The IMD and JNK pathways drive the functional integration of the immune and circulatory systems of mosquitoes

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Abstract

The immune and circulatory systems of animals are functionally integrated. In mammals, the spleen and lymph nodes filter and destroy microbes circulating in the blood and lymph, respectively. In insects, immune cells that surround the heart valves (ostia), called perioistal hemocytes, destroy pathogens in the areas of the body that experience the swiftest hemolymph (blood) flow. An infection recruits additional perioistal hemocytes, amplifying heart-associated immune responses. Although the structural mechanics of perioistal hemocyte aggregation have been defined, the genetic factors that regulate this process remain less understood. Here, we conducted RNAseq in the African malaria mosquito, *Anopheles gambiae*, and discovered that an infection upregulates multiple components of the IMD and JNK pathways in the heart with perioistal hemocytes. This upregulation is greater in the heart with perioistal hemocytes than in the circulating hemocytes or the entire abdomen. RNAi-based knockdown then showed that the IMD and JNK pathways drive perioistal hemocyte aggregation and alter phagocytosis and melanization on the heart, thereby demonstrating how these pathways regulate the functional integration between the immune and circulatory systems. Understanding how insects fight infection lays the foundation for novel strategies that could protect beneficial insects and harm detrimental ones.

Introduction

Insect immune cells, called hemocytes, produce pattern recognition receptors that detect microbial invaders, activate immune signaling pathways, and kill pathogens via phagocytosis, lysis, melanization and other mechanisms (Hillyer, 2016; Hillyer & Strand, 2014; Strand, 2008). These hemocytes exist in a dynamic body cavity called the hemocoel, where the flow of hemolymph constantly circulates them throughout the body. However, not all hemocytes circulate; one quarter of hemocytes are attached to tissues and remain sessile (King & Hillyer, 2013). A sub-population of these sessile hemocytes concentrates on the outer surface of the heart and, more specifically, in the regions surrounding heart valves called ostia (King & Hillyer, 2012; Sigle & Hillyer, 2016). These heart-associated hemocytes, called periostial hemocytes, reside in the locations of the body that experience the highest hemolymph flow, and intensely phagocytose bacteria and malaria parasites within seconds of their entry into the hemocoel. As this is happening, additional hemocytes leave circulation and aggregate in the periostial regions of the heart, thereby augmenting the heart-associated immune response (King & Hillyer, 2012; Sigle & Hillyer, 2016). Although the kinetics and structural mechanics of periostial immunity have only been described in the African malaria mosquito, *Anopheles gambiae* (King & Hillyer, 2012; League & Hillyer, 2016; Sigle & Hillyer, 2016, 2018b, 2018c), the functional integration between the immune and circulatory systems is conserved across the entire insect lineage (Yan & Hillyer, 2020).

In mosquitoes, pattern recognition receptors in the thioester-containing protein family, together with adhesion and phagocytosis factors in the Nimrod protein family, positively regulate immune responses on the heart (Sigle & Hillyer, 2018a; Yan &

Hillyer, 2019). However, heart-associated immune responses are triggered by both bacterial and malarial infections, and by both peptidoglycan and B-1,3-glucan (King & Hillyer, 2012; Sigle & Hillyer, 2016). Hence, the regulatory networks that drive perioistial hemocyte aggregation undoubtedly extend beyond pattern recognition receptors and adhesion molecules. In insects, immune responses are primarily driven by pathways such as Toll, immune deficiency (IMD), c-Jun N-terminal kinase (JNK), and Jak/Stat (Hillyer, 2016). The Toll pathway acts via the transcription factor Rel1, and primarily responds to Gram(+) bacteria, fungi and rodent malaria parasites, whereas the IMD and interlinked JNK pathways act via the transcription factors Rel2 and JNK, respectively, and primarily respond to Gram(–) bacteria and human malaria parasites (King, 2020; Shaw & Catteruccia, 2019). In fruit flies, the Toll pathway is also involved in hemocyte differentiation, and its constitutive activation disrupts sessile hemocytes (Banerjee, Girard, Goins, & Spratford, 2019; Schmid et al., 2016). The IMD pathway has not been directly linked to cellular immunity but its canonical activator, PGRP-LC, positively regulates phagocytosis of *Escherichia coli* but not *Staphylococcus aureus* (Rämet, Manfrueilli, Pearson, Mathey-Prevot, & Ezekowitz, 2002). The Jak/Stat pathway acts via the Stat family of transcription factors. In mosquitoes, this pathway responds to malaria parasites and viruses (King, 2020; Shaw & Catteruccia, 2019), and in fruit flies it controls hemocyte maturation and differentiation (Krzemień et al., 2007; Minakhina, Tan, & Steward, 2011).

