Mosquito cellular immunity at single-cell resolution

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Hemocytes limit the capacity of mosquitoes to transmit human pathogens. Here we profile the transcriptomes of 8506 hemocytes of Anopheles gambiae and Aedes aegypti mosquito vectors. Our data reveal the functional diversity of hemocytes, with different subtypes of granulocytes expressing distinct and evolutionarily conserved subsets of effector genes. A previously unidentified cell type in An. gambiae, which we term “megacyte,” is defined by a specific transmembrane protein marker (TM7318) and high expression of lipopolysaccharide-induced tumor necrosis factor–α transcription factor 3 (LL3). Knockdown experiments indicate that LL3 mediates hemocyte differentiation during immune priming. We identify and validate two main hemocyte lineages and find evidence of proliferating granulocyte populations. This atlas of medically relevant invertebrate immune cells at single-cell resolution identifies cellular events that underpin mosquito immunity to malaria infection.

Anopheles mosquitoes transmit Plasmodium parasites to humans and are responsible for an estimated 219 million cases of malaria, leading to more than 400,000 deaths annually (1). Parasites taken up by female mosquitoes from the blood of an infected human transform into motile ookinetes, which traverse the mosquito midgut and establish an infection. The mosquito’s immune system limits Plasmodium infection in several ways (2–4), and hemocytes (insect white blood cells) are key players in these defense responses (5, 6). Ookinite invasion triggers a strong nitration response in invaded midgut epithelial cells and their basal lamina (7, 8). Hemocytes that come in contact with a nitrated midgut basal lamina release microvesicles into the epithelial basal labyrinth and promote local complement activation, inducing parasite lysis (6). An infection with Plasmodium primes mosquitoes to mount a stronger immune response to subsequent infections (9). Primed mosquitoes release hemocyte differentiation factor (HDF) into the hemolymph (9); this factor consists of a complex of lipoxin A4 bound to evokin, a lipocalin carrier (10). HDF increases the proportion of circulating hemocytes of the granulocyte type (2), promotes microvesicle release, and enhances complement-mediated parasite lysis (6). Enhanced immunity is lost if HDF synthesis is blocked (10).

Three hemocyte types have been described in Anopheles gambiae morphologically (11). Granulocytes are highly phagocytic cells of about 10 to 20 μm in diameter. Oenocytoids are 8- to 12-μm round cells that produce melanin, an insoluble pigment involved in wound healing and pathogen containment by encapsulation. Prohemocytes are round cells (4 to 6 μm) with a high nuclear-to-cytoplasmic ratio and are thought to be precursors of the other two cell types. Hemocytes alternate between circulatory and tissue-resident (sessile) states (12, 13). However, the full functional diversity of mosquito hemocytes and their developmental trajectories has not been established, and it is not clear to what extent morphologically similar hemocytes are functionally equivalent.

In this study, we used single-cell RNA sequencing (scRNA-seq) to analyze the transcriptional profile of individual mosquito hemocytes in response to blood feeding or infection with Plasmodium. Circulating hemocytes were collected from adult An. gambiae M-form (An. coluzzii) females that were either kept on a sugar meal or allowed to feed on a healthy or a P. berghei–infected mouse (Fig. 1A). Transcriptomes from 5383 cells (collected 1, 3, and 7 days after feeding) revealed nine major cell clusters (Fig. 1B and fig. S1). Two clusters originate from adipose tissue [fat-body clusters (FBCs) 1 and 2] and one from muscle tissue (MusC) (Fig. 1, B and C). FBC1 cells express several immune-modulatory genes such as CLIPs (CLIP1A1, -7, -8, -9, and -14), LRIMs (LRIM1, -4A, -8A, -8B, -9, and -17), lectins (CTL4 and MA2), and SRRP2 (Fig. 1, B and C, fig. S2, and table S1), whereas FBC2 cells express high levels of vitellogenin, a canonical fat-body marker (14), after blood feeding. On the basis of their distinctive transcriptional profiles, we identified six hemocyte clusters (HCs) (Fig. 1, B and C). Hemocyte cluster 1 (HC1) has high mRNAs levels of prophenoloxidases, including PPO4 (Fig. 1, B and C) and PPO9, characteristic of oenocytoids. This expression profile is consistent with reported scRNA-seq data for 25 hemocytes (15). To select markers for major hemocyte lineages, we used bulk RNA-seq data from different tissues to identify hemocyte-specific genes (fig. S3 and tables S2 and S3). HC1 contains low levels of leucine-repeat protein 8 (LRR8) mRNA, whereas HC2 to HC4 have an inverse pattern (i.e., low or absent PPO4 and high LLR8 levels) (Figs. 1, B and C, and fig. S2).

