. 4*D*). These results suggest that hemocytes respond to ookinete invasion and that LL3 plays a critical role in this process. Although hemocytes in *LL3*-silenced mosquitoes no longer respond to *Plasmodium* infection, no differences were detected in total hemocyte numbers (Fig. S4*B*) or in phagocytic activity (Fig. S5) compared with control *GFP*-silenced mosquitoes.

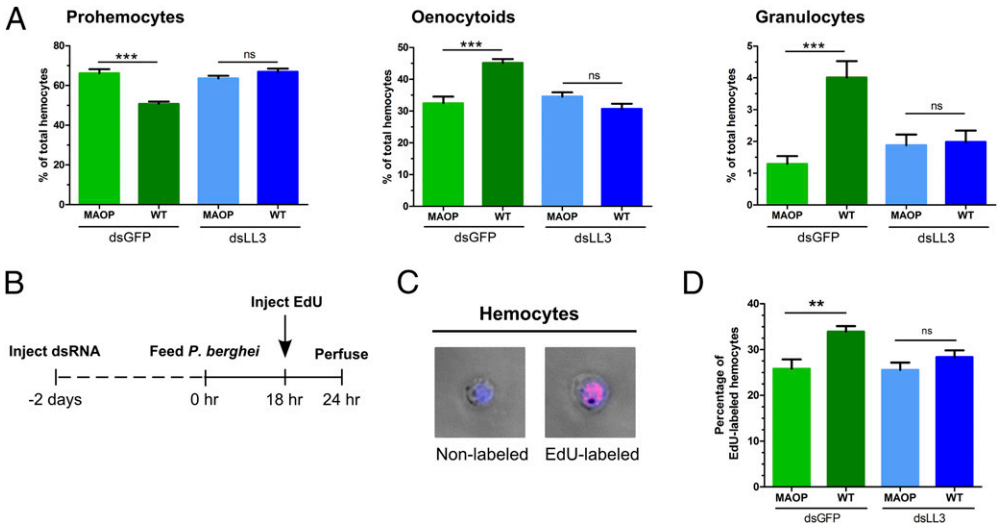

Fig. 4. LL3 is an important regulator of hemocyte differentiation. (*A*) To test the role of *LL3* gene expression in hemocyte differentiation, GFP- (control) or LL3-silenced mosquitoes were perfused 4 d postinfection with MAOP or WT *P. berghei* parasites. The percentage of the prohemocytes, oenocytoids, and granulocytes (Fig. S4*A*) in the total hemocyte population is displayed for each experimental treatment. Hemocyte percentages are displayed as the mean ± SEM of two independent biological replicates (∼10 individual mosquitoes for each experimental condition). Statistical analysis was performed using the Mann–Whitney test to determine significance as denoted by asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; ns, not significant). (*B*) Overview of the hemocyte EdU incorporation assay in *GFP*- or *LL3*-silenced mosquitoes. (*C*) Representative images of nonlabeled and EdU-positive hemocytes. (*D*) Percent EdU-positive hemocytes in *GFP*- or *LL3*-silenced mosquitoes infected with noninvasive MAOP or wild-type (WT) *P. berghei* parasites. The percentage of EdU-positive hemocytes is displayed as the mean ± SEM of two independent biological replicates (eight individual mosquitoes for each experimental condition; $n = 16$). Statistical analysis was performed using the Mann–Whitney test to determine significance as denoted by asterisks (**$P < 0.01$; ns, not significant).

Hemocyte Differentiation Is a Major Determinant of the Mosquito Late-Phase Immune Response. The involvement of LL3 in the late-phase immune response (Fig. 1) and its role in hemocyte differentiation (Fig. 4) suggest that hemocytes play an important role in limiting oocyst survival. The STAT pathway has also been implicated in the late-phase immune response (8), although the role of hemocytes in this response has yet to be determined. The following experiments sought to determine if STAT pathway components similarly regulate hemocyte function.

