Published in final edited form as:

Dev Comp Immunol. 2016 February ; 55: 119–129. doi:10.1016/j.dci.2015.10.020.

***Anopheles gambiae* hemocytes exhibit transient states of activation**

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Abstract

Hemocytes are crucial players of the mosquito immune system and critically affect transmission of pathogens including malaria parasites. We and others discovered previously that a blood meal is a major immune stimulus for mosquito hemocytes. To determine whether these blood meal-induced hemocyte changes in *Anopheles gambiae* constitute steps in cell differentiation or demonstrate transient cell activation, we analyzed the temporal pattern of these changes over the first three days post blood meal (dpbm). Flow cytometry and immunofluorescence analyses revealed a global shift of the entire hemocyte population, peaking at 1dpbm. All hemocyte activation markers returned to pre-blood meal baseline levels within the following 24 to 48 hours. Our observations are consistent with *An. gambiae* hemocytes undergoing transient activation rather than terminal differentiation upon blood feeding. Interestingly, the temporal pattern followed the gonotrophic cycle of the mosquito, strongly suggesting hormonal control of mosquito hemocyte activation and deactivation.

Abstract

Transient Hemocyte Activation

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Keywords

cellular immunity; innate immunity; mosquito; host-pathogen interactions; malaria

1. Introduction

Malaria continues to be a major health problem worldwide, especially in sub-Saharan Africa, where *Anopheles gambiae* is one of the major vectors for the protozoan *Plasmodium falciparum* (Oduola et al., 2013; Sinka et al., 2012). A major determinant for vector competence is the mosquito's immune system, which provides numerous defense strategies against a range of microbes (Severo and Levashina, 2014). Mechanistic insight into the *An. gambiae* immune system thus harbors the opportunity to potentially devise new malaria intervention strategies that aim to interrupt the malaria life cycle within its mosquito vector (Michel and Kafatos, 2005; Yassine and Osta, 2010). In addition, thorough understanding of mosquito immunity will be crucial for the development and maintenance of current cutting edge vector control methods that use infection with either *Wolbachia* (Frentiu et al., 2014) or entomopathogenic fungi (Blanford et al., 2011) to limit vector-borne diseases.

The mosquito immune system can be categorized broadly into humoral and cellular responses, the latter of which is executed by mosquito blood cells, called hemocytes. Cellular immune responses, including encapsulation and phagocytosis, do not significantly affect parasite development within the mosquito host (Hillyer et al., 2003; Paskewitz et al., 1988). However, hemocytes are central to humoral immune responses against malaria parasites, as they produce the majority of molecules for melanization and complement-like pathways, including prophenoloxidase (PPO) and thioester-containing protein (TEP1), respectively (Hillyer and Strand, 2014).

An adult female mosquito harbors anywhere between 500 to 4,000 hemocytes, and this number decreases with age in females maintained on sugar water (Hillyer and Strand, 2014). Mosquito hemocytes are classified into three main cell types based on morphology and function (Castillo et al., 2006). Granulocytes represent approximately 80-95% of adult hemocytes, are highly phagocytic, and measure approximately 9 μ m in diameter when in circulation and up to 35 μ m when allowed to spread on glass. Oenocytoids represent 10% of all hemocytes, are non-phagocytic, and are major producers of PPO. Oenocytoids are approximately 9 μ m in diameter and do not seem to spread significantly on surfaces. Prohemocytes represent the remainder of the hemocyte population in adult mosquitoes. Prohemocytes are significantly smaller than oenocytoids or granulocytes, roughly 4-6 μ m in diameter, and are proposed to be the hemocyte stem cell lineage in adult mosquitoes (Hillyer and Strand, 2014). A recent study reported that prohemocytes are substantially smaller in size and constitute about 50-60% of the entire hemocyte population in adult *An. gambiae* (Rodrigues et al., 2010). However, these observations are not reported in other studies, including earlier publications by the same authors (reviewed in Hillyer and Strand, 2014). Most recently, hemocytes have also been classified based on DNA-content, with on average 60% of hemocytes being euploid, and 40% being polyploid (Bryant and Michel, 2014). It is

currently unclear in how far these two classification systems relate to each other, as ploidy levels for the individual hemocyte cell types have not been described.

In unchallenged mosquitoes, roughly three fourths of all hemocytes circulate freely in the hemocoel, while the remaining cells are attached to multiple tissues including midgut, fat body, and tracheae (Hillyer and Strand, 2014). However, this distribution is altered upon infection. For example, infection with the rodent malaria parasite, *Plasmodium berghei* recruits hemocytes to the mosquito midgut (Rodrigues et al., 2010; Volz et al., 2005), the primary site of parasite infection, as well as the mosquito heart (King and Hillyer, 2012).