The goal of this study was to identify genes and regulatory pathways that drive perioistial hemocyte aggregation in *A. gambiae*. Using an unbiased RNA sequencing (RNAseq) approach that was followed by empirical testing with RNA interference

(RNAi), we uncovered that the IMD and JNK pathways drive periostial hemocyte aggregation and immune responses on the heart, thereby regulating the functional integration between the immune and circulatory systems of mosquitoes.

Results

Infection upregulates immune genes in periostial hemocytes

To identify genes that may drive periostial hemocyte aggregation, we undertook an unbiased RNAseq approach where we sequenced the transcriptome of tissues from mosquitoes that were naïve, injured, or infected with Gram(–) GFP-*E. coli* or Gram(+) *S. aureus* (Figure 1A-B; *Dataset S1*). Three tissues were isolated at 4 hr after treatment: (i) the heart containing periostial hemocytes, (ii) the hemolymph containing circulating hemocytes, and (iii) the entire abdomen. In these experiments, most mosquitoes were used for sequencing, but a subset of mosquitoes was used to confirm that infection induces heart-associated cellular immune responses. Indeed, a resident population of periostial hemocytes was present in both naïve and injured mosquitoes, and the number of periostial hemocytes increased 1.8- and 1.6-fold after GFP-*E. coli* and *S. aureus* infection, respectively (Figure 1B-C).

RNAseq revealed that, relative to the heart and periostial hemocytes of naïve and injured mosquitoes, 73 and 132 genes are upregulated more than 4-fold ($P < 0.05$) in the heart and periostial hemocytes of mosquitoes infected with GFP-*E. coli* and *S. aureus*, respectively (Figure 1D). Because periostial hemocyte aggregation is a dynamic immune response that occurs during any bacterial infection, we hypothesized that its core molecular drivers must be preferentially upregulated in periostial hemocytes

108 following both *E. coli* and *S. aureus* infection. We found that 55 genes met this overlap
 109 criterion (Figure 1E; Figure 1–figure supplement 1). Comparing the heart and peristial
 110 hemocyte RNAseq data to RNAseq data from (i) circulating hemocytes and (ii) the
 111 entire abdomen then revealed that infection upregulates more genes in the heart with
 112 peristial hemocytes than in the circulating hemocytes or the abdomen (Figure 1D-E;
 113 Figure 1–figure supplement 1-3).

114 **Figure 1. Infection upregulates immune genes in peristial hemocytes.** (A) The
 115 heart with peristial hemocytes, the circulating hemocytes, and the abdomen of
 116 mosquitoes that were naïve, injured, or infected with GFP-*E. coli* or *S. aureus*
 117 were sequenced by RNAseq at 4 hr post-treatment. (B) Fluorescence and DIC overlay shows
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that the perioistal hemocytes (red) remain attached to a resected heart (green). Image is modified from Sigle and Hillyer (Sigle & Hillyer, 2018b), and reproduced according to Creative Commons Attribution License CC BY. (C) Naïve and injured mosquitoes have resident perioistal hemocytes, but infection for 4 hr with GFP-*E. coli* or *S. aureus* induces the aggregation of additional hemocytes at the perioistal regions. Whiskers show the standard error of the mean (S.E.M.; n = 14 for all groups). (D) Bar plots show the number of genes significantly upregulated or downregulated at 4 hr after GFP-*E. coli* or *S. aureus* infection in the perioistal hemocytes and heart (left), the circulating hemocytes (middle), and the entire abdomen (right). (E) Venn diagrams show that 55, 21 and 15 genes are significantly upregulated in the perioistal hemocytes, the circulating hemocytes and the abdomen, respectively, during both GFP-*E. coli* and *S. aureus* infections. (F) Table listing the immune genes that are among the 55 genes that were upregulated in the heart with perioistal hemocytes following both GFP-*E. coli* or *S. aureus* infection. Genes that participate in the IMD/JNK pathway are in bold.