Similar to our results for LL3 (Fig. 4*A*), *STAT-A* silencing renders the hemocytes nonresponsive to wild-type *Plasmodium* infection (Fig. 5*A*) in agreement with previous results that STAT-A contributes to hemocyte differentiation (26). Moreover, when *SOCS* (a negative regulator of the STAT pathway) is silenced, hemocytes respond normally to *Plasmodium* infection while displaying higher basal percentages of granulocytes (Fig. 5*B*), suggesting that STAT pathway activation may promote granulocyte differentiation. However, when *LL3* and *SOCS* are double-silenced, hemocytes are nonresponsive to *Plasmodium* infection and basal levels of granulocytes are restored to those in control noninfected mosquitoes (Fig. 5*C*). Therefore, LL3 and STAT-A are both important components of hemocyte differentiation.

In addition to hemocyte differentiation, we sought to determine the connection between the LL3 and STAT-A late-phase immune response components in the modulation of oocyst numbers. As previously reported (12), *LL3* silencing increases oocyst numbers, whereas silencing of *SOCS* limits parasite survival (8) (Fig. 5*D*). When *LL3* and *SOCS* were double-silenced, oocyst numbers were significantly higher relative to mosquitoes silenced with *SOCS* alone (Fig. 5*D*). This correlates with the loss of hemocyte differentiation produced by the double knockdown. However, restoration of the dsLL3 phenotype is incomplete, implying that other targets of the STAT pathway may be at work when SOCS is silenced that are independent of hemocyte differentiation.

LL3 and the STAT Pathway Are Independently Regulated. To determine whether the LL3 and the STAT pathways are interconnected, we quantified *LL3*, *STAT-A*, and *SOCS* gene expression after individually silencing each of these genes in dissected midgut samples (Fig. 6 *A*–*C*). The levels of *STAT-A* expression were unaltered following *LL3* silencing (Fig. 6*A*), suggesting that LL3 and the STAT pathway act independently. Conversely, *STAT-A* (Fig. 6*B*) or *SOCS* (Fig. 6*C*) silencing did not significantly influence LL3 expression. Together, these results suggest that LL3 and the STAT pathways are not directly interconnected.

Influence of LL3 Silencing on NOS Expression. To further validate the hypothesis that LL3 does not influence STAT expression, we evaluated *NOS* expression, which is regulated by the STAT pathway and is an important effector of STAT-mediated late-phase immunity. Overactivation of the STAT pathway via *SOCS* silencing significantly increases *NOS* expression in midgut and carcass tissues, contributing to the near-refractory phenotype (8). The following experiments sought to determine if *LL3* silencing influenced *NOS* expression.

Whereas *LL3* silencing resulted in an increase in *NOS* expression (~twofold) in the midgut 4 d postinfection, no significant differences were detected either in hemocyte or in carcass tissues (Fig. 6*D*). Similar to results by Gupta et al. (8), *SOCS* silencing also produced significant changes in *NOS* expression in the midgut and carcass tissues, whereas expression in hemocytes was widely variable between experiments (Fig. 6*D*). These results imply that hemocyte-derived *NOS* is not a significant determinant of oocyst survival. In experiments where *LL3* and *SOCS* were double-silenced, *NOS* levels largely followed the dsSOCS pattern of increased *NOS* expression (Fig. 6*D*). Although we cannot explain the increase in midgut *NOS* expression, the decreased oocyst attrition when *LL3* is silenced suggests that midgut *NOS* expression may not be a critical determinant of late-phase oocyst survival. However, the increased *NOS* expression in the carcass of *SOCS* and *LL3*/*SOCS* double-silenced mosquitoes presumably