The majority of mosquito species are anautogenous, where the adult female must obtain nutrients from a blood meal for egg production (Hansen et al., 2014). This obligatory blood-feeding requirement exposes female mosquitoes to a variety of microorganisms, and causes major transient changes in the mosquito gut microbiota. Not surprisingly, recent studies have led to renewed appreciation of the blood meal as a major immunostimulant for female mosquitoes (Bryant and Michel, 2014; Upton et al., 2015). Specifically, cellular immunity undergoes major shifts in the female mosquito after she blood feeds. For *Aedes aegypti*, hemocyte numbers transiently increase after a blood meal, which are at least in part regulated by insulin signaling (Castillo et al., 2011). We recently reported multiple blood meal-induced changes in *An. gambiae* hemocytes (Bryant and Michel, 2014) including (i) their proliferation, (ii) shifts in abundance of euploid and polyploidy cell types, (iii) dramatic morphological changes in size and granularity, and (iv) up-regulation of the TEP1 and PPO6 immune factors, which are critical mosquito immunity factors responsible for complement and melanization, respectively (Frolet et al., 2006; Muller et al., 1999). In addition, we observed increased levels of phosphorylated extracellular signal-regulated kinase (pERK) in hemocytes (Bryant and Michel, 2014), a highly conserved marker for Ras-MAPK signaling across multiple taxa (Downward et al., 1990; Sinenko et al., 2011). However, we currently lack information on the temporal pattern of these blood meal-induced changes in cellular immunity in *Anopheles gambiae*, and therefore do not know whether these changes constitute steps in cell differentiation or transient cell activation.

To address this question, the current study assessed changes in hemocytes after a blood meal temporally and quantitatively with regards to proliferation, cell populations, morphology, size, and activation markers.

2. Materials and Methods

2.1. Mosquito rearing and maintenance

The *An. gambiae* G3 strain was reared according to our standard protocol (An et al., 2011). For blood feeding, mosquitoes were starved for approximately 6-8 h before blood feeding and subsequently provided with heparinized horse blood (Plasvacc, Templeton, CA, USA) through an artificial feeding system using parafilm as the membrane. Only fully engorged females were used for subsequent analyses. Some females were removed prior to blood feeding and used as age- and population-matched controls.

2.2. Hemocyte collection

Hemocytes were collected by perfusion using a modified protocol. Mosquitoes were injected with anticoagulant buffer (60% Schneider's medium, 10% FBS, 30% citrate buffer [98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA, 41 mM citrate]) and incubated on ice for 10-15 minutes. Using dissection forceps, a small tear was made into the penultimate abdominal segment and ~6-10 μ l of anticoagulant buffer was perfused through the hemocoel at a rate of 1 μ l/dispension using a Hamilton syringe system. Perfused hemocytes were processed for quantification or other analyses as described below.

2.3. Hemocyte counts

Hemocytes were collected as described above, but perfusions were performed with 50 μ l of anticoagulant buffer. The entire perfusate was put on a slide, and cells were allowed to adhere at 4°C for 45 min. Cells were subsequently fixed with 4% paraformaldehyde in PBS. Cells were washed briefly in PBS and embedded in Vectashield® with DAPI (Vector Laboratories, Burlingame, CA, USA). All DAPI-positive cells were counted immediately.

2.4. 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay

To assess DNA replication and, by proxy, cell proliferation, EdU incorporation was monitored. Assays were performed with the Click-iT® EdU Alexa Fluor® 488 Imaging kit (Invitrogen, Grand Island, NY, USA) following manufacturer's instructions as described previously (Bryant and Michel, 2014). For temporal analysis of EdU incorporation, a single population cage from a highly synchronized mosquito population was blood fed 4 days post emergence. 25-30 mosquitoes were injected once with EdU at one of the following time points: 8, 20, 32, and 44 hours post-blood meal (hpbm). After a four hour incubation period, each group of mosquitoes was processed for EdU incorporation. EdU incorporation was expressed as the fraction of positive cells in a pool of at least 300-400 hemocytes. Experiments were performed with two independent biological replicates.

2.5. Hemocyte population and morphometric analysis by flow cytometry

Hemocytes were collected and pooled from 60-75 mosquitoes by perfusion using anticoagulant buffer. Cells were resuspended in 200 μ l PBS with 0.1% FBS. 700 μ l of 70% ethanol was added drop wise to the cells and incubated for 1 h at room temperature (RT). Cells were centrifuged again at $2350 \times g$ at 4°C and resuspended in 200 μ l of Propidium Iodide (PI) solution (50 μ g/ml PI, 100 μ g/ml RNaseA, 0.1% Triton X-100, 0.1 mM EDTA in PBS). Cells were incubated for at least 1 h at 4°C, and pushed through a 40 μ m nylon filter (Becton Dickinson Falcon, San Jose, CA, USA) to remove large cell aggregates. Processed cells were run on a FACSCalibur cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and data were analyzed using CellQuest software (Becton Dickinson, Franklin Lakes, NJ, USA). For cell cycle analysis, cells were gated and their corresponding histograms were obtained. For morphometric analysis, cell populations were distinguished based on DNA content and backgated to determine size (FSC-H) and granularity (SSC-H). To backgate cells, first a dot plot based on area and width of PI (DNA) signal was drawn up. Cell populations were gated on ploidy levels of interest, then back-gated, and density dot plots and overlaying histograms were drawn for analysis of size (FSC-H) or granularity

(SSC-H). All experiments were performed in triplicate with three independent biological replicates. To quantify cell size, 5-, 10-, and 15-micron iCyt AccuSize beads (SONY, San Jose, CA, USA) were run as size standards on the flow cytometer in parallel to processed hemocytes. Beads were run for each biological replicate immediately before and after the hemocyte samples, 20,000 events were counted, and histograms on forward scatter (FSC, size) were drawn.