We then scrutinized the reported function of the 55 candidate genes. Twelve have immune function (Figure 1F), and strikingly, 6 are part of IMD and JNK pathway: *PGRP-LA* (Gendrin et al., 2017), *IAP2* (Myllymäki, Valanne, & Rämet, 2014), *rel2* (Garver et al., 2012; Meister et al., 2005), *cec3* (Meister et al., 2005), *def1* (Garver, Dong, & Dimopoulos, 2009; Luna et al., 2006), and *JNK3* (Boutros, Agaisse, & Perrimon, 2002; Souvannaseng et al., 2018). Multiple aspects of the IMD/JNK pathway are represented: upstream components such as *PGRP-LA* and *IAP2*, and downstream elements from both branches of the pathway, with the classical IMD cascade represented by *rel2*, *cec3* and *def1*, and the bifurcation into the JNK pathway represented by *JNK3* (Myllymäki et al., 2014; Silverman et al., 2003; Zhuang et al., 2006) (Figure 1—figure supplement 4). Relevant to this finding, activating the IMD pathway induces the expression of *eater* (Wong et al., 2017), which positively regulates hemocyte adhesion in fruit flies and perioistal hemocyte aggregation in mosquitoes (Bretscher et al., 2015; Sigle & Hillyer, 2018a). The IMD pathway also induces the expression of *TEP1*, which is involved in phagocytosis and perioistal hemocyte

aggregation (Yan & Hillyer, 2019). The JNK pathway is associated with cell adhesion and phagocytosis by regulating the cytoskeleton (Boutros et al., 2002; Etchegaray et al., 2012). The JNK pathway also induces the expression of *draper* (Weavers, Evans, Martin, & Wood, 2016) and *TEP1* (Souvannaseng et al., 2018), which affect peritostial hemocyte aggregation in mosquitoes (Sigle & Hillyer, 2018a; Yan & Hillyer, 2019).

All six IMD and JNK pathway genes identified in our unbiased screen were more highly upregulated in the heart and peritostial hemocytes than in the other tissues (Figure 1–figure supplement 5). The remaining six candidate genes with immune function that were identified in our screen – *CLIPB17*, *DDC*, *SRPN16*, *CLIPB11*, *PGRP-LB* and *upd3k* – do not participate in a common pathway. Therefore, based on the RNAseq analyses we hypothesized that the main driver of peritostial hemocyte aggregation is the IMD/JNK pathway.

The IMD pathway positively regulates peritostial hemocyte aggregation

To determine whether the IMD pathway regulates peritostial hemocyte aggregation, we synthesized double-stranded RNA (dsRNA) to target the IMD cascade transcription factor, *rel2*, and its negative regulator, *caspar*, and achieved RNAi-based silencing of 43% and 37%, respectively, compared to the *dsbla(Ap^R)* control mosquitoes (Figure 2–figure supplements 1-2). Moreover, because *Tep1* is (i) activated by the Rel2 arm of the IMD pathway (Feng et al., 2021; Frolet, Thoma, Blandin, Hoffmann, & Levashina, 2006) and (ii) a positive driver of peritostial hemocyte aggregation (Yan & Hillyer, 2019), we measured its mRNA abundance in *rel2* and *caspar* RNAi mosquitoes. Silencing of *rel2* did not alter *TEP1* mRNA abundance but silencing of *caspar* increased

it. Because *cec3* and *def1* are also activated by Rel2 (Garver et al., 2009; Luna et al., 2006; Meister et al., 2005), we measured their mRNA abundance in RNAi mosquitoes. Silencing of *rel2* decreased *cec3* and *def1* mRNA abundance whereas silencing of *caspar* increased them (Figure 2–figure supplement 1). Overall, these data are consistent with the role that the Rel2 arm of the IMD pathway plays in regulating *TEP1*, *cec3* and *def1* expression.