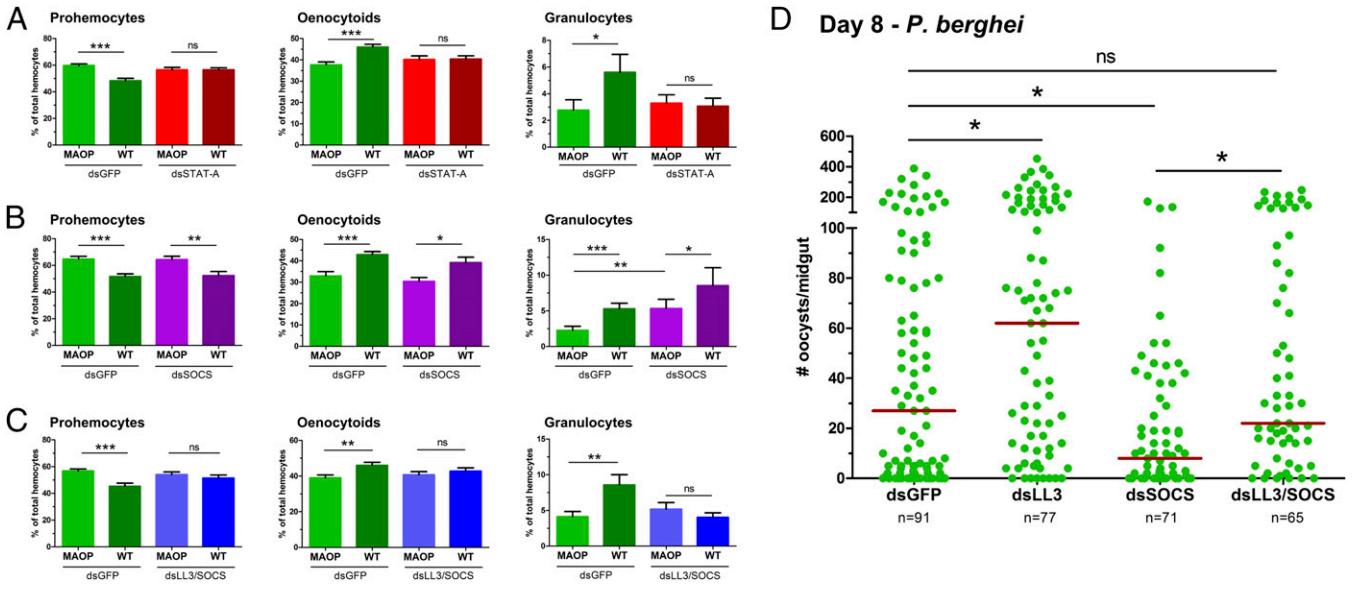

Fig. 5. Hemocyte differentiation is a major component of mosquito late-phase immunity. (*A*–*C*) The prevalence of prohemocytes, oenocytoids, and granulocytes in the total hemocyte population was examined in mosquitoes 4 d postinfection with MAOP or wild-type (WT) *P. berghei* parasites following dsRNA silencing of *STAT-A* (*A*), *SOCS* (*B*), and *LL3*/*SOCS* (*C*). Hemocyte percentages are displayed as the mean ± SEM of two independent biological replicates (~10 individual mosquitoes for each experimental condition). Statistical analysis was performed using the Mann–Whitney test to determine significance as denoted by asterisks (*$P <$ 0.05, **$P < 0.01$, ***$P < 0.001$). (*D*) Oocyst numbers were determined at 8 d postinfection with wild-type *P. berghei* after dsRNA silencing of *GFP*, *LL3*, *SOCS*, or *LL3*/*SOCS* expression. Each dot represents the number of oocysts in an individual midgut, and median oocyst numbers are denoted by a red horizontal line. Statistical analysis was performed using Kruskal–Wallis with a Dunn's posttest to determine significance. Data were pooled from three independent biological replicates. The total number of mosquitoes analyzed (n) is indicated at the bottom of the figure. Asterisks denote significance (*$P < 0.05$). ns, not significant.