In situ hybridization using PPO4 and LRR8 as markers revealed that the morphology of circulating HC1 (PPO4high/LRR8low) cells is typical of oenocytoids, with round cells that have few pseudopodia and granules (Fig. 2B), whereas the morphology of HC2 to HC4 (PPO4low/LRR8high) cells is typical of prohemocytes and granulocytes (Fig. 2B). HC2 and HC3 shared markers such as SPARC, cathepsin-L, and LRR8 (Fig. 1C). However, HC2 had 73% fewer unique molecular identifiers (UMIs) (mean UM1 of 413) than HC3 (mean UM1 of 1516), which suggests that HC2 cells are less differentiated and probably constitute prohemocytes. Our observation that cells of prohemocyte morphology did not express markers of the more differentiated HC1 or HC5 cells is consistent with this notion (Fig. 2B). HC3 cells have a typical granulocyte morphology, with prominent pseudopodia and abundant granules (Fig. 2B).

HC4 shares markers with HC3, and a correlation analysis shows these cells to be granulocytes (fig. S6C). However, HC3 cells are also characterized by a specific subset of markers (Fig. 1C). HC4 cells express cyclin B, aurora kinase, and other mitotic markers, which suggests that they are proliferating hemocytes (fig. S6, C to E). Cells in HC5 and HC6 both are negative for PPO4 (Figs. 1C and 2A). HC5 cells express high levels of an uncharacterized transmembrane protein AGAAP007318 (TM7318) and lipopolysaccharide-induced tumor necrosis factor–α transcription factor 3 (LL3) (Figs. 1C and 2A), whereas HC6 is negative for those markers and expresses antimicrobial peptides such as defensin 1, cecropins 1, and C-type lysozyme (Fig. 1C). Cells in HC5 and HC6 have both low levels of LRR8 and no PPO4 but have two distinct morphologies (Fig. 2, A and B). Cells negative for TM7318 (HC6) are small granulocytes that express antimicrobial genes (AM Gran) (16.4% of granulocytes), whereas TM7318-positive cells (HC5) are in low abundance (0.5% of granulocytes) and represent a separate giant cell type (25 to 40 μm) that we named “megacytes” (Fig. 2B).

To investigate the differentiation dynamics of An. gambiae hemocytes, we reclustered the cellular transcriptomes at higher resolution and performed lineage-tree reconstruction

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The granulocyte lineage tree should be treated with caution owing to the paucity of distinctive markers. Prohemocytes are proposed to be stem cell precursors of both granulocytes and oenocytoids (II). However, oenocytoids are transcriptionally disconnected from other hemocyte subtypes, and we did not observe transcriptional markers of cell proliferation in oenocytoids, which suggests that they may represent a separate lineage originating from either larval stages or other adult tissues. Alternatively, oenocytoids could be derived from granulocytes (Fig. 1B), but the differentiation rate may be very low when melanization responses are not elicited, which could result in too few cells at intermediate stages of differentiation to be captured in our transcriptomic analysis.

To assess which of the newly discovered putative cell types are shared between anopheline and culicine mosquitoes, we analyzed the transcriptomes of 3123 hemolymph cells from *Aedes aegypti*, a vector for several viral diseases including yellow fever, dengue, chikungunya, and Zika. As with *Anopheles*, a dimensional reduction plot shows both canonical hemocytes and other cell types with mostly fat-body signatures (Fig. 3A and fig. S7). A cross-species correlation analysis reveals two clusters (AaHC1 and AaHC2) with conserved transcriptome signatures for oenocytoids (99 and 77% correlation, respectively, with AgHC1) (Fig. 3, A and B) and different granulocyte types, including antimicrobial peptide–expressing cells (94% with AgHC6) and proliferating granulocytes (87% with AgHC4) (Fig. 3, A and B, and table S4). Granulocytes and prohemocytes are again positioned along a continuum of transcriptomic similarity, with four different cell states, including a proliferating S-phase granulocyte cluster (AaHC9) without a clear *Anopheles* equivalent (Fig. 3, A and B). Granulocytes express laminins, leucine-rich repeat proteins, scavenger receptors, Toll-like receptor 5, and the transcription factor Rel2 (fig. S8 and table S5). However, megacytes (AgHC5) lack an obvious counterpart in *Aedes*, and their characteristic marker (TM7318) is present only in anophelines of the *Cellia* subgenus (malaria vectors in Africa and Asia; fig. S9).

