The Role of Hemocytes in *Anopheles gambiae* Antiplasmodial Immunity

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Key Words

Hemocyte differentiation · Plasmodium · Malaria · Immune priming

Abstract

Hemocytes synthesize key components of the mosquito complement-like system, but their role in the activation of antiplasmodial responses has not been established. The effect of activating *Toll* signaling in hemocytes on *Plasmodium* survival was investigated by transferring hemocytes or cell-free hemolymph from donor mosquitoes in which the suppressor *cactus* was silenced. These transfers greatly enhanced antiplasmodial immunity, indicating that hemocytes are active players in the activation of the complement-like system, through an effector/effectors regulated by the *Toll* pathway. A comparative analysis of hemocyte populations between susceptible G3 and the refractory L3-5 *Anopheles gambiae* mosquito strains did not reveal significant differences under basal conditions or in response to *Plasmodium berghei* infection. The response of susceptible mosquitoes to different *Plasmodium* species revealed similar kinetics following infection with *P. berghei*, *P. yoelii* or *P. falciparum*, but the strength of the priming response was stronger in less compatible mosquito-parasite pairs. The *Toll*, *Imd*, *STAT* or

JNK signaling cascades were not essential for the production of the hemocyte differentiation factor (HDF) in response to *P. berghei* infection, but disruption of *Toll*, *STAT* or *JNK* abolished hemocyte differentiation in response to HDF. We conclude that hemocytes are key mediators of *A. gambiae* antiplasmodial responses.

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Introduction

Hemocytes are important immune effector cells that are thought to participate in pathogen recognition, mediate fat body production of immune peptides and mediate and coordinate the mosquito systemic immune response [1–3]. Recently, hemocyte differentiation has also been shown to play an important role in innate immune memory responses in *Anopheles gambiae* mosquitoes that enhance the response to subsequent *Plasmodium* infections [4].

The *Toll*, *Imd* and *JNK* pathways have been shown to mediate antiplasmodial immune responses that target the

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ookinete stage of the parasite [5–8] while the *STAT* pathway limits oocyst survival through activation of nitric oxide synthase [9]. For example, overactivation of the *Toll* pathway, by silencing the suppressor *cactus*, has been shown to result in a strong immune response that greatly reduces *Plasmodium berghei* infection in *A. gambiae* mosquitoes [5, 6]. The mosquito complement-like system mediates this response, because cosilencing of the thioester-containing protein 1 (TEP1) and *cactus* prevents the antiplasmodial effect of silencing *cactus* alone [5]. TEP1 is produced by hemocytes and is released into the mosquito hemolymph [10]; however, because systemic injection of double-stranded RNA (dsRNA) into the hemocoel silences *cactus* expression in many organs, the mechanism by which *cactus* silencing promotes TEP1 activation and the identity of the cells or tissue mediating this response have not been established.

The genetically selected *A. gambiae* L3-5 strain is refractory to infection with most *Plasmodium* parasites [11] and expresses a dominant allele of TEP1 that mediates the refractory phenotype [10, 12]. The refractory strain is in a chronic state of oxidative stress that is exacerbated after blood feeding [13], and recent studies revealed that refractory females have reduced longevity, faster utilization of lipid reserves, impaired mitochondrial state 3 respiration, increased rate of mitochondrial electron leak and higher expression levels of several glycolytic enzyme genes [14]. The status of hemocytes in the refractory strain and their potential participation in the antiplasmodial responses of this refractory strain has not been investigated.

An innate immune memory priming response is triggered in *A. gambiae* mosquitoes when *Plasmodium* ookinetes invade the midgut and disrupt the barriers that normally prevent direct contact between the microbiota and midgut epithelial cells. *Plasmodium* infection, in the presence of gut bacteria, triggers the release of a hemocyte differentiation factor (HDF) in the mosquito hemolymph that increases the granulocyte population of challenged mosquitoes and confers enhanced immunity to subsequent *Plasmodium* infections [4]. Furthermore, the transfer of cell-free hemolymph from challenged mosquitoes (which contains HDF) induces hemocyte differentiation in recipient mosquitoes and renders them more resistant to *Plasmodium* infection [4]. The signaling cascades involved in the induction of HDF synthesis or the response of hemocytes to HDF have not been established.

In this study, we explore two different aspects of the participation of hemocytes in antiplasmodial immunity: (1) the initial activation of the complement-like system in

response to *Plasmodium* infection, and (2) the activation of hemocyte differentiation as part of a priming response that enhances immunity to subsequent infections. The strength of the priming response to infections with different *Plasmodium* species that have variable levels of compatibility with a given mosquito strain were compared, and a functional screen was carried out to establish the participation of different signaling pathways in HDF synthesis and on hemocyte responses to HDF.