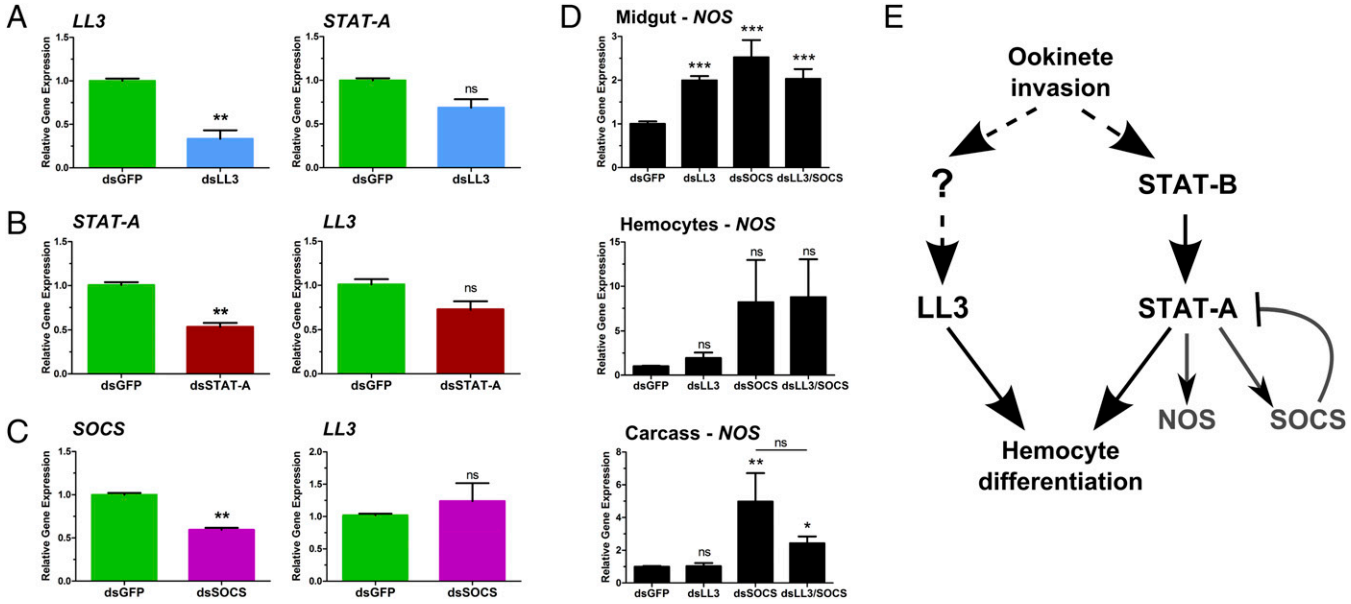

Fig. 6. LL3 and STAT-A promote hemocyte differentiation through independent pathways. (*A*) Effect of *LL3* silencing on *LL3* and *STAT-A* expression. (*B*) Effect of *STAT-A* silencing on *STAT-A* and *LL3* expression. (*C*) Effect of *SOCS* silencing on *SOCS* and *LL3* expression. (*D*) *NOS* expression was evaluated in midgut, hemocyte, and carcass tissues in mosquitoes that were silenced with ds*GFP*, ds*LL3*, ds*SOCS*, or ds*LL3*/*SOCS*. In *A*–*D*, gene expression was examined in midgut tissues 4 d postinfection with wild-type *P. berghei*. All data are shown as mean ± SEM of three or more independent biological replicates, and statistical analysis was performed using the Mann–Whitney test (*A*–*C*) or Kruskal–Wallis with a Dunn's posttest (*D*) to determine significance. Significant differences are indicated by asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (*E*) Proposed model for the signaling of the LL3 and STAT pathways leading to hemocyte differentiation.

has greater effects on oocyst survival and may account for the differences in oocyst numbers between the *LL3*/*SOCS* double knockdown and *LL3* alone (Fig. 5*D*).

The finding that *LL3* silencing alone does not reduce the expression of *NOS* (Fig. 6*D*) suggests that additional immune components contribute to oocyst survival. LL3 and the STAT pathways appear to act independently to promote hemocyte differentiation and to limit oocyst survival in response to ookinete invasion (Fig. 6*E*).

Discussion

Plasmodium killing via the mosquito innate immune response is a major determinant of success of parasite development in its vector and disease transmission (1). To date, a number of components of the mosquito innate immune response have been described that modulate *Plasmodium* development (1, 27, 28), yet our understanding of the mechanisms that limit *Plasmodium* development remains limited. Importantly, most studies have evaluated *Plasmodium* oocyst numbers at a single endpoint, thus missing the mechanistic detail of the early and late responses that play distinct roles in defending the mosquito from parasite infection.