The transcription factor LL3 can be detected in granulocytes from *Plasmodium*-infected *An. gambiae*, and silencing LL3 expression disrupts priming (20). However, it is not clear whether LL3 is essential for HDF synthesis or for hemocytes to respond to HDF. We found that LL3 is highly expressed in megacytes (HC5) (Figs. 1C and 4A) and explored whether silencing LL3 affects the HDF response. Transfer of hemolymph from primed *An. gambiae* donors had HDF activity and elicited a strong priming response in control recipients injected with *lacZ* double-stranded (ds) RNA, resulting in a prominent increase in circulating granulocytes, a modest increase in oenocytoids, and a decrease in prohemocytes (Fig. 4B and tables S6 to S9). This response was abolished when LL3 expression was silenced in the recipients.
by injection of dsLL3 RNA (Fig. 4B and tables S6 to S9). We cannot rule out the possibility that hemocytes other than megacytes express LL3 at levels below the detection limit of scRNA-seq, which could have led to the notion that LL3 is up-regulated more broadly in granulocytes after Plasmodium infection (20), in which case LL3 silencing might directly control hemocyte differentiation in response to HDF. However, the discovery of megacytes expressing high levels of LL3 as a defining feature now raises the possibility that this cell type plays a key role in orchestrating hemocyte responses to HDF.

The proportion of circulating granulocytes is low (1 to 3%) under normal conditions but increases in response to Plasmodium infection (2), in which case LL3 silencing might directly control granulocyte proliferation, differentiation from prohemocytes, or mobilization of sessile hemocytes was unknown. Transmission electron microscopy of sugar-fed mosquitoes showed individual sessile hemocytes bathed by hemolymph and attached to the basal lamina of the tissues through pseudopods (Fig. 4C), indicative of a dynamic and potentially transient association.

Using whole-tissue–mount in situ hybridization, we found that most sessile hemocytes are PPO4low/LLR8high granulocytes (89.3 ± 6.2% SEM), whereas PPO4high/LLR8low oenocytoids are less abundant (4.2 ± 3.1% SEM) and TM7318–positive megacytes are even rarer (2.7 ± 2.3% SEM) (Fig. 4, D and E, and tables S10 and S11). Furthermore, we found a reduction of sessile PPO4low/LLR8high granulocytes in response to Plasmodium infection (P < 0.0001, Welch
granulocytes are closely related cells, and we
transcriptome. We show that prohemocytes and
cyte types in mosquitoes (oenocytoids and
firms the existence of two canonical hemo-
culating granulocyte numbers in response to
differentiation can all contribute to boost cir-
activation and proliferation, and prohemocyte
Combined, our results suggest that hemocyte
crease in FBN29831-positive cells (fig. S11).
LLR8low oenocytoids (Fig.
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megacytes, or TM7318

Fig. 4. An. gambiae cellular immune responses to Plasmodium infection. (A) UMAP visualization of all hemocytes by LL3 expression. Cells with more than 1 UMI are shown in red. The arrow denotes a megacyte cluster. (B) Percentage of circulating granulocytes, oenocytoids, and prohemocytes of LL3-silenced mosquitoes injected (+) or not (−) with HDF versus double-stranded lacZ RNA—
jected mosquitoes used as negative controls (mean ± SEM, ****P < 0.0001, unpaired t test; two independent experiments), ns, not significant. (C) False-color transmission electron microscopy images depicting an An. gambiae (M form) granulocyte (purple) attached via pseudopodia (inset) to the abdominal fat body (blue). Scale bar: 1.5 μm. (D) RNA in situ hybridization of a longitudinal section (top) or carcass whole-mounts (bottom) of control blood-fed (CI) and P. berghei–infected (Inf) An. gambiae mosquitoes. (Top) Glyceraldehyde phosphate dehydrogenase, green; LRR8 in red. (Bottom) LRR8, green. Scale bars: 20 μm. (E) Quantification of Anopheles hemocytes attached to the mosquito fat body with blood feeding or P. berghei infection, normalized by surface area of fat bodies. All, all hemocytes; Oen, oenocytoids; Gra, granulocytes; Meg, megacytes; AM Gr, antimicrobial granulocytes (mean ± SEM, ****P < 0.0001, Welch t test; three independent experiments). (F) Percentage of circulating oenocytoids (LRR8+PPO4+) and (G) granulocytes (LRR8+PPO4+) positive for FBN29831 in control (C) and P. berghei–infected (I) mosquitoes 48 hours after feeding (right panels), n, number of cells examined. Representative RNA in situ hybridization images of oenocytoids (F) and granulocytes (G) with low and high expression of FBN29831 (left panels). Actin, green; FBN29831, magenta; nuclei, blue. Scale bars: 5 μm (****P < 0.0001, χ2 test; two independent experiments).

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The conservation of diverse and molecularly well-defined hemocyte types between distantly related mosquito genera and the apparent absence of megacytes in our Ae. aegypti mosquito dataset raise questions as to how the immune systems of these mosquito species have evolved to limit their capacity to transmit parasites and arboviruses to humans. This knowledge will ultimately underpin immunological strategies aimed at interrupting disease transmission by rendering mosquitoes resistant to such pathogens.
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SUPPLEMENTARY MATERIALS

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Materials and Methods

Figs. S1 to S11

Tables S1 to S18

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