2.6. Immunofluorescence analysis

Hemocytes were harvested as described above and processed for activation markers (pERK, TEPI1, and PPO6). Harvested cells were allowed to adhere on PTFE printed glass slides (Electron Microscopy Sciences, Hatfield, PA, USA) at 4° C for 1 h, fixed with 4% formaldehyde for 15 min, blocked in blocking buffer (5% BSA, 0.3% Triton X-100 with PBS as diluent) for 1 h, and subsequently incubated overnight at 4° C with primary antibody in antibody dilution buffer (1% BSA, 0.3% Triton X-100 in PBS). Primary antibody dilutions were 1:30 for pERK (rabbit anti-pERK, Cell Signaling, Boston, MA, USA); 1:350 for TEPI1 [rabbit anti-TEPI1, kindly provided by M. Povelones and G. Christophides, Imperial College, London, UK (Povelones et al., 2009)]; and 1:1000 for PPO6 [rat anti-PPO6, provided by H.-M. Muller H-M, Heidelberg University (Muller et al., 1999)]. Following primary antibody incubation, cells were washed in PBS three times, and incubated with secondary antibody in antibody dilution buffer in the dark for 1-2 h at RT. Secondary antibody dilutions were 1:100 for goat anti-rabbit (pERK); 1:500 dilution for goat anti-rabbit (TEPI1); and 1:1000 for goat anti-rat (PPO6). All secondary antibodies were IgG (H + L) conjugated with Alexa Fluor® 594 (Invitrogen, Grand Island, NY, USA). For double antibody staining, hemocyte samples were incubated simultaneously with TEPI1 and PPO6 primary antibodies and subsequently with both secondary antibodies using the same conditions as described above. Cross reactivity between the secondary antibodies was not observed. Following secondary antibody incubations, cells were rinsed in PBS three times, mounted in Vectashield with DAPI (Vector labs, Burlingame, CA), sealed using nail polish, and stored at 4° C until further analysis. Experiments were performed with three independent biological replicates.

2.7. Quantification of Immunofluorescence Staining

To quantify hemocyte activation markers, TIFF images were obtained with an Axioplan2 fluorescent light microscope (Zeiss, Jena, Germany) equipped with a camera and processed using the imaging software Image J (<http://rsb.info.nih.gov/ij/>). For temporal comparison, identical magnification, fluorescence intensities, and exposure times were used to obtain images for each individual marker. TIFF image files were imported into Image J, circles were drawn around cells, and raw intensity values were obtained. Background levels were obtained by measuring a blank space in the image, which was subtracted from foreground values. Experiments were performed with three biological replicates, which when pooled together resulted in a range of 162 to 207 cells per time point. Frequency distributions of the fluorescence signals for pERK, PPO6, and TEPI1 were obtained by binning with GraphPad Prism (GraphPad Software, Inc.). Bins were chosen using the automated setting on the program based on the following two principles: (i) bin number equal to the log base 2 of sample size, and (ii) bin width being a round number.

2.8. Data analyses

All statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc.). Data were analyzed initially for normal distribution using the Shapiro-Wilk normality test. For non-normally distributed time course data, Kruskal-Wallis test was applied followed by Dunn's multiple comparison test.

3. Results

3.1. Blood feeding induces transient hemocyte proliferation

To determine temporal dynamics of hemocyte proliferation after a blood meal, we first counted absolute numbers of hemocytes that can be obtained by perfusion. The total number of hemocytes per mosquito changed significantly between sugar fed and blood fed mosquitoes at 24 hpbm, while returning to pre-blood meal levels at 48 hpbm (Fig. 1A, Kruskal-Wallis test followed by the Dunn's post test, $P = 0.0003$, $P < 0.05$ at 24 hpbm as compared to SF and 48 hpbm). To determine how these temporal changes of hemocytes related to cell proliferation, we assayed DNA replication in hemocytes by monitoring EdU incorporation into DNA over a four hour time window at 12, 24, 36, and 48 hpbm. The percentage of EdU-positive hemocytes obtained from sugar-fed mosquitoes was low, with a median of 1.0% (Fig. 1B). This number increased significantly to 13.9% and 15.5 % at 12 and 24 hpbm, respectively (Kruskal-Wallis test followed by the Dunn's post test, $P < 0.0001$). However, after 24 hpbm, the percentage of EdU-positive hemocytes dropped to 1.8 % and 3.1 % at 36- and 48-hpbm, respectively (Fig. 1). The data revealed that DNA replication was elevated significantly in hemocytes between 12-24 hpbm, suggesting that hemocyte proliferation undergoes a burst after blood meal that is limited to the first 1-1.5 days of the gonotrophic cycle. As a consequence, hemocyte numbers doubled and peaked around 24 hpbm, returning to pre-blood meal baseline levels with 48 hpbm.

3.2. Blood feeding induces transient hemocyte population changes

To assess changes in hemocyte populations based on DNA content, PI staining was used to differentiate between euploid and polyploid hemocyte populations from sugar-fed, 1 and 2 day pbm (dpbm) mosquitoes. PI-stained hemocytes were displayed in dot plots as fluorescence signal area versus width (Fig. 2A), cell aggregates were excluded by gating (Givan, 2001), and histograms were obtained (Fig. 2B). As we reported previously, hemocytes from sugar-fed mosquitoes contained distinct hemocyte populations with varying DNA content ranging from 2C to 16C. Two markers were placed on the histograms delineating hemocytes based on ploidy levels. Euploid (dotted marker line) were defined by the characteristic G0/G1 (2C) and G2 (4C) peaks, and polyploid cells (dashed marker line) showed peaks at 8C or 16C. Euploid cells represented the dominant hemocyte population in sugar fed as well as blood fed mosquitoes, 1 and 2 dpbm (Figs. 2B, C). However, the ratio between euploid and polyploid cells within the entire hemocyte population depended strongly on the physiological state of the mosquito. While on average, 58 % of hemocytes in sugar fed mosquitoes were euploid cells, their proportion increased to 91 % within 24 h post blood meal (Fig. 2B, C). In addition, the number of hemocytes that fell outside of the drawn gate due to high PI fluorescence width, indicative of cell aggregates, was elevated at 1dpbm

(Fig. 2A). The strong shift in cell population distribution and cell aggregation propensity observed at 1dpbm reverted back to sugar-fed levels by 2 dpbm (Fig 2 and Fig. S1).