Materials and Methods

Mosquito Maintenance and Plasmodium spp. Infections

A. gambiae (G3 and L3-5 strains) mosquitoes were reared at 27°C, 80% humidity with a 12-hour light/dark cycle, following standard laboratory conditions. *P. berghei* and *P. yoelli* infections were conducted using the *P. berghei* GFP-CON transgenic 259cl2 strain and GFP *P. yoelii yoelii* 17X nonlethal transgenic strain [15], respectively. Infections were maintained by serial passage in 3- to 4-week-old female BALB/c mice, and parasitemias were assessed by light microscopy from methanol-fixed blood smears that were stained with 10% Giemsa and air dried prior to visualization. Female mosquitoes were exposed to GFP *P. berghei*- or *P. yoelii*-infected mice that had a 3–6% parasitemia and with 1–2 exflagellations/field. In turn, *P. falciparum* infections were conducted as previously described [4]. Briefly, *P. falciparum* NF54 strain gametocyte culture was maintained in O⁺ human erythrocytes using RPMI-1640 medium supplemented with 25 mM, 50 mg/l hypoxanthine, 25 mM NaHCO₃ and 10% (v/v) heat-inactivated type O⁺ human serum. *A. gambiae* (G3 strain) mosquitoes were fed on gametocyte cultures (stages IV and V, 14–16 days old) using artificial membrane feeders at 37°C for 30 min. Mosquitoes were then kept at 27°C and 80% relative humidity until the day of hemolymph collection.

Hemocyte Collection and Quantification

Hemocytes were collected as previously described [4]. Briefly, mosquitoes were injected into the thorax with an anticoagulant solution consisting of 60% Schneider's Insect medium, 10% fetal bovine serum and 30% citrate buffer. A fine incision was made between the last 2 abdominal segments using sterile fine-tip forceps, and additional anticoagulant buffer was added until 10 µl was recovered using a pipettman with a sterile pipette tip. For gene expression of the total hemocyte population, perfusions were collected in TRIzol reagent (Invitrogen-Life Technologies) and subjected to the method for gene expression analysis (see below). For quantification, the collected sample was deposited in a sterile disposable hemocytometer slide (10 µl capacity, Neubauer Improved, iNCYTO C-Chip DHC-N01). Hemocyte numbers were then counted using a light microscope (40× objective), differentiating the three hemocyte cell types (granulocytes, prohemocytes and oenocytoids) [16]. Total number of cells and proportions were then determined from these numbers for each individual mosquito. To evaluate any differences in hemocytes between the susceptible (G3) and refractory (L3-5) *A. gambiae* strains, hemolymph was collected at 2, 4, 6, 8, 10, 12 and 16 days after emergence in

sugar-fed mosquitoes. To evaluate any species-specific effects of *Plasmodium* infection on hemocyte differentiation, female *A. gambiae* G3 strain mosquitoes were infected with either *P. berghei*, *P. yoelii* or *P. falciparum*, and their hemolymph was collected and hemocytes counted as described above.

Hemolymph (Cell-Free) and Hemocyte Transfers

For transfer of cells or cell-free hemolymph from silenced donors into naïve recipients, 1- to 2-day-old donor mosquitoes were injected with dsRNA against *cactus* or the non-target LacZ control. At 2–3 days after silencing, 2 µl hemolymph was perfused from each of 25–40 silenced donors using the modified anticoagulant buffer, collected using a siliconized pipet tip into siliconized microcentrifuge tubes on ice. Each sample was monitored by light microscopy for the presence of bacteria and discarded if bacteria were observed. Samples were centrifuged at 4°C, 6,000 rpm for 20 min, and the supernatant was moved to a new tube. Cells were resuspended in modified anticoagulant buffer. Then, 1- to 2-day-old recipient female mosquitoes were immediately injected with 207 µl of either cell-free hemolymph or cells in fresh buffer. Recipient mosquitoes were allowed to recover at 28°C for 3 days after transfer and were then either fed on a *P. berghei*-infected mouse, maintained, dissected and counted as indicated above or homogenized in RNeasy lysis buffer (Qiagen) and subjected to RNA extraction for gene expression analysis indicated below (fig. 1b, c).