We propose that TEP1 and components of the complement-like immune cascade solely function in the early-phase response to eliminate invading ookinetes and have no role in defining subsequent oocyst survival. This is supported by observations that complement-like components circulate in the mosquito hemolymph in anticipation of immune challenge and are likely only replenished after *Plasmodium* challenge (5–7). Our findings provide mechanistic insight into the events of a subsequent late-phase immune response where the LL3 and the STAT pathways play major roles in determining oocyst survival.

Previous experiments have described the role of LL3 as a transcription factor in *An. gambiae* that, when silenced, resulted in a significant increase of oocyst numbers (12). Here we show that this increase is not the result of killing at the ookinete-to-oocyst transition but by events that occur later, during oocyst maturation. In agreement with these results, SRPN6, which is regulated by LL3 (12), has been described to have a delayed oocyst clearance phenotype (13). However, the observation that oocyst numbers change after LL3 but not after SRPN6 silencing suggests that SRPN6 does not play a major role in the late-phase response.

The role of hemocytes in mosquito anti-*Plasmodium* immunity remains largely unexplored. Hemocytes appear to be the primary phagocytic cells that eliminate bacterial pathogens (29) and are thought to produce several hemolymph components involved in pathogen recognition and immune clearance (4, 30). Evidence suggests that hemocytes undergo proliferation and an increase in overall hemocyte numbers in response to a blood meal (19, 23), as well as aggregate in response to immune challenge (24). Following a *Plasmodium*-infected blood meal, mosquito hemocyte transcripts respond specifically to parasite infection, suggesting that hemocytes may have an integral role in modulating mosquito immunity (22, 31). In agreement with previous reports (21, 26), we detect increased proportions of oenocytoids and granulocytes following *Plasmodium* infection and demonstrate that ookinete midgut invasion is a requirement for hemocyte differentiation. In support of the proposed role of hemocytes in immune function, evidence suggests that increased proportions of circulating granulocytes render the mosquito more resistant to *Plasmodium* infection (21), and transferring hemocytes in which *Toll* signaling was activated (by silencing cactus, a suppressor of *Toll*) to naïve mosquitoes resulted in fewer oocysts when challenged with *Plasmodium* (26). Although both reports implicate a role of hemocytes in anti-*Plasmodium* immunity, our data directly connect hemocyte differentiation to oocyst survival through the requirement of LL3 and the STAT pathway.

Our experiments suggest that hemocyte differentiation is triggered by epithelial damage during ookinete invasion, in view of the inability of the MAOP mutant to trigger the hemocyte response. When fed to mosquitoes, the MAOP mutant produces a normal number of ookinetes that attach to the midgut epithelium but are unable to invade (17). Ookinete invasion produces considerable

damage to midgut cells and promotes apoptosis (32). Similar to the suggested role of hemocytes in *Drosophila* (33, 34), mosquito hemocyte differentiation may promote a wound-healing response that in turn negatively influences parasite survival. In support of this hypothesis, genes involved in wound healing have been implicated in *P. falciparum* killing (35). Alternatively, we cannot exclude that oocyst recognition may itself trigger hemocyte differentiation. Future work is needed to better define the steps that lead to hemocyte differentiation and its respective contributions to oocyst survival.

Whereas NOS (and subsequent NO) production has been proposed to be a major anti-*Plasmodium* effector gene (8–11), it does not appear to significantly contribute to the late-phase phenotype produced by *LL3* silencing. Despite a significant increase in *NOS* expression in midgut tissues when *LL3* is silenced, oocyst survival increased compared with control mosquitoes. This increase in midgut NOS may be involved in signaling additional tissues to defend against the parasite, as previously proposed (36). However, this does not exclude the role of NOS in other tissues (hemocytes and carcass) in anti-*Plasmodium* immunity. Increased NOS expression in the carcass of *SOCS* and *LL3*/*SOCS*-silenced mosquitoes reduces oocyst survival, in agreement with previous reports (8). Most likely, aside from NOS, other yet unidentified components may contribute to oocyst survival. Moreover, late-phase immunity in *An. gambiae* appears to be conserved across *Plasmodium* species, as it is activated by both *P. berghei* and *P. falciparum*.