To assess whether the IMD pathway drives heart-associated immune responses, we compared the number and activity of perioistial hemocytes in *rel2* and *caspar* RNAi mosquitoes, relative to *dsbla(Ap^R)* control mosquitoes (Figure 2). As expected, infection for 4 hr induced the aggregation of hemocytes at the perioistial regions of *dsbla(Ap^R)* mosquitoes, and this aggregation remained in place at 24 hr. In uninfected mosquitoes, knocking down *rel2* did not change the number of perioistial hemocytes, but at 4 hr following infection, knockdown of *rel2* decreased the number of perioistial hemocytes by 18%, relative to *dsbla(Ap^R)* mosquitoes (Figure 2A-B). At 24 hr post-infection, the effect of *rel2* RNAi on perioistial hemocyte aggregation was diminished. When we instead knocked down *caspar*, the number of perioistial hemocytes increased by 39% in uninfected mosquitoes, and infection for 4 and 24 hr increased the number of perioistial hemocytes by 30% and 19%, respectively, relative to similarly treated *dsbla(Ap^R)* mosquitoes. This shows that *rel2* positively regulates perioistial hemocyte aggregation whereas *caspar* negatively regulates this process.

Figure 2. The IMD pathway drives periostial hemocyte aggregation. (A)

Fluorescence images show periostial hemocytes (CM-Dil; red) surrounding a single pair of ostia (asterisks) on a segment of the heart (outlined by dotted lines) of *dsbla(Ap^R)*, *dsrel2* and *dscaspar* mosquitoes that were not infected or had been infected with GFP-*E. coli* for 4 or 24 hr. Anterior is on top. (B-F) Graphs for *dsbla(Ap^R)*, *dsrel2* and *dscaspar* mosquitoes that were not infected or had been infected with GFP-*E. coli* for 4 or 24 hr. The graphs show: (B) average number of periostial hemocytes; (C) average number of sessile hemocytes outside of the periostial regions in the tergum of abdominal segments 4 and 5; (D) pixel area of GFP-*E. coli* in the periostial regions; (E) pixel area of melanin in the periostial regions; and (F) the systemic GFP-*E. coli* infection intensity. Graphs in B-F show the mean and S.E.M. The data were analyzed by two-way ANOVA (bottom box), followed by Dunnett's multiple comparison test. N indicates sample size. Asterisks in graphs indicate post-hoc P < 0.05.

To determine whether the IMD pathway specifically affects periostial hemocytes and not sessile hemocytes in general, we counted the number of sessile hemocytes outside of the periostial regions in the tergum of abdominal segments 4 and 5 in the same mosquitoes examined for periostial hemocytes (Figure 2C). Knockdown of *rel2* or *caspar* did not alter the number of non-periostial sessile hemocytes in uninfected mosquitoes or mosquitoes infected for 4 hr. However, at 24 hr following infection, *caspar* RNAi decreased non-periostial sessile hemocytes by 22%. These results demonstrate that the IMD pathway regulates periostial hemocyte aggregation while having a minimal effect on the rest of the sessile hemocytes.

Periostial hemocytes phagocytose bacteria, leading to their accumulation at the periostial regions (King & Hillyer, 2012; Sigle & Hillyer, 2016). To determine whether the IMD pathway affects the phagocytic activity of periostial hemocytes, we measured the GFP-*E. coli* fluorescence pixel area in the periostial regions of infected mosquitoes (Figure 2D; Figure 2–figure supplement 3). At 4 hr following infection, the GFP-*E. coli* pixel area was similar regardless of dsRNA treatment. At 24 hr after infection, this area decreased in *dsbla(Ap^R)* and *dscaspar* mosquitoes, indicating that periostial hemocytes efficiently destroyed the pathogens. However, at 24 hr following infection, *rel2* RNAi significantly increased GFP-*E. coli* accumulation at the periostial regions. Periostial hemocytes also phagocytose melanized bacteria (King & Hillyer, 2012; Sigle & Hillyer, 2016). Therefore, we measured melanin accumulation in the periostial regions (Figure 2E; Figure 2–figure supplement 3). Melanin was absent in uninfected mosquitoes and mosquitoes that were infected for 4 hr. However, at 24 hr following infection, *rel2* RNAi

increased melanin deposits at the periostial regions whereas *caspar* RNAi had no effect.