To assess whether the innate immune signaling pathways are required for the production of the HDF, four groups of mosquitoes were allowed to feed on the same *P. berghei*-infected mouse 3 days after silencing of the main pathway components. Two groups of mosquitoes (one control and one experimental) were placed at 28°C soon after blood feeding to prevent infection (naïve set) while the two other groups (challenged set) were maintained at 21°C for 48 h to proceed with normal midgut infection and then transferred to 28°C to reduce parasite load. Seven days after infection (dpi), the cell-free hemolymph was collected from the naïve and challenged sets and transferred into four recipient sugar-fed mosquito groups. Hemocyte collection and counts were then performed 4 days after transfer (fig. 4).

To assess the effect of immune signaling pathway silencing on the hemocyte responses to HDF, cell-free hemolymph transfers were conducted as previously described [4]. In short, hemolymph was collected from naïve and challenged mosquitoes 7 days after priming by perfusion using a modified anticoagulant buffer (95% Schneider's medium and 5% citrate buffer). The collected hemolymph was then centrifuged at 4°C, 10,000 rpm for 10 min, and the cell-free supernatant was transferred to a new microcentrifuge tube. The cell-free hemolymph was then injected in a total volume of 210 µl per mosquito. Hemocytes in recipient mosquitoes were counted 4 days after transfer following the methodology described above (fig. 5).

RNAi Silencing

RNAi-directed silencing of immune signaling pathways was performed as previously described [17]. In short, 2- to 3-day old female *A. gambiae* mosquitoes were cold anesthetized and injected with 69 µl of a 3-µg/µl dsRNA. The MEGAscript RNAi kit (Ambion) was used to synthesize the dsRNA solution using as a template cDNA from *A. gambiae* mosquitoes. Primers used for gene silencing have been previously designed and are listed in online

supplementary table S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000353765). RNAi-treated mosquitoes were then used for subsequent assays at 3 dpi. The whole-body silencing efficiency of *cactus*, *Rel1*, *Rel2*, *STAT* and *JNK* after dsRNA injection was, 80, 64, 45, 75 and 75%, respectively, relative to *dsLacZ* controls.

Gene Expression

Hemocytes were collected in TRIzol as indicated above and processed for RNA according to the manufacturer's instructions. Similarly, whole bodies were homogenized in RNeasy lysis buffer (Qiagen) and processed for RNA using the RNeasy RNA extraction kit (Qiagen) according to the manufacturer's instructions. RNA from either method was assessed for concentration and quality using a NanoDrop (Thermo Scientific) and used in synthesis of cDNA using the QuantiTect reverse transcription kit with DNA Wipeout (Qiagen). Generated cDNA was used as template for real-time quantitative PCR assays using the DyNamo SYBR green qPCR kits (Thermo Scientific) with gene-specific primers given in online supplementary table S1 and run using a CFX96 Real Time PCR Detection System (Bio-Rad). Data were collected and analyzed using the CFX96 software, and statistical significance was determined using Student's t test (GraphPad, La Jolla, Calif., USA).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 5 (GraphPad). Analyses derived from at least two independent biological replicates. Hemocyte numbers and proportion analyses were performed using Student's t test, and error bars represent the SEM. Significance was assessed at $p < 0.05$.

Results

Activation of the Toll Pathway in Hemocytes and Antiplasmodial Immunity

We explored the role of the *Toll* pathway specifically in the hemocyte compartment. The effect of overactivating the *Toll* pathway, by silencing the suppressor *cactus*, on the relative abundance of different hemocyte populations was investigated. *cactus* silencing significantly increased the proportion of oenocytoids from 34 to 51% ($p < 0.0001$, t test), with a concomitant reduction in the prohemocyte population from 62 to 46% ($p < 0.0001$, t test), relative to *dsLacZ*-injected controls (fig. 1a). However, no significant changes in the proportion of granulocytes or the total number of hemocytes were observed (fig. 1a). The injection of *dsLacZ* did not affect the proportion or the total number of hemocytes relative to uninjected controls (online suppl. fig. S1).