We propose the following model of parasite killing in the mosquito host (Fig. 7). As invading ookinetes reach the basal lamina, they are recognized by circulating components of the complement-like pathway to initiate an early-phase response leading to lysis or melanization. In addition, ookinete damage to the midgut epithelium generates unknown signals that promote hemocyte differentiation in a process that requires the action of LL3 and genes of the STAT pathway. Furthermore, by analogy to *Drosophila*, other cellular and humoral responses that may also involve the fat body are likely to influence oocyst survival. In *Drosophila*, hemocytes are key intermediate components required for interorgan communication between the midgut and fat body and are required for activation of humoral immunity and pathogen elimination (36, 37).

Although LL3 and the STAT pathway are both necessary to promote hemocyte differentiation in response to *Plasmodium* invasion, gene silencing experiments indicate that the two pathways act independently. The signals that lead to their activation have yet to be elucidated. To a varying extent, components of both pathways are expressed in the midgut, hemocytes, and fat body of the mosquito. However, due to the systemic nature of dsRNA silencing, we were unable to assign a role for LL3 and the STAT pathway separately for each of these specific tissues. The advent of improved genetic tools may allow this question to be addressed in greater detail.

In summary, our results demonstrate the critical role of LL3 in the innate immune response, while providing further insight into the "two phase" model of mosquito anti-*Plasmodium* immunity in *An. gambiae*. Independent of complement-like immune function, LL3 is an essential component of the molecular mechanisms that regulate oocyst attrition. Integrating previously characterized components of the STAT pathway, we establish that LL3 and STAT-A are key elements in the signals that lead to hemocyte differentiation that ultimately modulates anti-*Plasmodium* immunity. These pathways (LL3 and STAT) appear to be independently regulated, pointing to the complexity of the late-phase immune response. Limited studies suggest that responses that limit oocyst survival also occur in the field (38). Our results provide substantial insights into the important mechanisms of oocyst killing.

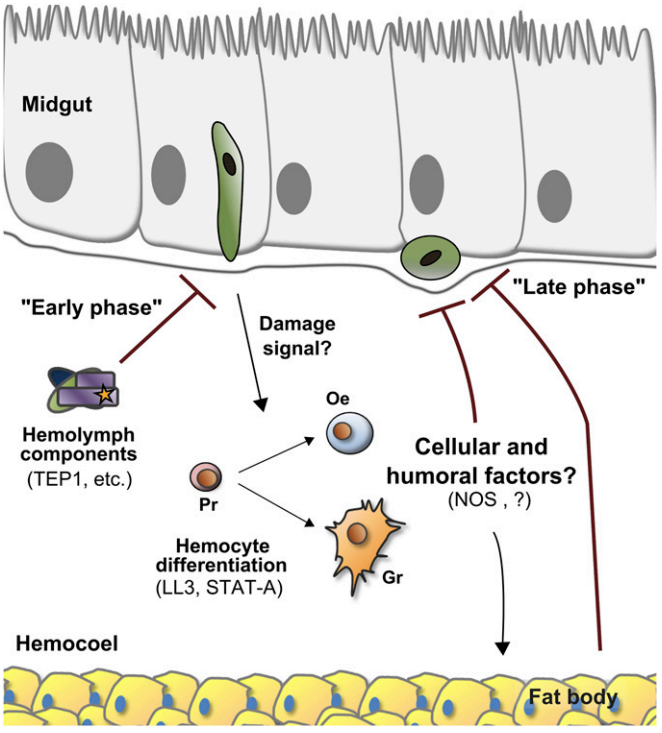

Fig. 7. The two-phase model of parasite clearance in *An. gambiae*. In the first wave, or early phase of parasite killing, complement-like components (including TEP1) circulating in the mosquito hemolymph bind to ookinetes in a response ultimately leading to parasite killing. Surviving parasites are later subjected to a second wave, or late phase, of immune responses that act on the developing oocyst and are triggered by epithelial damage produced by ookinete invasion of the midgut. Central to this response, mosquito blood cells (hemocytes) undergo differentiation, a process that requires LL3 and genes of the STAT pathway. Additional cellular and humoral responses are likely to influence oocyst survival. Gr, granulocyte; Oe, oenocytoid; Pr, prohemocyte.

