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Mosquito hemocytes preferentially aggregate and phagocytose pathogens in the periostial regions of the heart that experience the most hemolymph flow

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A B S T R A C T

When a mosquito acquires an infection in the hemocoel, dedicated immune cells called hemocytes aggregate around the valves of the heart. These sessile hemocytes are called periostial hemocytes. In the present study we scrutinized the immune response mounted by the periostial hemocytes of the malaria mosquito, *Anopheles gambiae*, against bacterial pathogens, and tested the relationship between periostial hemocyte aggregation, immune activity, and hemolymph flow. Initially, we quantified the process of periostial hemocyte aggregation and found that hemocytes migrate to the periostial regions in response to infection with *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Micrococcus luteus* (all infections tested). Then, we investigated whether the periostial hemocytes are evenly distributed along the six periostial regions of the heart, and found that they preferentially aggregate in the periostial regions of the mid-abdominal segments (4, 5 and 6). This distribution perfectly correlates with the spatial distribution of phagocytic activity along the surface of the heart, and to a lesser extent, with the distribution of melanin deposits. Finally, experiments measuring circulatory physiology found that the majority of hemolymph enters the heart through the ostia located in the periostial regions of abdominal segments 4, 5, and 6. These data show that periostial hemocytes aggregate on the surface of the heart in response to diverse foreign stimuli, and that both hemocytes and immune activity preferentially occur in the regions that experience the swiftest hemolymph flow. Thus, these data show that two major organ systems – the immune and circulatory systems – interact to control infections.

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1. Introduction

Mosquitoes have evolved robust innate immune responses that destroy pathogens (Bartholomay, 2014; Blair and Olson, 2014; Hillyer, 2010; Severo and Levashina, 2014). These immune responses act via phagocytic, lytic, and melanization pathways, and are driven by hemocytes, the fat body, and other tissues. Hemocytes are immune cells that are present in the hemocoel, and can be found both in circulation with the hemolymph as well as attached to tissues (Hillyer and Strand, 2014). Humoral factors that are secreted by hemocytes also circulate in the hemolymph, as well as pathogens that have penetrated the cuticle or have crossed the gut epithelium (Hillyer et al., 2007; Paulson and Grimstad, 1989; Povelones et al., 2011). While many pathogens succumb to immune responses, others escape or survive these assaults, as is the case of malaria parasites in susceptible mosquitoes (Mitri and Vernick, 2012). A further understanding of the dynamics of the immune processes that confer the resistance of mosquitoes to infection may lead to the development of new strategies to control vector-borne disease (Dong et al., 2011; Franz et al., 2006).

Because hemocytes, humoral immune factors and pathogens are present in the hemocoel, their biology is impacted by the circulation of hemolymph. Mosquitoes have an open circulatory system that propels hemolymph via the contractile action of a muscular tube called the dorsal vessel (Glenn et al., 2010; League et al., 2015). This dorsal vessel extends the length of the body and is comprised of two contiguous sections: the aorta in the thorax and the heart in the abdomen. Wave-like contractions of the heart propel the hemolymph, and these contractions periodically reverse the direction in which they propagate. When the contractions propagate toward the head (anterograde), hemolymph enters the dorsal vessel

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through 6 pairs of ostia (valves) that are sequentially located in the anterior portion of abdominal segments 2 through 7, and exits the vessel in the head. When the contractions propagate toward the posterior of the abdomen (retrograde), which occurs a minority of the time, hemolymph enters the dorsal vessel through a single pair of ostia located at the thoraco-abdominal junction and exits the vessel in the 8th abdominal segment (Glenn et al., 2010; League et al., 2015).

Pathogens in the hemocoel, such as malaria parasites and arboviruses, circulate with the hemolymph as they attempt to complete an obligate migration across the mosquito hemocoel (Hillyer et al., 2007; Paulson and Grimstad, 1989). Recently, a novel, antimalarial and antibacterial cellular immune response that occurs on the surface of the heart was described (King and Hillyer, 2012). Specifically, in the absence of infection, mosquitoes possess sessile hemocytes that are attached to the heart and heart-associated tissues at the location of the ostia. Because of their location at the periostial regions, these sessile hemocytes were named periostial hemocytes. Periostial hemocytes rapidly phagocytose *Escherichia coli* and *Plasmodium berghei* that are circulating with the hemolymph, and the periostial regions are the only locations where sessile hemocytes increase in number in response to infection (King and Hillyer, 2012, 2013). This increase in cell number is at least partly due to the directed migration of hemocytes to the periostial regions, which is advantageous as it places immune cells in the areas of highest hemolymph flow, thus increasing the probability that they will encounter circulating pathogens.