The hypothesis that overactivation of *Toll* signaling in hemocytes mediates the enhanced antiplasmodial response that is observed when *cactus* expression is silenced by systemic dsRNA injection was explored. Hemolymph from *cactus*-silenced mosquitoes was collected 2–3 days

Fig. 1. Effect of activation of the *Toll* pathway in hemocyte anti-plasmodial immunity. n.s. = Not significant; *Cact* = *cactus*-silenced control; *LacZ* = *dsLacZ* controls. **a** Total hemocyte cell count and proportions of granulocytes, oenocytoids and prohemocytes in individual mosquitoes from *cactus*-silenced and *dsLacZ* controls. Error bars indicate SEM. *** $p < 0.001$. **b, c** *P. berghei* oocyst numbers in mosquito midguts that received either washed cells or cell-free supernatant from *cactus*-silenced or *dsLacZ* control donors at 12 h before feeding (**b**) or 3 days before feeding (**c**). Horizontal red lines indicate median infection intensity. SN = Supernatant; Prev. = prevalence.

after dsRNA injection using the low-pressure flushing method previously described [4]. Cells were collected by centrifugation, washed with buffer and injected into recipient mosquitoes that received the equivalent of half of the hemocytes present in one mosquito. The cell-free hemolymph was also injected into recipient mosquitoes, and they received the equivalent of 1/10 of the hemolymph present in one mosquito. The effect of transferring cells or cell-free hemolymph from donors that had been injected with either *dsLacZ* or *dsCactus* on the susceptibility of the recipients was evaluated. The number of oocysts present at 7 dpi was significantly lower in mosquitoes that received the cell-free hemolymph from *cactus*-silenced donors 12 h before feeding, relative to the *dsLacZ* controls, but the transfer of cells had no effect on infection (fig. 1b). However, the transfer of either cell-free hemolymph or cells from *cactus*-silenced donors 3 days before feeding on an infected mouse greatly reduced *P. berghei* infection (fig. 1c). We confirmed that there was no difference in *cactus* expression levels in the mosquitoes that received either hemolymph or cells from a *cactus*-silenced donor (online suppl. fig. S2).

Because TEP1, a key component of *Plasmodium* lysis mediated by the complement-like system, is known to be synthesized by hemocytes [10], we explored whether the observed immune enhancement in the recipients could be due to an overall increase in TEP1 expression. However, *cactus* silencing significantly reduced TEP1 expression in hemocytes collected at 3 dpi, the time when they were transferred, relative to the *dsLacZ* controls ($p < 0.05$, t test; online suppl. fig. S3), indicating that the phenotype of transferring *cactus*-silenced hemocytes was not due to higher steady-state levels of TEP1 expression.

Hemocyte Populations in Susceptible and Refractory *A. gambiae* Strains

TEP1 is known to be a critical mediator of *Plasmodium* elimination in the *A. gambiae* refractory strain, and susceptible and refractory mosquitoes are known to express different alleles of TEP1 [10]. Furthermore, genetic studies revealed that TEP1-R1, the allele present in the refractory strain, has a dominant effect in *P. berghei* melanization. Because TEP1 is synthesized in hemocytes, we investigated whether the refractory (L3-5) and susceptible (G3) *A. gambiae* strains may differ in the distribution of their hemocyte populations or in the total number of cells. No significant difference in the proportion of hemocytes was observed between these mosquito strains in samples collected from sugar-fed females during the first 16 days after emergence (fig. 2a, online suppl. fig. S4).

However, the total number of hemocytes was significantly lower in the refractory strain at 8 and 12 days after emergence (fig. 2b).

Changes in hemocyte populations in response to *P. berghei* infection were also compared between susceptible and refractory females. Both refractory and susceptible strains exhibited a similar pattern of hemocyte population changes between infected and non-infected mosquitoes: a prominent increase in granulocytes beginning at 2 dpi that persisted up to 12 dpi (fig. 2c), concurrent with a significant reduction in prohemocytes that was more prominent at 2 and 6 dpi (fig. 2c). Neither refractory nor susceptible mosquitoes displayed a drastic difference in the proportion of oenocytoids in response to infection, and a slight reduction in the total number of hemocytes in the infected group was observed for both strains (online suppl. fig. S5).

A. gambiae Hemocyte Differentiation in Response to Infection with Different *Plasmodium* Species

We investigated whether the immune response to an initial infection with *Plasmodium* affects the strength of the hemocyte differentiation response that mediates immune priming and enhances the immune response to a subsequent infection. To this end, we compared the hemocyte proliferation response of susceptible G3 females following infection with *P. berghei*, *P. yoelii* and *P. falciparum*, three strains that differ in their compatibility with this mosquito strain. It has been previously established that silencing TEP1 has a dramatic effect on *P. yoelii* infection (increasing the median number of oocysts by 32 fold) [18], an intermediate effect on *P. berghei* infection (increasing the median number of oocysts by 4–6 fold) [19], while silencing TEP1 has no effect on infection with *P. falciparum* NF54 strain [20]. We found that infection with *P. yoelii* or *P. falciparum* induced a robust increase in granulocytes, very similar to what we observed with *P. berghei* infection, which was already apparent at 2 dpi and was still present at 12 dpi (fig. 2c, 3a). In general, the in-