Materials and Methods

Mosquito Rearing. The Keele colony of *An. gambiae* (39) was maintained at 27 °C and 80% relative humidity with a 14/10 h light/dark cycle. Larvae were reared on a diet of cat food pellets, whereas adult mosquitoes were maintained on 10% (wt/vol) sucrose and fed on anesthetized mice for egg production.

To produce aseptic mosquitoes, newly eclosed adults were given 10% (wt/vol) sucrose containing 10 units/10 mg of Penicillin–Streptomycin (Pen–Strep) (Sigma) 2 d before feeding and were subsequently maintained on 10% (wt/vol) sucrose/Pen-Strep (10 units/ml–10 mg/ml) solution for the duration of the experiments.

***Plasmodium* Infections.** For *P. berghei* infections, Swiss Webster mice were infected with wild-type *P. berghei*-mCherry (40) or MAOP mutant (17) parasites as described previously (12). At 2 d or 8 d following infection with the *P. berghei*-mCherry parasites, midgut dissections were performed in 1× PBS and oocyst numbers were counted by fluorescence.

P. falciparum infections were performed by diluting mature NF54 gametocytes to 0.1% gametocytemia, and mosquitoes were fed using an artificial membrane feeder as previously described (15). At 2 d postinfection, oocyst numbers were quantified by IFA similar to that previously described (8) using a mouse anti-Pfs28 antibody (13). At 8 d postinfection, oocyst numbers were determined by dissecting midguts in 1× PBS and stained with 0.1% mercurochrome before visualization with a compound microscope.

dsRNA Synthesis and Gene Silencing. PCR products for TEP1, STAT-A, and SOCS were produced with slight modification from previous reports (8, 41) to create plasmid constructs for dsRNA production. PCR products were subcloned into a pJet1.2 vector using the CloneJet PCR cloning kit (Thermo Scientific) and used as a template for amplification with T7 promoter sequences to amplify T7-PCR products. Following PCR purification with the DNA Clean and Concentrator (Zymo Research), resultant T7-PCR templates were used for dsRNA production using the MEGAscript RNAi kit (Life Technologies). All dsRNA products, including those for GFP and LL3, were prepared as previously described (12).

Primers used for amplification and the production of T7-PCR templates are listed in Table S1. Two days postinjection, surviving mosquitoes were fed on *P. berghei*- or *P. falciparum*-infected blood and maintained at 19 °C or 25 °C, respectively. The efficiency of dsRNA-mediated silencing was examined in mosquito tissues (midgut, hemolymph, and carcass) 4 d after *P. berghei* infection (6 d postsilencing) and analyzed by qRT-PCR as described below.

Hemolymph Perfusion and Cell Counting. Hemocytes were collected as previously described (21, 26) using an anticoagulant solution of 60% (vol/vol) Schneider's Insect medium, 10% (vol/vol) FBS, and 30% (vol/vol) citrate buffer (98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, and 41 mM citric acid; buffer pH 4.5). In brief, mosquitoes were injected into the thorax with anticoagulant solution and the perfusion collected (~10 μL) from a small perforation in the abdomen using Sigmacote (Sigma)-treated pipet tips and a pipetman. For hemocyte quantification, the collected perfusion sample was added to a disposable Neubauer Improved hemocytometer slide (iNCYTO C-Chip DHC-N01), and the hemocyte subtypes were distinguished by morphology using a light microscope with a 40× objective and counted. To determine total cell numbers and hemocyte proportions, ~500 cells were analyzed per individual mosquito as previously described (21, 26).