In this study we scrutinized the immune response mounted by the periostial hemocytes of the malaria mosquito, *Anopheles gambiae*, against multiple bacterial pathogens. We found that hemocytes aggregate at the periostial regions of the heart in response to all infections tested, and that periostial hemocytes preferentially aggregate in the mid-abdominal segments. This asymmetric distribution of hemocytes correlates with spatial differences in immune activity on the surface of the heart and with spatial differences in the rate of hemolymph flow through the ostia. Thus, these data show the correlative activities of the mosquito circulatory and immune systems, and support the hypothesis that periostial hemocyte aggregation is a basal immune response employed by mosquitoes against foreign invaders.

2. Materials and methods

2.1. Mosquito rearing and maintenance

A. gambiae Giles sensu stricto (G3 strain) were reared and maintained at 27 °C and 75% relative humidity under a 12 h: 12 h light/dark photoperiod, and adults were fed a 10% sucrose solution as previously described (Estévez-Lao et al., 2013). All experiments were carried out using female mosquitoes at 5 or 6 days post-eclosion.

2.2. Mosquito injections and bacterial infections

For injections, mosquitoes were anesthetized on ice, a finely pulled glass needle was inserted through the thoracic anepisternal cleft, and 0.15–0.20 μl of a solution was injected into the hemocoel. For bacterial infections, tetracycline resistant/GFP-expressing *E. coli* (DH5 alpha), *Micrococcus luteus*, *Staphylococcus aureus* (RN6390), and *Staphylococcus epidermidis* (HIP 04645) were grown and injected into mosquitoes as previously described (Coggins et al., 2012). Across experimental trials, infection doses per mosquito averaged 54,720 for *E. coli*, 45,733 for *S. epidermidis*, and 67,789 for *S. aureus*. Because *M. luteus* does not form individual colonies under our growing conditions, the exact dose per mosquito is unknown. However, all *M. luteus* infections were performed with cultures at $OD_{600} = 5.0$. Control mosquitoes were injected with sterile LB broth (injured) or were left untreated (naïve).

2.3. Hemocyte labeling and mosquito dissections

Periostial hemocytes were stained and observed as previously described (King and Hillyer, 2012). Briefly, mosquitoes were anesthetized and injected 75 mM Vybrant CM-DiI Cell-Labeling Solution (Invitrogen, Carlsbad, CA, USA) and 0.75 mM Hoechst 33342 (Invitrogen) in PBS. Live mosquitoes were incubated at 27 °C for 20 min, which allows the dye to incorporate into the hemocytes. Mosquitoes were anesthetized again, injected 16% formaldehyde, and the dorsal abdomens were placed in PBS containing 0.1% Tween-20 before being bisected along the coronal plane. The dorsal abdomens, which contain the heart and the periostial hemocytes, were then either mounted between a microscope slide and a coverslip using Aqua Poly/Mount (Polysciences; Warrington, PA, USA) or further processed to stain muscle tissue. For the staining of muscle (including the heart), dorsal abdomens were incubated in 4% formaldehyde for 5 min, suspended in 0.6 μM phalloidin-Alexa Fluor 488 (Invitrogen) in PBS for 10 h at 4 °C, washed twice in PBS, and mounted on microscope slides.

2.4. Bright field and fluorescence microscopy: acquisition of still images

A Nikon 90i compound microscope (Nikon Corp., Tokyo, Japan) equipped with a Nikon Intensilight C-HGFI fluorescence illumination unit and a Nikon DS-Qi1Mc CCD camera was used for the acquisition of still images. Samples were visualized under bright field and/or epi-fluorescence illumination, and Z-stacked images were acquired using a linear encoded Z-motor and Nikon Advanced Research NIS-Elements software. For the rendering of two-dimensional images from three-dimensional Z-stacks, all images in a Z-stack were combined to form a single focused image using the Extended Depth of Focus tool of NIS-Elements.

2.5. Counting of periostial hemocytes

Immediately after a dorsal abdomen was mounted on a microscope slide, it was visualized at 200–400× magnification and the periostial hemocytes present in the second through seventh abdominal segments were counted. A cell was considered a periostial hemocyte if it resided in a region immediately adjacent to an ostium or was aggregated with hemocytes that were associated with an ostium. Furthermore, for a cell to be considered a periostial hemocyte it had to be 9–18 μm in diameter, and it had to be labeled with both CM-DiI and Hoechst 33342. For each treatment group (naïve, injured, and infected), a minimum of 20 mosquitoes were analyzed across 11 independent trials.

2.6. Quantification of phagocytosis and melanization of live *Escherichia coli*

At 4, 9 and 24 h post-treatment, live mosquitoes were anesthetized and the dorsal abdomens were dissected and mounted on microscope slides. Fluorescence and brightfield Z-stacked images were acquired sequentially at 40× magnification, using identical microscope and camera settings for all samples.

Phagocytosis of *E. coli*-GFP was quantified by measuring fluorescence pixel intensity. Briefly, extended depth of focus images from naïve, injured and infected mosquitoes were used to determine the threshold of background fluorescence, and pixels with values below the threshold were assigned a value of zero as they

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