3.3. Morphological changes of hemocytes within the first 48h of the gonotrophic cycle

The increased number of hemocyte aggregates suggested changes in hemocyte surfaces as a consequence of blood feeding. To assess additional potential morphological changes in hemocytes induced by blood feeding, we backgated euploid and polyploid cell populations to determine their size and granularity properties (Fig. 3, individual replicates are shown in Fig. S2). The forward scatter (FSC) vs. side scatter (SSC) dot plot of hemocytes from sugar fed mosquitoes, showed nearly three quarters of all euploid cells in the lower left quadrant (small size, low granularity, Fig 3D, G). At 1 dpbm, nearly half of all euploid hemocytes shifted to the upper right-quadrant, (Fig 3E, G), indicating larger cells with increased granularity. By 2 dpbm, cells returned to sugar-fed morphological properties with approximately 60 % in the lower left quadrant (Fig 3F, G). The histograms of FSC (Fig. 3H) and SSC (Fig. 3I) revealed that the whole euploid hemocyte population shifted within the first 24 hpbm and returned to base line by 48 hpbm.

Approximately half of the polyploid hemocytes from sugar-fed mosquitoes were found in the lower left quadrant, indicative of small cell size and low granularity (Fig 3J, M). At 1dpbm polyploid cells shifted to 67 % in the upper right-quadrant (Fig 3K, M). However, the polyploid population was significantly reduced when compared to polyploid cells from sugar fed mosquitoes or euploid hemocytes at 1dpbm. The polyploid population at 2 dpbm was similar to those from sugar fed mosquitoes (Fig 3L, M). In contrast to euploid hemocytes, FSC (Fig 3N) and SSC (Fig. 3O) histograms indicated no obvious population shift possibly due to low cell numbers.

To quantitatively assess size changes of hemocyte populations after a blood meal, we ran multiple micron sized beads (5 μ m, 10 μ m, and 15 μ m) for each biological replicate immediately prior and after the hemocyte samples on the flow cytometer (Fig. 4A). The overlaying FSC histograms of beads for technical replicates demonstrated cytometer accuracy for cell size determination (Fig. S3). To compare hemocyte size to standardized beads, we drew markers on the bead histograms designating size ranges (M1 5 μ m, M2 6-10 μ m, M3 11-15 μ m, and M4 >15 μ m; Fig. 4A). These markers were overlaid on histograms of backgated hemocytes (gates are shown in Fig. 2A, 3A-C). We first analyzed the entire hemocyte population, encompassing euploid and polyploid cells (total hemocytes). Approximately 25 % of total hemocytes were under 5 μ m in diameter (M1), 30 % were between 6 μ m and 15 μ m (M2, M3), and 45 % were over 15 μ m in diameter (Fig. 4B and Table 1). To determine cell size for euploid and polyploid cells separately, we backgated each cell type and reanalyzed cell size parameters. Nearly, 27% of euploid cells were smaller than 5 μ m, 37 % were between 6 μ m and 15 μ m, and 37% were larger than 15 μ m in diameter (Fig. 4C and Table 1). In contrast, the polyploid cell population contained very few hemocytes smaller than 5 μ m (9 %), while 25 % were between 6 μ m and 15 μ m, and 66 % were larger than 15 μ m (Fig. 4D and Table 1).

Importantly, blood meal induced a transient increase in hemocyte size in both, euploid and polyploid hemocytes. The total hemocyte population larger than 15 μ m increased by 30% 1

dpbm, and returned to sugar fed baseline levels at 2 dpbm (Table 1). The percent of polyploid cells larger than 15 μm increased by 25 %, while the percent of euploid cells in the same size group almost doubled. (Table 1). The percent of euploid and polyploid cells in the largest size range returned to baseline levels at 2 dpbm (Fig. 4 and Table 1).

3.4. Expression of hemocyte activation markers follows the gonotrophic cycle

We reported previously that blood feeding increased pERK, TEP1, and PPO6 protein levels in hemocytes at 1 dpbm. To determine how these blood meal-induced hemocyte activation markers act over the course of a gonotrophic cycle, protein levels were assessed by IFA in hemocytes from sugar fed mosquitoes and at multiple time points after blood feeding. Similar to previous findings, pERK, TEP1, and PPO6 protein levels per hemocyte more than doubled at 1 dpbm, and their expression peaked at this time point (Kruskal-Wallis test $P < 0.0001$, followed by Dunn's multiple comparison test, $P < 0.05$, Fig. 5, S4). pERK protein levels returned to sugar-fed baseline by 2 dpbm. The frequency distribution of the pERK signal per hemocyte in sugar fed as well as blood fed mosquitoes was unimodal and followed a Gaussian curve (goodness of fit, $R^2 = 0.98, 0.891, 0.950$, and 0.897 , for SF, 1, 2, and 3 dpbm, respectively), indicating that the pERK signal increased similarly across the entire hemocyte population.