RNA Isolation and Gene Expression Analysis. Approximately 20 individual mosquitoes were dissected in 1× PBS to obtain midgut and carcass samples, whereas ~50 mosquitoes were perfused with anticoagulant buffer directly into TRIzol reagent (Invitrogen-Life Technologies) to obtain hemocyte samples. RNA was extracted with TRIzol reagent according to the manufacturer's protocol and further purified with the RNeasy MinElute Cleanup kit (Qiagen). cDNA was prepared using SuperScriptIII (Invitrogen) according to the manufacturer's protocol and used for quantitative real-time PCR as previously described (12). Mosquito gene expression was analyzed using gene-specific primers for LL3, STAT-A, SOCS, and NOS or using universal 16S bacteria primers to monitor bacterial content as previously described (42, 43). All qRT-PCR primers are listed in Table S1.

IFA Assays. Hemocytes were perfused from naïve sugar-fed mosquitoes or *P. berghei*-infected mosquitoes 4 d after infection. Hemocytes were collected directly onto a glass slide and then incubated at room temperature for 30 min before paraformaldehyde fixation. After washing with 1× PBS, fixed cells were treated with 0.1% Triton X-100 for 1 h before blocking overnight in 1% BSA at 4 °C. Cells were stained using a LL3 peptide-derived antibody (12) or FITC-conjugated WGA (Sigma) using 1:500 dilutions. A secondary goat anti-mouse Alexa Fluor 568 antibody was added at a 1:1,000 dilution for detection of LL3. DNA staining was performed using ProLong Gold antifade reagent with DAPI (Invitrogen), and hemocytes were visualized on a Nikon 90i compound fluorescence microscope.

EdU Incorporation Assays. EdU assays were performed in *GFP*- or *LL3*-silenced mosquitoes with the Click-iT EdU Alexa Fluor 594 Imaging kit (Invitrogen) similar to the procedures previously described (19). Approximately 18 h after infection with MAOP or wild-type *P. berghei* (corresponding to the onset of ookinete invasion), mosquitoes were injected with 20 μM EdU in PBS and allowed to recover under standard insectary conditions. Four hours postinjection, hemocytes were perfused directly onto glass slides and incubated at room temperature for 30 min before fixation with 4% (wt/vol) paraformaldehyde. Following fixation, cells were treated according to the manufacturer's specifications and then mounted using ProLong Gold antifade reagent with DAPI (Invitrogen). EdU incorporation was measured as the percentage of EdU-positive cells over the total number of cells detected by DAPI staining (~200 per mosquito analyzed) using a Nikon 90i compound fluorescence microscope.

Phagocytosis Assays. In vivo phagocytosis assays were performed on 3–5-d-old mosquitoes by silencing GFP (control) or LL3 as previously described (12). Two days later, mosquitoes were injected with red fluorescent FluoSpheres (1 μm, Molecular Probes) at a 1:100 dilution in 1× PBS. Following injection, mosquitoes were allowed to recover for 1 h under standard insectary conditions and the hemolymph contents were perfused onto a glass slide. Hemocytes were incubated at room temperature for 30 min to promote attachment and were then fixed in 4% (wt/vol) paraformaldehyde. Cell nuclei were stained with ProLong Gold antifade reagent with DAPI (Invitrogen) and then visualized by fluorescence. The percentage of hemocytes undergoing phagocytosis was defined as the number of hemocytes containing fluorescent beads divided by total hemocyte number determined by DAPI staining (~200 per mosquito were analyzed). In addition, the number of fluorescent beads per cell (phagocytic index) was also determined. Data from two independent biological replicates were pooled for each dsRNA treatment ($n = 5$ per experiment) and displayed as the mean ± SEM. Statistical analysis was performed using the Mann–Whitney test.

VectorBase Gene Accession Numbers. The following VectorBase gene accession numbers were used: LL3, AGAP009053; TEP1, AGAP010815; STAT-A, AGAP000099; SOCS, AGAP011042.

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