Unbiased classification of mosquito blood cells by single-cell genomics and high-content imaging


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Mosquito blood cells are immune cells that help control infection by vector-borne pathogens. Despite their importance, little is known about mosquito blood cell biology beyond morphological and functional criteria used for their classification. Here, we combined the power of single-cell RNA sequencing, high-content imaging flow cytometry, and single-molecule RNA hybridization to analyze a subset of blood cells of the malaria mosquito Anopheles gambiae. By demonstrating that blood cells express nearly half of the mosquito transcriptome, our dataset represents an unprecedented view into their transcriptional program. Analyses of differentially expressed genes identified transcriptional signatures of two cell types and provide insights into the current classification of these cells. We further demonstrate the active transfer of a cellular marker between blood cells that may confound their identification. We propose that cell-to-cell exchange may contribute to cellular diversity and functional plasticity seen across biological systems.

Significance

Mosquito blood cells are central players of immunity against the vector-borne pathogens that devastate the lives of millions of people worldwide. However, their molecular identity and classification remain controversial. By applying single-cell RNA sequencing and high-content imaging flow cytometry, we defined the molecular fingerprint of a subset of mosquito blood cells and characterized two transcriptionally distinct blood cell populations that resemble previously described cell types. Surprisingly, cell population analyses at a single-cell level uncovered an active molecular transfer between the two cell types that may contribute to cellular diversity and plasticity seen across biological systems.


The authors declare no conflict of interest.

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Data deposition: The sequences reported in this paper have been deposited in the European Nucleotide Archive (accession no. PRJEB23372). Analyses were performed in R, and scripts are available on Github (https://github.com/mssevero/hemo-scRNASeq). The expression data can be accessed at https://svb.sanger.ac.uk/#base/main for single gene visualization.

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stain only weakly for reactive nitrogen and oxygen species, but all cell types can bind lectins (9). A few studies have also identified mosquito plasmocytes as a cell type distinct from granulocytes due to their fibroblast-like shape, lack of granules, and a well-developed RER (8, 21). Although an increase in hemocyte numbers was reported upon blood feeding and infection (23, 29, 31), the pathways underlying their differentiation into these classes remain unknown. Whether the current classification represents true cell types or states, and if cell subpopulations exist, are also yet to be explored. In mammals, single-cell transcriptomics has recently begun to tackle similar questions. It is increasingly evident that cell populations long considered to be of the same type display significant functional differences and considerable variability in gene expression (32–34). The use of single-cell approaches to explore cellular heterogeneity in nonmodel organisms holds the promise to uncover unforeseen complexity and to identify cellular populations that would be undetectable in bulk measurements.

Here, we unravel the molecular fingerprint of a subset of mosquito blood cells at a single-cell level and show that naive hemocytes express nearly half of the mosquito transcriptome. By applying fluorescence-activated cell sorting (FACS), single-cell RNA sequencing (scRNA-seq), and high-content imaging flow cytometry, our unparalleled study identifies two distinct cell populations that resemble granulocytes and oenocytoids. Our findings further reveal active molecular exchange between blood cells and the presence of extracellular vesicles (EVs) in the mosquito hemolymph. Altogether, our study contributes molecular insights into the established classification of mosquito blood cells.

**Results**

**scRNA-Seq of Blood Cells from PPO6::RFP Transgenic Mosquitoes.**

We chose to explore mosquito blood cells using a transgenic strain expressing a red fluorescence reporter (tdTomato, herein RFP) under the control of the prophenoloxidase 6 (PPO6) melanization-related gene (PP06::RFP) (35). Melanization is a well-established immune response of invertebrates that controls infection against bacteria and parasites (36–39). Several reports suggest that melanization is mostly mediated by a specific cell population called oenocytoids, which represents ~10% of the blood cells. We focused on cells obtained in the absence of infection or blood feeding as a baseline for analysis of cell-to-cell variation. We first confirmed that RFP-positive hemocytes were present in the circulation by the identification of cells displaying RFP fluorescence in hemolymph perfusate (Fig. 1A). RFP signal was also observed in the hemocytes attached to the inner abdominal wall of dissected adult female mosquitoes, where fat body cells are most prominent (Fig. 1B, arrow). As expected, the hemolymph perfusate was heavily contaminated with a mixture of cells and subcellular/tissue debris (9). To purify live RFP-positive cells, we developed a FACS approach based on RFP expression and Hoechst nuclear staining and validated our method by microscopic inspection of sorted cells. The sorted cell population corresponded to 0.1% of the total events measured (n = 100,000) in the perfusate of at least 10 mosquitoes (Fig. 1C and D). This is in accordance with previous work indicating that only a small subset of adult mosquito hemocytes produces PPO (39). We FACS-sorted single blood cells and performed scRNA-seq to capture the transcriptome of single PPO-producing blood cells (Fig. 1E). Hemocytes were sorted into a 96-well plate and, after sample processing and quality assessment, we obtained successful cDNA amplification for 56 single cells in addition to two pools of 30 cells each, from which 28 high-quality cDNA libraries were sequenced, representing 26 single hemocytes and the two pooled samples (SI Appendix, Fig. S1 A–D). As a single mosquito can contain as few as 500 blood cells in the circulation (23, 40), we believe the small number of cells analyzed reflects a combination of technical limitations inherent to our approach. Mosquito blood cells reportedly vary in size from as little as 2 to 20 μm (8–10, 41), and variability in cell size can affect RNA recovery, as small cells contain small amounts of RNA. The adaptation of the protocol to the study of an invertebrate system (e.g., the choice of lysis buffer and chemistry) may also have influenced our results, especially since different biological cell types show distinct technical quality features in scRNA-seq (42). A small number of cells have, nevertheless, been used in other scRNA-seq studies (43, 44), and this does not preclude identification of cellular types when an adequate sequencing depth is used. We therefore prioritized the deep sequencing of individually curated, very high-quality samples representing a small subset of cells obtained ex vivo.