Fig. 2. Hemocyte population in sugar-fed susceptible (S) and refractory (R) strain of *A. gambiae* and in response to *P. berghei* infection. **a** Proportion of granulocytes (Gr), prohemocytes (Pr) and oenocytoids (Oe) in the sugar-fed susceptible and refractory strain. **b** Total number of hemocytes in the susceptible and refractory strain of sugar-fed females during the first 16 days after emergence. **c** Granulocyte and prohemocyte proportions in the susceptible and refractory strain during the first 12 days after *P. berghei* infection. C = Uninfected controls; I = infected. Error bars indicate SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

crease in granulocytes was more robust and consistent when susceptible females were infected with *P. yoelii*. Both *P. berghei* and *P. yoelii* infection also resulted in a significant reduction in prohemocytes, while only a slight reduction was observed 2 days after *P. falciparum* infection (fig. 3b). *P. yoelii* infection also resulted in a drastic and persistent reduction in the total number of hemocytes, while a milder and occasional reduction was observed after infection with *P. berghei* or *P. falciparum* (fig. 3c). Slight, occasional increases in oenocytoids were observed after *P. yoelii* infection (online suppl. fig. S6), but no significant changes were observed after *P. berghei* or *P. falciparum* infection (online suppl. fig. S5, S6).

Immune Signaling Pathways and Induction of HDF in Mosquito Hemolymph

To assess whether innate immune signaling pathways are required for the induction of HDF synthesis following a challenge with *P. berghei*, we disrupted signaling of the *Toll*, *Imd*, *STAT* and *JNK* pathways (online suppl. fig. S7) by silencing Rel1, Rel2, STAT-A or JNK, respectively, before mosquitoes were infected. Two groups of *dsLacZ*-injected controls and silenced mosquitoes were fed on a *P. berghei*-infected mouse and placed at either a non-permissive temperature (naïve group) or permissive (challenged group) for parasite development in the mosquito. Cell-free hemolymph from each of the four silenced treatments was transferred into recipient mosquitoes (fig. 4a). Transfer of hemolymph from all the *dsLacZ*-injected control groups that were challenged resulted in a significant increase in granulocytes relative to the naïve controls (fig. 4b). Disruption of the *Toll*, *Imd*, *STAT* or *JNK* pathways did not affect the proliferation response (fig. 4b, online suppl. table S2), indicating that signaling from these pathways is not essential for the induction of HDF production and release into the mosquito hemolymph.

Effect of Immune Signaling Pathway Silencing on Hemocyte Responses to HDF

To assess whether the immune signaling pathways were essential for hemocyte differentiation in response to HDF, each of them was disrupted in the recipient mosquitoes by dsRNA-mediated silencing (fig. 5a). A robust response to HDF was observed in control recipients that were injected with *dsLacZ* (fig. 5b). However, mosquitoes in which the *Toll*, *STAT* or *JNK* pathways have been compromised no longer responded to the transfer of hemolymph from challenged mosquitoes that contained HDF, and the proportion of granulocytes was not significantly different from those that received hemolymph from naïve

Fig. 3. Hemocyte differentiation in response to *P. yoelii* and *P. falciparum* infection. Granulocyte proportions (a), prohemocyte proportions (b) and total number of hemocytes (c) between uninfected controls (C) and infected (I) *A. gambiae* mosquitoes during the first 12 days following *P. yoelii* or *P. falciparum* infection. Error bars indicate SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

mosquitoes (fig. 5b, online suppl. table S3). In contrast, when hemolymph from challenged donors was transferred into mosquitoes with compromised *Imd* signaling (via *Rel2* silencing), a robust increase in the proportion of granulocytes, similar to that of the *dsLacZ*-injected con-