The increased accumulation of GFP-*E. coli* and melanin in *rel2* RNAi mosquitoes at 24 hr after infection could be due to either (i) enhanced phagocytosis by periostial hemocytes, or (ii) higher bacterial proliferation in the hemocoel, which places increased pressure on the phagocytosis response. To differentiate between these two scenarios, we measured the systemic GFP-*E. coli* infection intensity and observed that, at 4 hr after infection, the bacterial intensity was similar for all dsRNA treatments, but at 24 hr, knockdown of *rel2* resulted in a higher infection intensity than treatment with *dscaspar* or *dsbla(Ap^R)* (Figure 2F). This suggests that *rel2* is essential for proper bacterial killing in the hemocoel, and therefore, knocking it down increases infection intensity in a manner that leads to increased phagocytosis in the periostial regions. However, because silencing *rel2* and *caspar* had opposite effects on periostial hemocyte aggregation at 4 hr, a time when dsRNA treatment does not impact infection intensity, we conclude that the IMD pathway is a positive regulator of periostial hemocyte aggregation.

PGRP-LC is the canonical activator of the IMD pathway (Hillyer, 2016), but PGRP-LA (i) activates the IMD pathway in the midgut of mosquitoes and in the barrier epithelia of fruit flies (Gendrin et al., 2017; Gendrin et al., 2013), (ii) is expressed in *Drosophila* hemocytes (Werner et al., 2000), and (iii) is upregulated in the heart and periostial hemocytes (Figure 1F). *PGRP-LA* has three splice forms. To knock it down, we synthesized *dsPGRP-LA-RARB* to target the *RA* and *RB* splice forms, and *dsPGRP-LA-RC* to target the *RC* splice form. Using these dsRNAs, we achieved RNAi-based silencing that ranged from 56% to 77%, compared to the *dsbla(Ap^R)* control mosquitoes

(Figure 3—figure supplement 1). *PGRP-LA* knockdown with any dsRNA did not alter the number of periostial hemocytes, the number of non-periostial sessile hemocytes, melanin accumulation at the periostial regions, or systemic infection intensity (Figure 3). However, knockdown of *PGRP-LA-RC* increased phagocytosis of GFP-*E. coli* in the periostial regions, which suggests that the RC splice form is involved in the phagocytosis response.

Figure 3. The RC splice form of PGRP-LA regulates phagocytosis by periostial hemocytes. (A-E) Graphs for *dsbla(Ap^R)*, *dsPGRP-LA-RARB* and *dsPGRP-LA-RC* mosquitoes that were not infected or had been infected with GFP-*E. coli* for 4 or 24 hr. The graphs show: (A) average number of periostial hemocytes; (B) average number of sessile hemocytes outside of the periostial regions in the tergum of abdominal segments 4 and 5; (C) pixel area of GFP-*E. coli* in the periostial regions; (D) pixel area

of melanin in the periostial regions; and (E) the systemic GFP-*E. coli* infection intensity. Graphs show the mean and S.E.M. The data were analyzed by two-way ANOVA (bottom box), followed by Dunnett's multiple comparison test. N indicates sample size. Asterisk in graph indicate post-hoc $p < 0.05$.

The JNK pathway positively regulates periostial hemocyte aggregation

We next tested whether the JNK pathway regulates periostial hemocyte aggregation. *A. gambiae* encodes two JNK genes: *JNK1* and *JNK3* (Horton et al., 2011). Therefore, we synthesized double-stranded RNA (dsRNA) to target the JNK cascade transcription factors, *JNK1* and *JNK3*, and their negative regulator, *puckered* (*puc*). Because of high sequence identity between *JNK1* and *JNK3*, ds*JNK1/3* simultaneously targeted both JNK genes. RNAi-based knockdown resulted in 33%, 44% and 31% reduction in mRNA abundance of *JNK1*, *JNK3* and *puc*, respectively (Figure 4—figure supplement 1). Moreover, because the JNK pathway has also been implicated in the expression of *TEP1* (Garver, de Almeida Oliveira, & Barillas-Mury, 2013), we measured the mRNA abundance of *TEP1*, *cec3* and *def1* in *JNK1/JNK3* and *puc* RNAi mosquitoes. Manipulating the JNK pathway did not have a clear impact on effector gene expression (Figure 4—figure supplement 1). The lack of clarity on how the JNK pathway controls the expression of *TEP1*, *cec3* or *def1* is likely due to their co-regulation by other pathways.