Our sequencing generated on average 4.5 million reads per sample, well-above the minimum of one million reads previously suggested as a requirement for adequate single-cell studies (45). Over 70% of the reads were successfully mapped, with exonic reads comprising of more than 40% (SI Appendix, Fig. S1E and Table S1). All samples achieved saturation at 2 million reads, comparable with that previously observed for mammalian cells (45). For further analyses, we discarded one cell as it showed gene expression suggestive of a doublet (SI Appendix, Fig. S1F). Doublets have been reported in the circulation of adult female mosquitoes (29) and may fall within a size range comparable with larger hemocytes. Around 3,800 genes were detected in each pool whereas single cells expressed between 450 and 1,400 genes. This large transcriptional variability likely results from the diverse functions hemocytes exhibit as they circulate in the open body cavity, exposed to biochemical changes and in close contact with internal organs. This is also in agreement with recent findings of cellular heterogeneity in subtypes of immune cells of other organisms, such as humans (46), mice (47), and fish (48).

Similar expression profiles were obtained for pools and single cells with comparable numbers of detected genes (1,100 and 1,200 genes, respectively) (Fig. 1F and G). The marker genes used for FACS sorting (PPO6 and tdTomato) were identified in both single cells and pools (Dataset S1), confirming the efficiency of our method. Altogether, our results showed that the transcriptome of mosquito hemocytes comprises over 6,000 genes, of which more than half (3,022) had not been identified in earlier studies (SI Appendix, Fig. S1G) (24, 25). As previous studies were based on pooled samples, their datasets were also compared with our pools and over 2,200 genes were identified only by our scRNA-seq approach (SI Appendix, Fig. S1H). In both single cells and pools, the absolute number of revealed sequences for over 80% (n = 914) of the proteins reported by an earlier proteomics study based on magnetic beads isolation of Anopheles gambiae phagocytes (26) (SI Appendix, Fig. S1J). Most of the identified genes were present in more than one cell, but a few genes were detected in only one cell (SI Appendix, Fig. S1J). About 140 genes were found in 90% of the cells sequenced (Dataset S2), and gene ontology (GO) analyses indicated that this core transcriptome represents metabolism, biosynthesis, translation, and immune defenses (Datasets S5 and S6).

Mosquito hemocyte biology has been mostly studied in the context of immunity. We thus inspected our dataset for previously identified immune genes. Naïve hemocytes expressed low levels of such immune genes as the transcription factors REL1 (AGAP009515) and REL2 (AGAP006747), Cactus (AGAP007938), IsbI (AGAP009166) and IsbY (AGAP005933), and the receptors PGR-P-LC (AGAP005203) and PGRPS-I (AGAP008536) (Dataset S1). Components of the complement cascade [e.g., TEPI (AGAP010815), APLIC (AGAP007033), LRMI (AGAP006348), and HPX2 (AGAP009033)] were also detected in some cells, along with the LPS-induced TNF receptor family member (LITAF)-like 3 (AGAP009053) described to control Plasmodium survival in the gut (28). The phagocytic and antibacterial activities of these cells can be illustrated by the expression of Eater (AGAP012386), Ninjurin (AGAP006745), and Ninnod (AGAP009762), alongside that of several fibrinogen-related proteins (FREPs/FBNs), such as FBN8.
Identification of Blood Cell Populations. To account for the technical noise arising from the small amounts of RNA, we included in our samples External RNA Controls Consortium (ERCC) spike-ins before cDNA amplification (55). We analyzed the percentage of ERCC and mitochondrial counts as a proxy for sequencing efficiency, RNA degradation, or incomplete lysis and potential cell death. As anticipated, variation was observed (SI Appendix, Fig. S1 K–L), but caution was taken in applying these criteria and attributing them biological meaning because variability could have arisen from true cell type-related processes. As differences in the total number of expressed genes could also have stemmed from different morphologies and cell types, we manually curated their individual mappings to confirm that the samples corresponded to potentially true representations of blood cells. To estimate technical noise, we applied the variability threshold based on the square of the coefficient of variation (CV2) of the ERCCs (56) and identified 148 genes whose expression exceeded the threshold (Fig. 2A). These highly variable genes included scavenger receptor, fibronectin-related, and leucine-rich repeat-containing proteins, as well as genes involved in vesicle transport, metabolism, and transcription (Dataset S4). No genes directly associated with cell cycle had high variability although several cyclin genes were detected in specific cells (Dataset S1), corroborating previous reports of the potential of mosquito hemocytes to undergo cellular division (23, 29, 41).