TEP1 protein levels returned more slowly to baseline levels. While the median of TEP1 signal dropped between 1 to 2 dpbm, baseline levels were only reached at 3 dpbm (Kruskal-Wallis test $P < 0.0001$, followed by Dunn's multiple comparison test, $P < 0.05$, Fig. 5, S4). The TEP1 signal frequency distribution in hemocytes from sugar-fed mosquitoes was unimodal and followed a Gaussian curve (goodness of fit, $R^2 = 0.97$). Similar results were obtained from hemocytes of blood fed mosquitoes at 2 and 3 dpbm, albeit with a lower goodness of fit ($R^2 = 0.89$ at both time points). However, the TEP1 protein levels obtained from hemocytes 1 dpbm were not normally distributed ($R^2 = 0.75$) and appeared to follow a bimodal distribution (Fig. 5). In summary, these data revealed that TEP1 expression increased in all hemocytes within the first 24 hours of the gonotrophic cycle. However, the amplitude of increase was not uniform among the total hemocyte population and suggested that there are at least two hemocyte subpopulations that respond differentially to blood feeding with regards to TEP1 expression.

Similarly to TEP1, PPO6 protein levels remained elevated beyond 1dpbm, and in contrast to TEP1, continued to be significantly higher even at 3dpbm as compared to sugar fed baseline (Kruskal-Wallis test $P < 0.0001$, followed by Dunn's post test, <0.05 , Figs. 5, S4). The goodness of fit of PPO frequency data to a Gaussian distribution was marginal at each time point, with R^2 values of 0.93, 0.78, 0.89, and 0.88 for hemocytes from sugar fed and blood fed mosquitoes. While the median content of PPO6 of all hemocytes was elevated at 1dpbm, a smaller subpopulation of hemocytes expressed PPO6 at very high levels, independent of blood feeding (Fig. 5). The size of this subpopulation increased transiently at 1 and 2 dpbm and, similar to the median PPO6 level, began to approach base line levels at 3 dpbm.

Others and we showed previously that hemocytes are the sole source of TEP1 and PPO6 proteins in *Anopheles gambiae*. To determine whether TEP1 and PPO6 co-localize within hemocytes, we performed double-IFAs at 1dpbm, the peak of expression for both proteins

(Fig. 6). All hemocytes that were analyzed were positive for both, TEP1 and PPO6. Consistent with our quantitative analysis described above, protein levels for TEP1 as well as PPO6 varied considerably between cells. However, we did not observe a consistent ratio between TEP1 and PPO6 signal, e.g. the high-expressing PPO6 hemocyte subpopulation did not show increased levels of TEP1 protein. As described previously, the staining pattern for both proteins was punctate, consistent with these proteins being present in secretory vesicles or granules. The staining pattern of PPO6 in the high-expressing PPO6 hemocytes appeared less granular albeit intracellular throughout the cytoplasm. Overall, the staining patterns of TEP1 and PPO6 showed little overlap, demonstrating that these proteins are mostly contained within distinct cellular compartments.

4. Discussion

Mosquito hemocytes respond strongly to a variety of immune stimuli including bacteria, parasites, and a blood meal. Their numbers in circulation increase (Bryant and Michel, 2014; Castillo et al., 2011; King and Hillyer, 2012), and their molecular make-up changes significantly (Baton et al., 2009; Bryant and Michel, 2014; Frolet et al., 2006; King and Hillyer, 2012; Pinto et al., 2009). However, prior to this current study, it was unclear whether stimulated mosquito hemocytes undergo transient activation, or undergo cell differentiation and therefore remain in the activated state until cell death. We observed that within 1 dpbm *An. gambiae* hemocytes were at their peak of mitotic activity leading to an overall three-fold increase in absolute hemocyte numbers. At the same time point, hemocytes displayed an increased ratio of euploid to polyploid cells. Based on total hemocyte counts and ratio of euploid to polyploid cells, a sugar fed mosquito therefore contains on average 1320 euploid and 880 polyploid hemocytes, respectively, while at 1 dpbm 3820 euploid and 430 polyploid hemocytes, and at 2dpbm, 1110 euploid and 730 polyploid hemocytes are present. These numbers reveal that the adult hemocyte population undergoes highly dynamic changes within the first two days of the gonotrophic cycle. Euploid cells undergo increased cell proliferation within the first 24 hours after a blood meal, leading to a dramatic relative decrease in polyploid cells at 1 dpbm. To a lesser degree, increased cell death of polyploid cells within 1 dpbm may also contribute, reducing their absolute number from roughly 880 to 430 cells. Between 1-2 dpbm, euploid cells then cease their increased proliferation rate, and their absolute number decreases by more than 50%, possibly due to increased apoptosis. In addition, the absolute number of polyploid cells increases again to pre-blood meal levels. While the ontogeny of the polyploid cell population remains to be elucidated, it is likely that they originate from euploid hemocytes through endoreplication. This suggests that roughly 10% of the euploid cell population may undergo endoreplication between 1-2 dpbm. Overall however, the temporal changes seen in cell number and ploidy levels of hemocytes across the first two days of the gonotrophic cycle can be largely explained by elevated cell proliferation of euploid cells within a one day time window at the beginning of the gonotrophic cycle.