Fig. 4. Effect of disrupting the *Toll*, *Imd*, *STAT* or *JNK* pathways on HDF production and release into the mosquito hemolymph. **a** Model depicting the process of parasite invasion of the midgut epithelial cells and the release of HDF following midgut injury and exposure to gut microbiota. **b** Proportion of granulocytes in sugared mosquitoes that received either cell-free hemolymph from naïve (Nv) or challenged (Ch) *dsLacZ* controls or *cactus*, *Rel1*, *Rel2* or *JNK* silenced mosquitoes. Hemolymph was collected 7 days after *P. berghei* infection and transferred into recipient mosquitoes to assess hemocyte differentiation. Proportion of granulocytes in mosquitoes that received cell-free hemolymph from naïve or challenged donors of *dsLacZ* or immune pathway-silenced backgrounds. Error bars indicate SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Fig. 5. Participation of immune signaling pathways in hemocyte responses to HDF. **a** Model depicting the effect of cell-free hemolymph transfer from challenged mosquitoes, which contains HDF (red dots), on the proportion of granulocytes (blue oval cells). **b** Proportion of granulocytes in *dsLacZ* controls or silenced-replicant mosquitoes after transfer of cell-free hemolymph from naïve (Nv) or challenged (Ch) donors. Error bars indicate SEM. *** $p < 0.001$, ** $p < 0.01$.

trols, was observed (fig. 5b, online suppl. table S3), indicating that the *Imd* pathway is not essential for hemocytes to respond to HDF.

Discussion

Prohemocytes are thought to be precursor cells that differentiate into granulocytes and oenocytoids [21, 22]. The concomitant reduction in prohemocytes when oenocytoids increase in response to *cactus* silencing suggests that the overactivation of *Toll* signaling is favoring the differentiation of the oenocytoid lineage. The fact that transfer of a small amount of hemolymph (1/10 of the hemolymph in the donor) from a mosquito in which *cactus* has been silenced greatly enhances antiplasmodial immunity suggests that overactivation of the *Toll* pathway results in the release of a soluble factor into the mosquito hemolymph that promotes activation of the complement-like system. This factor is probably produced by hemocytes, because if mosquitoes are infected 12 h after the transfer, there is no effect on infection, but if more time is given (3 days), the recipients of hemocytes with enhanced *Toll* signaling also mount a stronger antiplasmodial response. Taken together, this indicates that besides their role in TEP1 synthesis, hemocytes also play an active role in regulating activation of the complement-like system.

No significant differences in the proportion of the different hemocyte populations were observed between susceptible and refractory mosquitoes between 2 and 12 days after emergence. However, in general, the total number of hemocytes was lower in the refractory strain, and this difference was highly significant 12 days after emergence. The refractory strain is known to be in a state of chronic oxidative stress and to have reduced longevity [13, 14], suggesting these markers of accelerated aging/reduced fitness may also be correlated with the reduced number of hemocytes observed in refractory females. Both susceptible and refractory *A. gambiae* females responded to *P. berghei* infection with a strong and persistent increase in granulocytes and a modest decrease in prohemocytes, suggesting that *P. berghei* infection favors differentiation of the granulocyte lineage. The lack of any differences in hemocyte lineage proportions between the susceptible (G3) and refractory (L3-5) strain of *A. gambiae* in the context of *P. berghei* infection suggests that the refractory phenotype is due to mechanisms other than the relative abundance of a subpopulation of hemocytes.

Comparative infections of susceptible females with different species of *Plasmodium* parasites revealed that *P.*

berghei, *P. yoelii* or *P. falciparum* all triggered a significant and sustained increase in granulocytes and a reduction in prohemocytes. However, the magnitude of the responses appears to be proportional with the compatibility, that is, the extent to which the complement-like system limits infection, between each *Plasmodium* species and susceptible *A. gambiae* females [7]. The most compatible combination, *P. falciparum*-*A. gambiae* susceptible strain, had the mildest response, especially in terms of changes in the proportion of prohemocytes and total hemocytes, while the least compatible pair (*P. yoelii* in *A. gambiae* susceptible strain) exhibited the strongest and most persistent response. This suggests that, besides the disruption of the midgut barriers in the presence of the gut microbiota, the vector-parasite compatibility, which is hallmarked by the degree of activation of the complement-like system, also affects the hemocyte differentiation response.

Recent experiments revealed that the *Pfs47* gene allows some *P. falciparum* parasites to evade the mosquito immune system [17, 20]. Expression of *Pfs47* in wild-type NF54 parasites, the same line as used in this study, prevents the activation of epithelial responses to *Plasmodium* invasion, such as the induction of NOX5 and HPX2 expression, two enzymes that potentiate epithelial nitration and promote complement activation [20]. The observation that the priming response with *P. falciparum* NF54 was the weakest suggests that the ability to mount an effective immune response to the initial infection is key to establishing a robust priming response. Interestingly, *P. falciparum* infection in humans is a chronic disease, in which infections are usually not completely resolved, and multiple reinfections are common, due to an inability to mount effective immune memory responses. It is possible that other proteins from *P. falciparum* could also be modulating the initial immune response and limiting the ability of the human immune system to 'remember' this pathogen.