Hierarchical clustering of the variable genes based on pairwise Pearson correlation suggested the presence of at least two groups of cells (Fig. 2B). Principal component analysis (PCA) also yielded two cell populations, supporting our clustering (Fig. 2C). Interestingly, PPO6 showed high variability, and the overlay of PPO6 expression onto the PCA plot suggested that the two clusters were largely characterized by low and high expression of PPO6. PCA analysis in the absence of outlier cells confirmed the existence of the cell groups (SI Appendix, Fig. S2A). Comparable results were also found when the whole dataset was used for similar analyses (SI Appendix, Fig. S2 B and C). Differences in the PPO6 expression have been previously described by immunofluorescence microscopy (41) but have not been associated with cell types. Moreover, from the 10 PPO genes encoded in the

normalized read counts from pools and single cells. r2 indicates Pearson correlation. (G) Venn diagram of genes detected in single cells and pools (normalized count ≥ 1). (Scale bars: A, 10 μm; B, 20 μm; D, 5 μm.) DNA is stained with DAPI (A and B) and Hoechst (D).
characterization of PPO6 cells represents transcriptionally distinct datasets. Among the highly variable genes, we detected PPO6 and LysI and signal. Fig. 3 shows that high expression levels of FBN10 (left) were observed in PPO6 cells whereas metabolism and RNA processing defined the PPO6 subset (datasets S5 and S6). Although not significant, PPO6 cells appeared to express more genes in total, but mitochondrial counts did not differ between the groups (SI Appendix, Fig. S2). We next compared the overall gene expression between PPO6high and PPO6low cells. Based on differentially expressed genes, GO analyses uncovered that melanization characterized PPO6high cells whereas metabolism and RNA processing defined the PPO6low subset (datasets S5 and S6). Although not significant, PPO6low cells appeared to express more genes in total, but mitochondrial counts did not differ between the groups (SI Appendix, Fig. S2).

**PPO6high and PPO6low Cells Represent Transcriptionally Distinct Subpopulations.** Among the highly variable genes, we detected several FBN sequences, such as FBN8 and -10. PPO6high cells showed high expression levels of FBN10 (Fig. 3A, Left) whereas PPO6low cells exhibited weak or lack expression of FBN8, -10, and -30 (Fig. S4). Although below the ERCC-defined variability threshold, likely due to the small number of cells analyzed, expression of the antimicrobial peptide gene lysozyme type I (LysI) (AGAP011119) was more characteristic of PPO6low cells (Fig. 3A, Middle). In the search for a panhemocyte marker, we also identified expression of phagocytic receptor Nimrod in both cell groups (Fig. 3 A, Right). The siRNA results were validated by single-molecule RNA fluorescence in situ hybridization (RNA-FISH), showing coexpression of tdTomato and PPO6 in all PPO6:RFP hemocytes, with no detection of tdTomato in blood cells isolated from WT mosquitoes (SI Appendix, Fig. S3 A and B). RNA-FISH accurately distinguished PPO6high and PPO6low hemocytes. Consistently, PPO6high cells showed high levels of FBN10, which were very low or absent in PPO6low cells. High levels of LysI were found in PPO6low cells, reinforcing the presence of PPO6/FBN10/LysIhigh cells; and Nimrod transcripts were observed in all perfused hemocytes (Fig. 3B). We took advantage of the high conservation of PPO6, LysI, and Nimrod genes in the closely related mosquito species Anopheles stephensi to examine the discovered blood cell groups in other anopheline mosquitoes. We detected PPO6high and PPO6low/LysIhigh cells, along with a low levels of Nimrod expression. No FBN10 was observed (SI Appendix, Fig. S3C), probably due to the specificity of the probe to A. gambiae and the large diversity of this gene family.

Fig. 2. Identification of mosquito blood cell subpopulations. (A) The expression variability of individual genes measured by the squared coefficient of variation (CV²) is plotted against the mean expression level (normalized counts). Magenta points indicate mosquito genes showing higher than expected expression variability compared with ERCC spike-ins (blue) (adjusted P value of <0.1). The red line is the fitted line of the spike-ins, and the dashed line (pink) marks the margin for genes with 50% biological CV. (B) Pearson correlation heat map of single hemocytes based on the expression of the highly variable genes identified in A. Correlation suggests the presence of two groups of cells (red and yellow). (C) PCA plot based on the highly variable genes. The first two principal components are shown, and each point represents one single hemocyte. Two clusters were identified and correspond to the subgroups in B. PPO6 expression, as log10 (normalized counts +1), is overlaid onto the PCA plot. (D) Violin plots of PPO6 and RFP expression in the identified groups.

**Fig. 3.** Characterization of PPO6high and PPO6low cell subpopulations. (A) Violin plots of the expression of putative population and panhemocyte markers. (B) RNA-FISH validation of identified PPO6high and PPO6low cell subpopulations in perfused cells based on markers shown in A. Cells were classified as PPO6high (Upper) or PPO6low (Lower) according to the expression of PPO6 (red). Arrows indicate lower PPO6 signal. (C-C’) PPO6high and PPO6low cell subpopulations can also be seen as tissue-resident blood cells attached to the inner abdominal wall of female mosquitoes. Arrow and arrowhead indicate PPO6high and PPO6low cell subpopulations, respectively. C’ shows higher amplification of the C’ boxed area. (Scale bars: B, 5 μm; C and C’, 50 μm; C’ and C”, 5 μm.) DNA is stained with DAPI.
Fig. S3D). These findings suggest that PPO\textsuperscript{high} cells are specialized for melanization responses, expressing genes involved in these processes at very high levels, whereas PPO\textsuperscript{low} cells execute a broader range of biological tasks. The identified differences between the cell groups may represent different cell lineages, mediate diverse melanization processes [e.g., metamorphosis and cuticle sclerotization (57–59)], or reflect localization patterns of the cells inside the mosquito body (29). To assess whether differences were related to tissue residency, we performed RNA-FISH in tissues and observed both cell populations in close contact with the fat body cells within the abdominal wall with no conspicuous cell clusters. The majority of the sessile cells were positive for Nimrod, independent of PPO expression (Fig. 3C), indicating that Nimrod is a potential marker for both circulating and tissue-resident blood cells. Altogether, these results demonstrate that both circulatory and tissue-resident hemocytes display transcriptional heterogeneity and that PPO\textsuperscript{high} and PPO\textsuperscript{low} cell populations are present in two mosquito species.