Within the same 1 dpbm, both euploid and polyploid cells increased in size, granularity, and aggregation propensity. Activation markers such as pERK and immune factors TEP1 and PPO6 also peaked at 1dpbm. All morphological and molecular markers returned to baseline (sugar fed) levels by 2 dpbm, with the exception of PPO6, which remained slightly elevated

even at 3 dpbm. Importantly, the flow cytometry data demonstrated that the entire euploid and polyploid hemocyte populations shifted, rather than a subset of cells, while the EdU data revealed that insufficient numbers of hemocytes undergo cell division past 1 dpbm in order to replenish the entire euploid hemocyte population. Our data parallel observations of mammalian macrophages, which adopt different activation states in response to their environment. Activated by a stimulus, macrophages express certain marker genes; however once the stimulus is removed, marker gene expression is reduced to pre-stimulation levels (Degrossoli et al., 2004; Giorgio, 2013; Ishii et al., 2009; Ruckerl and Allen, 2014; Stout et al., 2005). All together, the most parsimonious explanation for our observations is that the majority hemocytes behave similarly to macrophages upon stimulation and undergo a transient activation step rather than terminal differentiation upon blood feeding. In addition, hemocyte activation upon blood feeding is a global phenomenon rather than limited to a subset of hemocytes.

In contrast, *An. gambiae* hemocytes have been described to undergo cell differentiation upon malaria parasite infection within the first 24 hours after uptake of the infectious blood meal (Rodrigues et al., 2010). During this time, hemocyte progenitor cells called prohemocytes, characterized by their extremely small size 1-2 μ m diameter (Smith et al., 2015), differentiated into the professional phagocytes, called granulocytes (Rodrigues et al., 2010). This malaria infection-driven hemocyte differentiation, which leads to a reported doubling of the granulocyte population from 4 to 8% of all hemocytes (Rodrigues et al., 2010), also influences late-phase immune responses against established oocysts (Smith et al., 2015) and trains the immune system to respond more effectively to further infections (Rodrigues et al., 2010). Using flow cytometry, we have thus far not been able to observe a significant number of cells of 1 μ m in size. Previous studies described putative prohemocytes in the 5 μ m size range (Castillo et al., 2006), which is closer to the lower end of the hemocyte size range we observed by flow cytometry. We cannot exclude that a small proportion of hemocytes undergo cell differentiation immediately after a blood meal, as it is possible that the number of differentiating cells is too small and that this sub-population has overlapping phenotypes with the euploid hemocyte cell population. However, given our previous observation of mitotically active circulating hemocytes, the flow cytometry data presented herein, and our observation that all hemocytes are already immunocompetent cells expressing TEP1 and PPO6, we assume that any cells potentially undergoing differentiation are granulocytes rather than progenitor cells. Examples of such transdifferentiation exist in the literature: *Drosophila* larval hemocytes, specifically phagocytically and mitotically active plasmatocytes can differentiate into lamellocytes (Honti et al., 2010; Meister and Ferrandon, 2012) as well as crystal cells (Leitao and Sucena, 2015). Definitive experiments to address whether a blood meal induces mosquito hemocyte transdifferentiation require lineage-specific markers for mosquito hemocytes. Such markers are currently under development.

Interestingly, while we found no obvious evidence for hemocyte differentiation within 1 dpbm, the bi- or multimodal distribution of TEP1 and PPO6 titers across hemocytes suggest amplitude differences in the response to stimulation as well as differences in the release of these two proteins from hemocytes. The general decrease in TEP1 and PPO6 across all hemocytes by 2 and 3 dpbm, indicates that both proteins are released from hemocytes during

the first 3 dpbm. TEP1, whose primary sequence contains a signal peptide, and therefore most likely contained within vesicles of the secretory pathway (Levashina et al., 2001). Our temporal IFA analyses are consistent with this notion and suggest that TEP1 is continuously released from hemocytes through the secretory pathway. In contrast, the release of PPO6 must be distinct from that of TEP1, as this protein neither contains a signal peptide (Muller et al., 1999) nor have we found it located in the same granules as TEP1. Indeed, PPO in insects is released in response to injury or infection from a subset of hemocytes, called oenocytoids (or crystal cells in *D. melanogaster*) by cell rupture (Cerenius and Soderhall, 2004). In *D. melanogaster*, this process is regulated by the interplay of several signal transduction pathways (Bidla et al., 2007). In *An. gambiae*, oenocytoid cell rupture in response to infection has not been observed. Our IFA analyses reveal that PPO6, while very highly expressed in a small subset of hemocytes, presumably oenocytoids, is also expressed at moderate levels in all other hemocytes we examined. Together, our data suggest that PPO6 at least in part is released from hemocytes by exocytosis, as the overall transient increase and decrease in PPO6 titers cannot be explained by cell rupture and replenishment by hemocyte proliferation alone. Indeed, exocytosis of PPO has been observed in crayfish (Johansson and Söderhäll, 1985), and can be stimulated *ex vivo* by non-self molecules as well as calcium. In this context it is interesting to note that regulated secretion of TEP1 from hemocytes has been observed after malaria parasite infection, suggestive of exocytosis (Frolet et al., 2006). Future studies will have to determine if exocytosis of immune molecules from hemocytes is a global response to immune stimulation in *An. gambiae*.