Our functional studies indicate that none of the immune signaling pathways tested (*Toll*, *Imd*, *STAT* and *JNK*) are essential for the induction of HDF synthesis and release into the mosquito hemolymph. However, the *Toll*, *STAT* and *JNK* pathways are key players in the hemocyte differentiation response to HDF. These signaling cascades are known to participate in hemocyte differentiation in *Drosophila*. For example, the *Toll* pathway has been shown to be involved in hemocyte proliferation and differentiation during larval hematopoiesis and following an immune challenge in *Drosophila melanogaster* flies [23, 24]. Constitutive activation of the *STAT* pathway re-

sults in overproliferation of hemocytes [25], and hemocytes release *upd3*, a cytokine that activates the *STAT* pathway in the fat body [26]. Furthermore, increasing reactive oxygen species levels in *Drosophila* hematopoietic progenitor cells activates *JNK* signaling and triggers premature differentiation into all mature cell types [27].

Whether HDF is activating these immune pathways directly in hemocytes and/or it is activating signaling in other organs that produce cytokines that, in turn, modulate hemocyte differentiation remains to be defined. Each immune signaling pathway could also control different and specific aspects of hemocyte differentiation. For instance, some of these pathways might be involved in controlling hematopoietic proliferation and differentiation [23], while others might regulate genes involved solely in hemocyte differentiation [28, 29].

We conclude that hemocytes play a central role in mediating and modulating antiparasitoid immunity. Besides their role in the synthesis of TEP1 and other key components of the complement-like system, they also play an active role in modulating activation of this immune effector mechanism. The strength of the hemocyte

differentiation response to *Plasmodium* infection appears to be dependent on the level of compatibility between particular parasite-vector combinations. The synthesis and release of HDF in response to infection is under regulation of a new immune signaling pathway that remains to be defined, and the differentiation response to HDF is complex and involves multiple signaling cascades.

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References

- Bartholomay LC, Mayhew GF, Fuchs JF, Rocheleau TA, Erickson SM, Aliota MT, Christensen BM: Profiling infection responses in the haemocytes of the mosquito, *Aedes aegypti*. *Insect Mol Biol* 2007;16:761–776.
- Strand MR: The insect cellular immune response. *Insect Sci* 2008;15:1–14.
- Lavine MD, Strand MR: Insect hemocytes and their role in immunity. *Insect Biochem Mol Biol* 2002;32:1295.
- Rodrigues J, Brayner FA, Alves LC, Dixit R, Barillas-Mury C: Hemocyte differentiation mediates innate immune memory in *Anopheles gambiae* mosquitoes. *Science* 2010;329:1353–1355.
- Frolet C, Thoma M, Blandin S, Hoffmann JA, Levashina EA: Boosting NF- κ B-dependent basal immunity of *Anopheles gambiae* aborts development of *Plasmodium berghei*. *Immunity* 2006;25:677–685.
- Garver LS, Dong Y, Dimopoulos G: Caspar controls resistance to *Plasmodium falciparum* in diverse anopheline species. *PLoS Pathog* 2009;5:e1000335.
- Jaramillo-Gutierrez G, Molina-Cruz A, Kumar S, Barillas-Mury C: The *Anopheles gambiae* oxidation resistance 1 (OXR1) gene regulates expression of enzymes that detoxify reactive oxygen species. *PLoS One* 2010;5:e11168.
- Garver LS, Bahia AC, Das S, Souza-Neto JA, Shiao J, Dong Y, Dimopoulos G: Anopheles Imd pathway factors and effectors in infection intensity-dependent anti-*Plasmodium* action. *PLoS Pathog* 2012;8:e1002737.
- Gupta L, Molina-Cruz A, Kumar S, Rodrigues J, Dixit R, Zamora RE, Barillas-Mury C: The STAT pathway mediates late-phase immunity against *Plasmodium* in the mosquito *Anopheles gambiae*. *Cell Host Microbe* 2009;5:498–507.
- Blandin S, Shiao S-H, Moita LF, Janse CJ, Waters AP, Kafatos FC, Levashina EA: Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector *Anopheles gambiae*. *Cell* 2004;116:661–670.
- Collins FH, Sakai RK, Vernick KD, Paskewitz S, Seeley DC, Miller LH, Collins WE, Campbell CC, Gwadz RW: Genetic selection of a *Plasmodium*-refractory strain of the malaria vector *Anopheles gambiae*. *Science* 1986;234:607–610.
- Blandin SA, Wang-Sattler R, Lamacchia M, Gagneur J, Lycett G, Ning Y, Levashina EA, Steinmetz LM: Dissecting the genetic basis of resistance to malaria parasites in *Anopheles gambiae*. *Science* 2009;326:147–150.
- Kumar S, Christophides GK, Cantera R, Charles B, Han YS, Meister S, Dimopoulos G, Kafatos FC, Barillas-Mury C: The role of reactive oxygen species on *Plasmodium melanotic* encapsulation in *Anopheles gambiae*. *Proc Natl Acad Sci USA* 2003;100:14139–14144.
- Oliveira JHM, Gonçalves RLS, Oliveira GA, Oliveira PL, Oliveira MF, Barillas-Mury C: Energy metabolism affects susceptibility of *Anopheles gambiae* mosquitoes to *Plasmodium* infection. *Insect Biochem Mol Biol* 2011;41:349–355.
- Ono T, Tadakuma T, Rodriguez A: *Plasmodium yoelii yoelii* 17XNL constitutively expressing GFP throughout the life cycle. *Exp Parasitol* 2007;115:310–313.
- Castillo JC, Robertson AE, Strand MR: Characterization of hemocytes from the mosquitoes *Anopheles gambiae* and *Aedes aegypti*. *Insect Biochem Mol Biol* 2006;36:891–903.
- Molina-Cruz A, DeJong RJ, Ortega C, Haile A, Abban E, Rodrigues J, Jaramillo-Gutierrez G, Barillas-Mury C: Some strains of *Plasmodium falciparum*, a human malaria parasite, evade the complement-like system of *Anopheles gambiae* mosquitoes. *Proc Natl Acad Sci USA* 2012;109:E1957–E1962.