**PPO\textsuperscript{high} and PPO\textsuperscript{low} Cells Share Functional and Morphological Features.** Mosquito blood cells are separated into three classes—granulocytes, oenocytoids, and prohemocytes. Our GO analyses suggested the presence of a PPO-specialized cell population and a second cell subset of a less specific nature. We reasoned that PPO\textsuperscript{high} and PPO\textsuperscript{low} cell groups could be representatives of oenocytoids and granulocytes, respectively. As phagocytosis is a hallmark of granulocytes, we first explored functional differences using magnetic bead uptake as a means for “phagocyte” isolation, as suggested before (26). To this end, we injected mosquitoes with magnetic beads and either allowed them to rest at 28 °C before perfusion or incubated the mosquitoes at 4 °C to inhibit phagocytosis. To our surprise, both PPO\textsuperscript{high} and PPO\textsuperscript{low} cells were identified among magnetically isolated cells and under both conditions, suggesting that, instead of phagocytosis, both cell types endocytosed the beads (Fig. 4A, arrows). We next compared the gene profiles of PPO-producing cells to the proteomics results obtained by Smith et al. (26) using magnetic bead isolation. Our analyses revealed that similarities were the strongest when profiles were compared across all samples—PPO\textsuperscript{high}, PPO\textsuperscript{low}, phagocytes, and all cells. PPO\textsuperscript{high} and PPO\textsuperscript{low} shared expression of more genes with phagocytes when considered together rather than alone, indicating that both PPO\textsuperscript{high} and PPO\textsuperscript{low} cell types shared similarities with “phagocytes” at the gene/protein level (Fig. 4B). In agreement, nearly all PPOs were present in all samples (Fig. 4B). Based on this assay, we did not detect functional differences between the groups as both PPO\textsuperscript{high} and PPO\textsuperscript{low} performed endocytosis. This was in agreement with the identification of endocytosis-related genes in the transcriptome (Dataset S1) and with a previous observation that oenocytoids can internalize beads and bacteria (16).

Next, we sought to investigate the morphology of the cell populations using imaging flow cytometry and RFP fluorescence as a proxy for PPO\textsuperscript{high} expression. We measured a series of morphological features of 519 single RFP-positive cells, which were divided into RFP\textsuperscript{high} (PPO\textsuperscript{high}, n = 58) and RFP\textsuperscript{low} (PPO\textsuperscript{low}, n = 261) based on their fluorescence intensity (Fig. 4 C and D and SI Appendix, Fig. S4). Overall, RFP-positive cells had a mean area of 67 μm\textsuperscript{2}, ranging from 18 μm\textsuperscript{2} to nearly 140 μm\textsuperscript{2}. These measurements are in accordance with the reported cell sizes (9, 23, 41). Similar to recent studies based on flow cytometry of fixed cells (23, 41), we did not detect the cells of 2 μm in size described by other research groups based on label-free light microscopy alone (27, 60). When comparing the cell groups in terms of bright-field measurements of their cytoplasm, PPO\textsuperscript{low} cells showed smaller area, width, and minor axis than PPO\textsuperscript{high} cells (Fig. 4E and SI Appendix, Fig. S4). Our expectation was to find that PPO\textsuperscript{high} comprised oenocytoids: i.e., spherical cells with weak or no granularity. To our surprise, no differences between the groups were detected in granularity or cell shape, and PPO\textsuperscript{high} and PPO\textsuperscript{low} cells were equally circular. (SI Appendix, Fig. S4 and Dataset S7). Cells from both groups also displayed an elongated shape, typical of the cytoplasmic extensions seen in fusiform or spindle-shaped cells. This shape is characteristic of plasmatocytes described in other insects and mosquito species (2, 8, 21). These results failed to assign RFP-positive cells to any of the groups previously characterized based on light microscopic morphological and ultrastructural analyses.
granulocytes, plasmocytes, or oenocytoids. In fact, the highest discriminating factors (Fisher’s linear discriminant) separating PPO6high and PPO6low subpopulations relied on RFP intensity alone, with bright-field parameters scoring poorly and failing to establish a morphological distinction between the cells (Dataset S7). Importantly, our imaging flow cytometry approach relied on morphological analyses of cells in suspension, which is unbiased and likely more relevant for the identification of the cellular types found in the hemolymph circulation. Fluorescence microscopy upon cell attachment reinforced our finding that elongated cells are found in both groups, with no particular cell shape being attributed to either group (SI Appendix, Fig. S5A). Round and oval cells were also observed as PPO6high and PPO6low using anti-PPO6 antibodies. PPO6 and RFP signals overlapped, but we occasionally identified cells expressing spotty and cytoplasmic PPO6 patterns that did not show any RFP signal (SI Appendix, Fig. S5B), suggesting differences in regulation or stability between the mRNAs and/or proteins.