The regulation of cellular immune responses in *An. gambiae* is complex, involving insulin signaling and likely Ras-MAPK signaling mediating proliferation (Bryant and Michel, 2014; Castillo et al., 2011), as well as the Toll, STAT and JNK pathways influencing differentiation (Ramirez et al., 2014). Our analysis reveals that the time course of blood meal-induced transient activation mirrored the gonadotrophic cycle of the mosquito, strongly suggesting that *An. gambiae* hemocyte activation is regulated additionally by hormonal changes. Multiple examples exist in the literature of hormonal regulation of insect hemocytes during development and immune responses. The molting process affects hemocyte numbers, mitotic index, and population changes in many insect orders including Blattodea (*Periplaneta americana*), Orthoptera (*Locusta migratoria*), Hemiptera (*Rhodnius prolixus*), Lepidoptera (*Bombyx mori*), and Diptera (*Sarcophaga bullata*) (reviewed in Gupta, 1979). During metamorphosis, at a time where 20-Hydroxyecdysone (20E) pulses, *Drosophila* hemocytes increase in size and granularity. Ecdysone signaling through *Ecdysone Receptor* (*EcR*) is required for these morphological cellular changes as hemocytes from *EcR* deficient flies remain unchanged during metamorphosis (Regan et al., 2013). Strikingly similar to this *Drosophila* hemocyte regulation during metamorphosis, *An. gambiae* 20E levels are high at one day after blood feeding (Bai et al., 2010), the same time point where *An. gambiae* hemocytes peak in size and granularity. By 2 dpbm, *An. gambiae* 20E levels drop back to baseline levels (Bai et al., 2010), and hemocytes from this time point morphologically resemble those from sugar-fed mosquitoes. In addition, 20E induces the expression of immune molecules in hemocyte-like cell lines, specifically of antimicrobial peptides in *D. melanogaster* S2 cells (Dimarcq et al., 1997; Flatt et al., 2008), and several PPOs, including PPO6 in *An. gambiae* 4a-3B cells (Muller et al., 1999). This

suggests that 20E is likely to not only direct the transient changes in hemocyte morphology after blood meal but also in their immune repertoire.

Additional hormonal regulation of hemocyte activation after blood meal may be provided by juvenile hormone (JH). JH is responsible for negating hemocyte activation in *Pseudoplusia includens*, *Plutella xylostella*, *Spodoptera frugiperda*, and *Tribolium castaneum* (Clark et al., 2005; Hapat and Kim, 2014; Kim et al., 2008; Kwon and Yonggyun, 2007). JHIII synthesis drastically increases 48h after blood meal in *Ae. aegypti*, at the time where 20E levels drop (Shapiro et al., 1986). While JH hormone titers during the gonotrophic cycle in *An. gambiae* remain unknown, JH analogs such as methoprene can negate 20E action during the gonotrophic cycle when applied 6 hpbm (Bai et al., 2010). Collectively, the striking parallels between the time course of blood meal-induced hemocyte activation and 20E and JH titers during the gonadotrophic cycle for the mosquito reveal the likely hormonal regulation of hemocytes in *An. gambiae*. Work is currently underway to determine whether 20E and JH directly or indirectly regulate hemocyte activation.

In summary, the temporal analysis of activation markers revealed that blood meal-induced hemocyte activation in *An. gambiae* is transient, and provides no indication of hemocyte differentiation from a reservoir of progenitor cells. Furthermore, deactivation of hemocytes is likely accompanied by degranulation leading to the release of immune proteins. The regulation of hemocyte activation and deactivation after blood feeding is complex and probably also mediated by hormonal changes. In the future, flow cytometry with molecular markers, can be employed to elucidate the respective contributions of the different signaling pathways that partake transient hemocyte activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank all members of the Michel lab for their help with mosquito colony maintenance, and Drs. S. D. Fleming and M. Gorman (KSU) for constructive feedback on the manuscript. We are grateful to Drs. R. Clem and M. Herman (KSU) for continued access to their fluorescent microscopes. We further thank Dr. Philine Wangemann and Mr. Joel Sanneman at the Confocal Microfluorometry & Microscopy Core funded by the College of Veterinary Medicine at KSU. Flow cytometry analyses were performed at the Flow Cytometry core laboratory maintained by the College of Veterinary Medicine at KSU. Antibodies were kindly provided by Drs. M. Povelones and G. Christophides (anti-TEP1, Imperial College London, UK), and Dr. H.-M. Mueller (anti-PPO6, University of Heidelberg, Germany).

This study was supported by Grant Numbers R01-AI095842 (to K.M.) and F32-AI104154 (to W.B.B) from NIAID. This is contribution 16-128-J from the Kansas Agricultural Experiment Station. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the funders.

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Highlights

- Blood meal-induced hemocyte activation in *Anopheles gambiae* is transient.
- We do not find evidence of hemocyte differentiation upon blood meal.
- Hemocyte activation is likely accompanied by degranulation of immune proteins.
- The time course of transient hemocyte activation suggests hormonal control.

Figure 1. Blood feeding induces transient hemocyte proliferation

(A) Total hemocyte numbers increase significantly within the first 24 hpbm, and return to pre-blood meal levels at 48 hpbm. For each data point, hemocytes were collected from a single mosquito. (B) EdU-incorporation in hemocytes from sugar-fed, 12 and 24 hpbm mosquitoes was significantly transiently increased in hemocytes from blood fed mosquitoes. For each data point, 300-400 hemocytes were collected and pooled from two mosquitoes. Figure shows combined data from two biological replicates, with $N=5$ or 6 for (A) and $N=6$ (B) for each treatment group and biological replicate. Graphs show each data point with median and interquartile range.