- 18 Jaramillo-Gutierrez G, Rodrigues J, Ndi-kuyeze G, Povelones M, Molina-Cruz A, Barillas-Mury C: Mosquito immune responses and compatibility between *Plasmodium* parasites and anopheline mosquitoes. *BMC Microbiol* 2009;9:154.
- 19 Blandin S, Shiao S, Moita L, Janse C, Waters A, Kafatos F, Levashina E: Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector *Anopheles gambiae*. *Cell* 2004;116:661–670.
- 20 Molina-Cruz A, Garver LS, Alabaster A, Bangiolo L, Haile A, Winikor J, Ortega C, van Schaijk BCL, Sauerwein RW, Taylor-Salmon E, Barillas-Mury C: The human malaria parasite *Pf*s47 gene mediates evasion of the mosquito immune system. *Science* 2013;340:984–987.
- 21 Williams MJ: *Drosophila* hemopoiesis and cellular immunity. *J Immunol* 2007;178:4711–4716.
- 22 Meister M: Blood cells of *Drosophila*: cell lineages and role in host defence. *Curr Opin Immunol* 2004;16:10–15.
- 23 Qiu P, Pan PC, Govind S: A role for the *Drosophila* Toll/Cactus pathway in larval hematopoiesis. *Development* 1998;125:1909–1920.
- 24 Chiu H, Ring BC, Sorrentino RP, Kalamariz M, Garza D, Govind S: dUbc9 negatively regulates the Toll-NF- κ B pathways in larval hematopoiesis and drosomycin activation in *Drosophila*. *Dev Biol* 2005;288:60–72.
- 25 Dearolf CR: Fruit fly leukemia. *Biochim Biophys Acta* 1998;1377:M13–M23.
- 26 Agaisse H, Petersen U, Boutros M, Mathey-Prevot B, Perrimon N: Signaling role of hemocytes in *Drosophila* JAK/STAT-dependent response to septic injury. *Dev Cell* 2003;5:441–450.
- 27 Owusu-Ansah E, Banerjee U: Reactive oxygen species prime *Drosophila* haematopoietic progenitors for differentiation. *Nature* 2009;461:537–541.
- 28 Luo H, Rose P, Barber D, Hanratty WP, Lee S, Roberts TM, D'Andrea AD, Dearolf CR: Mutation in the Jak kinase JH2 domain hyperactivates *Drosophila* and mammalian Jak-Stat pathways. *Mol Cell Biol* 1997;17:1562–1571.
- 29 Remillieux-Leschelle N, Santamaria P, Rands-holt NB: Regulation of larval hematopoiesis in *Drosophila melanogaster*: a role for the multi sex combs gene. *Genetics* 2002;162:1259–1274.