In addition to intensity, the cell groups differed in their RFP area. PPO6high cells displayed an overall cytoplasmic distribution of the RFP signal whereas a more localized globular signal was detected in the cytoplasm of PPO6low cells (Fig. 4D and E). Microscopic examination also revealed that nearly half of the cells from both groups displayed internal structures and “budding” extensions of the cytoplasm suggestive of vesicles (Fig. 4F and G, arrowhead and arrow, respectively). To confirm that, we performed correlative scanning electron microscopy (SEM) and demonstrated the presence of membrane protrusions or “blebs” in RFP-positive cells (SI Appendix, Fig. S6C). Altogether, these results established that morphological plasticity of the mosquito blood cells is independent from their transcriptional profile and that mosquito blood cells have membrane vesicles and protrusions.

**Mosquito Blood Cells Exchange Molecular Information.** We were puzzled by the possibility that the RFP signal analyzed in our imaging flow cytometry could have originated from RFP-positive vesicles. Earlier reports used DiD, a lipophilic cyanine dye, to label both mosquito hemocytes and hemocyte-derived vesicles (61, 62). To test whether the localized RFP signal seen in our imaging was associated with vesicles, we first stained PPO-producing cells with DiD and observed that RFP-positive cells indeed contained DiD-positive membrane-bound and internal vesicles that were both RFP-positive and negative (SI Appendix, Fig. S6B and C). To identify EVs in the mosquito circulation, we performed imaging flow cytometry using DiD and a recently published approach (63). Both DiD-positive cells and EVs could be identified in hemolymph perfusate (Fig. 5A and B). EVs were detected based on their size, weak dark-field and positive DiD fluorescence, with a few EVs also displaying weak RFP signal (Fig. 5B, arrowhead). The degree of DiD intensity differed between cells and did not depend on RFP fluorescence. Differential centrifugation followed by EM confirmed the presence of EVs in hemolymph perfusate of naïve female mosquitoes (Fig. 5C). SEM of perfused cells also revealed that vesicles of different sizes and shapes, corresponding to the different vesicle types described in the literature—exosomes, microvesicles, and apoptotic vesicles (64)—could be indeed observed in association with naïve mosquito blood cells (SI Appendix, Fig. S6D). These findings suggested that EV production is a general phenomenon that is not limited to PPO-associated cells.

A growing body of evidence has demonstrated that RNA can be transferred between mammalian cells. As RNA can be found in EVs and our data showed that EVs are present in the mosquito hemolymph, we explored whether a potential exchange between PPO-positive and negative cells could be responsible for the identification of PPO6high and PPO6low cells. Strengthening this idea were the observations that (i) our scRNA-seq results uncovered cells with minute levels of PPO6 and RFP transcripts and (ii) expression of PPO6 by RNA-FISH was detected inside budding extensions associated with PPO6-positive cells (Fig. 5D). To test whether RFP mRNA can be transferred between naïve transgenic and WT blood cells, we developed a transwell assay using blood cells from PPO6::RFP transgenic and WT mosquitoes (Fig. 5E). Remarkably, after exposure to hemolymph perfusate from transgenic mosquitoes, RFP transcripts were observed by RNA-FISH inside WT blood cells (Fig. 5F, arrowhead). This result indicated that RFP mRNAs can be shuttled between blood cells and might account for the PPO6low population identified by our scRNA-seq and imaging. Taken together, our findings demonstrated that molecular exchange...
between cells, likely via EVs, impacts their transcriptional profile. As EVs have been shown to carry lipids, proteins, and RNA and can be secreted by virtually all cells, our results revealed an unappreciated role of intercellular molecular exchange in defining cellular identity.

**Discussion**

Understanding how transcriptional networks influence cell identity is a central problem in modern molecular biology. Our study describes mosquito blood cells as a source of key components of immunity, development, and tissue homeostasis and places them as a central hub coordinating mosquito biology at different levels. Using a combination of single-cell genomics and imaging, we reveal that hemocytes display an unexpected degree of complexity where two transcriptionally defined cellular “populations” suggestive of distinct cell types share morphological and functional features. We also demonstrate that mosquito blood cells exchange mRNA, leading to the detection, by RNA-FISH, of an “exogenous” gene in acceptor cells. Altogether, our results contribute insights into cellular cross-talk and cell type classification, in addition to illustrating the power of single cell-based approaches in discovering unappreciated events at the core of biological processes.

Using single-cell RNA sequencing, we describe the baseline expression of a mosquito blood cell in exceptional detail. An average mosquito blood cell under resting conditions expresses ~1,000 genes, or 7% of the mosquito transcriptome. In total, about half of the genes currently annotated in the mosquito genome were detected by RNA sequencing of naïve, unstimulated mosquito hemocytes. Our dataset represents a substantial gene expression resource for further studies of tissue-specific alternative splicing, RNA editing, and gene and transcript models. It also illustrates the importance of tissue-specific approaches and paves the road toward the detailed mapping of gene expression in cells and tissues of insects, with the ultimate goal of creating a comprehensive reference atlas of cellular diversity.