Figure 2. Blood feeding induces transient hemocyte population changes

Flow cytometry analysis of propidium iodide (PI)-stained hemocytes collected from sugar-fed, 1, and 2 dpbm mosquitoes. For each time point, hemocytes from 60-75 mosquitoes were pooled, and 20,000 PI-positive events were analyzed and plotted. (A) Dot plots of PI fluorescence signal area over width illustrate DNA content per cell. Gates were drawn to eliminate cell aggregates (solid line). (B) Histograms illustrate DNA content for the gated cells and drawn markers correspond to euploid (dotted line) and polyploid (dashed line) populations. The figure shows a representative from three independent biological replicates, the additional two replicates are shown in Fig. S1. (C) The percentage of all three biological replicates for euploid and polyploid cell percentages over the temporal analysis (means $\pm$ 1 SEM).

Figure 3. Blood feeding induces transient hemocyte morphological changes

Gates were drawn on dot plots of PI fluorescence signal area over width (A-C, same as in Fig. 2) delineating euploid (dotted line) and polyloid (dashed line) hemocytes from sugar-fed, 1, and 2 dpbm mosquitoes. The two cell populations were performed on to analyze their size (FSC-H) and granularity (SSC-H). Density dot plots (D-F for euploid, and J-L for polyloid) illustrate size over granularity with intensity on a red to white color scale. The quadrant percentage of all three biological replicates for euploid (G) and polyloid (M) illustrate their morphological changes over the time course analysis (means $\pm$ SEM). Overlaying histograms reveal a transient increase in size and granularity for euploid cells (H, I), whereas polyloid cells decrease in number at 1 dpbm and return by 2 dpbm (N, O), black, sugar-fed; blue, 1 dpbm; orange, 2 dpbm. The figure shows a representative dot blot and its analyses of three independent biological replicates; additional replicates are shown in Fig. S2.

Figure 4. Blood feeding induces transient hemocyte size changes

Size reference microspheres demonstrated the range of hemocyte size collected from sugar-fed, 1, and 2 dpbm mosquitoes. (A) Representative histogram of 5 μ m, 10 μ m, and 15 μ m beads. Markers designate four different size regions: M1, 5 μ m; M2, 6-10 μ m; M3, 11-15 μ m; M4, >15 μ m. Histograms for (B) total hemocytes, (C) euploid, and (D) polyploid cells isolated from sugar-fed, 1, and 2 dpbm mosquitoes, including M1 thru M4 size markers. Black, sugar-fed; blue, 1 dpbm; orange, 2 dpbm. The figure shows a representative of three independent biological replicates; results from all three replicates are summarized in Table 1. Reproducibility of the size reference data is shown in Fig. S3.

Figure 5. Blood feeding induces transient hemocyte activation

Hemocytes from sugar fed, 1, 2, and 3 dpbm mosquitoes were analyzed for blood meal-induced activation markers. Fluorescence signal per hemocyte were binned and are shown as frequency distributions. Blood feeding led to a significant increase in fluorescence for all activation markers after 1-day pbm (Kruskal-Wallis test followed by the Dunn's post test, $P < 0.0001$), with levels returning to sugar-fed levels by 2 dpbm for pERK (Kruskal-Wallis test followed by the Dunn's post test, $P < 0.0001$). For TEP1 and PPO6, values were significantly albeit slightly elevated by 2 and 3 dpbm, respectively (Kruskal-Wallis test followed by the Dunn's post test, $P < 0.0001$). Experiments were performed in triplicate and data was pooled and graphed. Unbinned data are shown in Fig. S4. $N=207$ for pERK, $N=162$ for TEP1, and $N=176$ for PPO6, pooled from three biological replicates.

Figure 6. PPO6 and TEP1 proteins are co-expressed but not co-localized in hemocytes
Hemocytes were co-stained with DAPI, anti-PPO6 and anti-TEP1 antibodies 24h after blood meal. Maximum intensity projections of triple-stained hemocytes reveal little overlap (white arrows) between the staining patterns, indicating that PPO6 and TEP1 proteins are present in distinct granules. Scale bar = 10 μ m

Table 1Size distribution of *An. gambiae* hemocytes isolated from sugar fed (SF), 1 and 2 dpbm mosquitoes

	All Hemocytes			Euploid Hemocytes			Polyploid Hemocytes		
	SF	1 dpbm	2 dpbm	SF	1 dpbm	2 dpbm	SF	1 dpbm	2 dpbm
M1 (5 µm)*	24.6 ± 1.9**	10.9 ± 3.7	25.2 ± 3.1	26.6 ± 4.4	7.6 ± 3.4	26.5 ± 2.4	8.7 ± 1.3	2.1 ± 0.6	10.4 ± 2.5
M2 (6-10 µm)	19.1 ± 0.9	14.8 ± 4.7	17.8 ± 1.6	21.5 ± 2.5	12.9 ± 4.9	18.0 ± 1.3	13.3 ± 2.3	5.0 ± 1.8	14.9 ± 2.5
M3 (11-15 µm)	11.4 ± 0.2	11.9 ± 2.7	10.8 ± 0.6	15.3 ± 0.4	14.4 ± 3.6	13.6 ± 0.2	12.1 ± 1.5	6.1 ± 1.8	12.3 ± 1.6
M4 (15 µm)	45.1 ± 2.9	62.4 ± 10.9	46.1 ± 4.5	36.8 ± 7.3	65.4 ± 11.8	42.2 ± 3.6	66.1 ± 5.1	86.8 ± 4.3	62.6 ± 5.5

* M1-4, markers indicating distinct size ranges as shown in Figure 4.

** all numbers represent the percentage of hemocytes (means ± 1 SEM) within the particular size marker, N=3