By successfully applying single-cell RNA sequencing to the study of mosquito blood cells, we demonstrated proof of the existence of at least two transcriptionally distinct cell groups that are similar to currently defined cell types. PPO6
transcripts that coordinate cell-to-cell and tissue communication not only in infection but also under physiological conditions. Disturbance of homeostasis, be it by infection, metabolic changes, tissue damage, or stress, may escalate secretion of vesicles containing virus-derived siRNAs have been identified in *Drosophila* and contribute to systemic antiviral immunity (73). Apoptotic vesicles released by hemocytes in the vicinity of invading parasites have been implicated in anti-*Plasmodium* responses by activating the complement pathway in *A. gambiae* mosquitoes (61). Interestingly, using a GFP reporter strain, Volohonsky et al. (74) reported that the antimalaria mosquito complement-like factor TEP1 is predominately expressed in the fat body as a transcript, but, at the protein level, it is found in hemocytes upon blood feeding and infection (74). The authors speculate that this is due to the uptake by the blood cells of TEP1 attached to bacterial cells. As in our sequencing only one cell contained low levels of TEP1, we suggest that EV-mediated delivery of TEP1 (mRNA or protein) may better explain these findings. We propose that vesicles found in the mosquito hemolymph contain proteins and transcripts that coordinate cell-to-cell and tissue communication not only in infection but also under physiological conditions. Disturbance of homeostasis, be it by infection, metabolic changes, tissue damage, or stress, may escalate secretion of vesicles containing an array of different cargo that can be targeted to specific tissues and complement systemic responses. We believe that, similar to how environment, microbiota, and genetic make-up influence phenotypic variation, cellular exchange can also drive cellular identity and represents an inventive and unexplored way through which nature coordinates who and what we are.

**Materials and Methods**

**Mosquito Rearing, Fluorescence Microscopy, and Hemolymph Perfusion.** *A. gambiae* sensu lato PPO6:RFP transgenic and WT strains were reared at 28 °C under 80% humidity and a 12/12-h day-night cycle. Larvae were fed with cat food, and adult mosquitoes were fed ad libitum with 10% sugar. For tissue microscopy, mosquitoes were dissected in 1x PBS, fixed in 4% parafomaldehyde (PFA), washed, and mounted using Vectashield mounting medium containing DAPI. For hemolymph perfusion, 3- to 5-d-old female mosquitoes were anesthetized, and 1-μl injection of exogenous PPO6::RFP transcripts, and potentially other PPO genes, contribute to the function of acceptor cells is another exciting question. PPO proteins lack the signal peptides required for their secretion, and it has been suggested that PPO6 is secreted by exocytosis as cell rupture has not been observed (41). It is plausible that PPO transcripts are shed by PPO6cells and processed by PPO6lownegative cells that locally activate melanization only under specific conditions: e.g., upon infection with specific pathogens or during wounding and tissue repair. Molecular signals exchanged between cells can, thus, coordinate cellular plasticity and contribute to the diversity of functional subsets or “hybrid” cells that express markers of different or multiple cell types.

Although we cannot rule out that RFP mRNAs were transferred between mosquito cells by means other than EVs, the demonstration of mosquito blood-borne EVs indicates that different cells and tissues likely communicate through vesicles secreted into the insect open circulatory system. Several recent reports have suggested EV-mediated immune responses in dip- teran insects. Exosome-like vesicles containing virus-derived PPO6low have been identified in *Drosophila* and contribute to systemic antiviral immunity (73). Apoptotic vesicles released by hemocytes in the vicinity of invading parasites have been implicated in anti-*Plasmodium* responses by activating the complement pathway in *A. gambiae* mosquitoes (61). Interestingly, using a GFP reporter strain, Volohonsky et al. (74) reported that the antimalaria mosquito complement-like factor TEP1 is predominately expressed in the fat body as a transcript, but, at the protein level, it is found in hemocytes upon blood feeding and infection (74). The authors speculate that this is due to the uptake by the blood cells of TEP1 attached to bacterial cells. As in our sequencing only one cell contained low levels of TEP1, we suggest that EV-mediated delivery of TEP1 (mRNA or protein) may better explain these findings. We propose that vesicles found in the mosquito hemolymph contain proteins and transcripts that coordinate cell-to-cell and tissue communication not only in infection but also under physiological conditions. Disturbance of homeostasis, be it by infection, metabolic changes, tissue damage, or stress, may escalate secretion of vesicles containing an array of different cargo that can be targeted to specific tissues and complement systemic responses. We believe that, similar to how environment, microbiota, and genetic make-up influence phenotypic variation, cellular exchange can also drive cellular identity and represents an inventive and unexplored way through which nature coordinates who and what we are.

**Materials and Methods**

**Mosquito Rearing, Fluorescence Microscopy, and Hemolymph Perfusion.** *A. gambiae* sensu lato PPO6:RFP transgenic and WT strains were reared at 28 °C under 80% humidity and a 12/12-h day-night cycle. Larvae were fed with cat food, and adult mosquitoes were fed ad libitum with 10% sugar. For tissue microscopy, mosquitoes were dissected in 1x PBS, fixed in 4% paraformaldehyde (PFA), washed, and mounted using Vectashield mounting medium containing DAPI. For hemolymph perfusion, 3- to 5-d-old female mosquitoes were anesthetized, and 1-μl injection of exogenous PPO6::RFP transcripts, and potentially other PPO genes, contribute to the function of acceptor cells is another exciting question. PPO proteins lack the signal peptides required for their secretion, and it has been suggested that PPO6 is secreted by exocytosis as cell rupture has not been observed (41). It is plausible that PPO transcripts are shed by PPO6high cells and processed by PPO6lownegative cells that locally activate melanization only under specific conditions: e.g., upon infection with specific pathogens or during wounding and tissue repair. Molecular signals exchanged between cells can, thus, coordinate cellular plasticity and contribute to the diversity of functional subsets or “hybrid” cells that express markers of different or multiple cell types. Although we cannot rule out that RFP mRNAs were transferred between mosquito cells by means other than EVs, the demonstration of mosquito blood-borne EVs indicates that different cells and tissues likely communicate through vesicles secreted into the insect open circulatory system. Several recent reports have suggested EV-mediated immune responses in dip- teran insects. Exosome-like vesicles containing virus-derived PPO6low have been identified in *Drosophila* and contribute to systemic antiviral immunity (73). Apoptotic vesicles released by hemocytes in the vicinity of invading parasites have been implicated in anti-*Plasmodium* responses by activating the complement pathway in *A. gambiae* mosquitoes (61). Interestingly, using a GFP reporter strain, Volohonsky et al. (74) reported that the antimalaria mosquito complement-like factor TEP1 is predominately expressed in the fat body as a transcript, but, at the protein level, it is found in hemocytes upon blood feeding and infection (74). The authors speculate that this is due to the uptake by the blood cells of TEP1 attached to bacterial cells. As in our sequencing only one cell contained low levels of TEP1, we suggest that EV-mediated delivery of TEP1 (mRNA or protein) may better explain these findings. We propose that vesicles found in the mosquito hemolymph contain proteins and transcripts that coordinate cell-to-cell and tissue communication not only in infection but also under physiological conditions. Disturbance of homeostasis, be it by infection, metabolic changes, tissue damage, or stress, may escalate secretion of vesicles containing an array of different cargo that can be targeted to specific tissues and complement systemic responses. We believe that, similar to how environment, microbiota, and genetic make-up influence phenotypic variation, cellular exchange can also drive cellular identity and represents an inventive and unexplored way through which nature coordinates who and what we are.
mosquitoes were anesthetized on ice for 10 min, microinjected with 700 nL of a buffer containing 60% Schneider's medium, 10% FBS, and 30% citrate buffer (pH 5.2). The injection was performed using a Picopette (World Precision Instruments). After injection, the mosquitoes were allowed to recover on ice for 1 h, and whole hemolymph was collected at 2 h after injection and used for RNA in situ studies. Hemocytes were purified from hemolymph by centrifugation (1000 × g, 5 min, 4 °C) and resuspended in RNase-free PBS and transferred to a microscopy slide. Cells were allowed to attach for 15 min and then fixed in PFA as described above. If needed, slides were dehydrated and kept in 100% ethanol at −20 °C until processing. Tissue samples were processed immediately after fixation in RNase-free PBS. All RNAseq probes were designed by Advanced Cell Diagnostics and are commercially available. Each probe was tested against a negative control before and during each analysis. Imaging was performed on an LSM 880 NLO and a TCS SP8 microscope equipped with 405, 488, 561, and 647-nm lasers and prepared for submission using the basic features of the LAS X software.

**RNA in Situ Hybridization using RNAscope.** RNA in situ studies were performed according to the RNAscope Multiplex Fluorescence manual (Advanced Cell Diagnostics). Cells were perfused onto glass slides, allowed to attach, and fixed in PFA as described above. If needed, slides were dehydrated and kept in 100% ethanol at −20 °C until processing. Tissue samples were processed immediately after fixation in RNase-free PBS. All RNAseq probes were designed by Advanced Cell Diagnostics and are commercially available. Each probe was tested against a negative control before and during each analysis. Imaging was performed on an LSM 880 NLO and a TCS SP8 microscope equipped with 405, 488, 561, and 647-nm lasers and prepared for submission using the basic features of the LAS X software.

**RNA-Seq Data Analysis.** Sequencing reads were demultiplexed using bcl2fastq (version 1.8.4) and mapped to an A_ gammaiae genome (P4), ERCC92 (Ambion), and dTomato sequence (35) with the STAR aligner (version 2.4.2a) (76). The genome index was generated with an A_ gammaiae genome file in gtf format (P4.4), and gene count tables were produced during mapping (quantMode Genecounts). They were next normalized with size factors calculated from the ERCCs using DESeq2 (77). A gene was considered expressed if at least one normalized read was identified in at least one sample. Genes were annotated using Vectorbase (78) and manual curation. For comparisons with previous studies (24–26), IDs were converted using Vectorbase and BioMart. Intersection analyses were performed in R using the VennDiagram and upsetR packages. Technical noise estimation and identification of the highly variable genes were performed as reported before (79) using the 60-percentile as a mean cutoff to include more ERCC genes in the technical fit. PCAs were done with the precomp function using the variable genes or the whole dataset: i.e., genes expressed in at least one cell. For the MDS analysis, only the expression of PPO genes was taken into consideration, and Euclidean distances and the cmdscale function were used. Differential expression analyses were based on DESeq2, using the ERCC size factors and PPO6' vs. PPO6'' as comparison. For GO analyses, we used topGO (78), and GO terms were obtained from the org.Agae.db package. Analyses were performed in R, and scripts are available at https://github.com/mssevero/hemo-scRNAseq. The sequencing results were deposited in the European Nucleotide Archive (accession no. PRJEB23372), and the expression data can be accessed at https://kcb.sanger.ac.uk/R/base/main for single gene visualization.

**Imaging Flow Cytometry.** Female mosquitoes (n = 10 to 12) were perfused into a final volume of 20 to 40 μL, and the samples were immediately analyzed on an Amnis ImageStreamX MKII (Merck). For PPO6**:RFP analyses, WT mosquitoes were used to set background fluorescence, and cells were measured with a 40× objective. Comparisons between populations were performed using the “Object” mask and based on the built-in function that uses Fisher’s discriminant ratio (RD) to determine the best statistical separation (largest RD) between identified populations. For the DIID analyses, cells were collected into FBS-free buffer containing 1 μM DIID and analyzed at 60×. Single staining controls representing RFP, DIID, and buffer alone were used for calibration and manual compensation. Experiments were repeated at least twice. Cell gating was confirmed considering the images and manually curated to exclude debris and doublets that could not be excluded by the gating alone. Vescicle detection and calibration, and sorting purity was validated by visualization of cells sorted onto a coverslip. Following washes, cells were mounted as described above and analyzed on a Zeiss Axiovert microscope.

**FACS and Single-Cell RNA Sequencing by SMART-Seq2.** Hemolymph from 10 to 12 mosquitoes was collected with the help of a pipette, transferred into a siliconized microtube, and diluted to a final volume of 500-fold buffer containing 2 μg/mL Hoechst 33342 (Molecular Probes). Cells were immediately analyzed in a BD ARIA II Cell Sorter equipped with lasers at 405 and 561 nm. Cells were first gated based on their RFP fluorescence, followed by positive Hoechst signal, each with area vs. width being used for doublet discrimination. The FACS machine was standardized with fluorochrome-containing beads, and samples were adjusted by visualization of cells sorted onto a coverslip. Cells were sorted into a 96-well PCR plate containing 5 μL of 0.2% Triton X-100 supplemented with 2 μL RNase inhibitor (Clontech), with two wells containing 30 cells (pool samples) and one column (eight wells) containing only the lysis buffer as a negative control. We added ERCC spike-ins (Ambion) at an 1:2:1 billion dilution into the plate before cDNA synthesis, according to the SMART-Seq2 protocol. cDNA was then amplified up to 22 PCR cycles for cDNA synthesis (75). PCR products were purified with AMPure XP beads (Beckman Coulter). Quality control was performed for each sample individually both as cDNA input and sequencing library by using a high sensitivity DNA kit (Agilent). A total of 125 pg of cDNA was used for library construction. Libraries were pooled at a 10-nM final concentration, and 100-bp paired-end sequencing was performed in one lane of a HiSeq2000 Sequencer (Illumina).

** bead Uptake Assay.** For magnetic isolation of hemocytes, we followed the protocol by Smith et al. (26). Briefly, 20 females were cold-anesthetized and injected with 300 μL of a 2 mg/mL suspension of MagneSil Carbonyl Derivatized Beads (Thermo Fisher Scientific) or MagneSil Derivatized Beads (PeproTech) (100 μL) for 2 h and perfused. Hemolymph was collected with a pipette tip and transferred into a 0.5-μL Eppendorf tube containing 100 μL of injection buffer. Samples were diluted to 200 μL and incubated in a magnetic stand for 20 min at 4 °C. Supernatant was removed by pipetting, and the pellet was resuspended in RNase-free PBS and transferred to a microscope slide. Cells were allowed to attach for 15 min and then processed for RNA-FISH.

** Transwell Assay.** Hemolymph was collected on the top of a glass coverslip placed inside a 24-well plate. A total of 100 μL of buffer was added to prevent dehydration. Cell inserts (Merck) were then placed over individual wells, and hemolymph from PPO6**:RFP females was gently pipetted onto the 1-μm membrane. Diluted hemolymph from at least two WT and four transgenic mosquito mosquitoes was used per treatment. Plates were kept at room temperature for 1 h, fixed with PFA, washed, and immediately processed based on the RNAscope manual. Images were obtained by confocal microscopy as described above. Experiments were repeated at least twice.

** Scanning Electron Microscopy.** Cells were perfused from at least two females directly onto coverslips and fixed with 4% PFA. To facilitate exosome imaging, poly-lysine-treated coverslips were used. For correlative SEM, cells were placed onto microscopio dishes with finder grids (ibidi) and imaged directly after fixation using a Zeiss Axiovert microscope, before SEM processing. Samples were postfixed in 2.5% glutaraldehyde, 0.5% osmium-tetroxide, tannic acid, and osmium-tetroxide again. The coverslips or optical membranes were then dehydrated in a graded ethanol series, dried in carbon dioxide at a critical point, and vacuum coated with 3 nm of Carbon-Platinum. Imaging was performed using a LEO 1550 (Zeiss) scanning electron microscope. Experiments were repeated at least twice.

** Transmission Electron Microscopy.** EVs were isolated as described before (80). Hemolymph of at least 20 mosquitoes was differentially centrifuged at 10,000 × g at 4 °C, and pellets were processed for negative staining electron microscopy. Aliquots were applied to freshly gloved dischared carbon- and pioloform film-coated copper grids and allowed to adsorb for 10 min. After washes, the grids were contrasted with 2% uranyl acetate, touched on filter paper, and air-dried. The grids were examined using a LEO 992 (Zeiss) AG electron microscope operated at 100 kV, and images were recorded with a Morada (SIS-Olympus) digital camera.

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