To assess whether the JNK pathway drives heart-associated immune responses, we compared the number and activity of periostial hemocytes in *JNK1/3* and *puc* RNAi mosquitoes, relative to ds*bla*(*Ap^R*) control mosquitoes (Figure 4). Regardless of infection status, the number of periostial hemocytes was statistically similar between ds*JNK1/3* and ds*bla*(*Ap^R*) mosquitoes, although ds*JNK1/3* mosquitoes averaged fewer

periostial hemocytes than control mosquitoes for all treatments (Figure 4A-B). Knockdown of *puc*, however, increased the number of periostial hemocytes 1.6-fold in uninfected mosquitoes, and 1.4- and 1.5-fold at 4 and 24 hr following GFP-*E. coli* infection, respectively, relative to similarly treated *dsbla*(*Ap^R*) mosquitoes. To further determine whether *puc* specifically affects periostial hemocytes or sessile hemocytes in general, we quantified the number of non-periostial sessile hemocytes on the tergum of the same mosquitoes (Figure 4C). *JNK1/3* RNAi mosquitoes showed a trend of fewer non-periostial sessile hemocytes than control mosquitoes regardless of infection status. Treatment with *dspuc*, however, increased the number of non-periostial sessile hemocytes for all treatments. This demonstrates that the JNK pathway positively regulates both periostial and non-periostial sessile hemocyte abundance.

We next measured whether the JNK pathway affects the phagocytic activity of periostial hemocytes (Figure 4D; Figure 4–figure supplement 2). At 4 hr following infection, the GFP-*E. coli* pixel area was similar regardless of dsRNA treatment. However, at 24 hr following infection, periostial hemocytes in *dspuc* mosquitoes phagocytosed more GFP-*E. coli* than the *dsbla*(*Ap^R*) mosquitoes. We then quantified the melanized bacteria that were sequestered by periostial hemocytes (Figure 4E; Figure 4–figure supplement 2). Melanin deposits were undetectable in uninfected mosquitoes and mosquitoes at 4 hr post infection; however, at 24 hr following GFP-*E. coli* infection, more melanin was present in *dspuc* mosquitoes than in *dsbla*(*Ap^R*) controls.

Figure 4. The JNK pathway drives periostial hemocyte aggregation. (A)

Fluorescence images show periostial hemocytes (CM-Dil; red) surrounding a single pair of ostia (asterisks) on a segment of the heart (outlined by dotted lines) of *dsbla(Ap^R)*, *dsJNK1/3* and *dspuc* mosquitoes that were not infected or had been infected with GFP-*E. coli* for 4 or 24 hr. Anterior is on top. (B-F) Graphs for *dsbla(Ap^R)*, *dsJNK1/3* and *dspuc* mosquitoes that were not infected or had been infected with GFP-*E. coli* for 4 or 24 hr. The graphs show: (B) average number of periostial hemocytes; (C) average number of sessile hemocytes outside of the periostial regions in the tergum of abdominal segments 4 and 5; (D) pixel area of GFP-*E. coli* in the periostial regions; (E) pixel area of melanin in the periostial regions; and (F) the systemic GFP-*E. coli* infection intensity. Graphs in B-F show the mean and S.E.M. The data were analyzed by two-way ANOVA (bottom box), followed by Dunnett's multiple comparison test. N indicates sample size. Asterisks in graphs indicate post-hoc P < 0.05.

The difference in GFP-*E. coli* and melanin accumulation in *dspuc* mosquitoes could be due to (i) enhanced phagocytosis by periostial hemocytes, or (ii) higher bacterial proliferation in the hemocoel. To differentiate between these two scenarios, we quantified the systemic *E. coli* infection intensity and found that the bacterial intensity was similar regardless of dsRNA treatment (Figure 4F). Therefore, this suggests that *puc* negatively regulates hemocyte adhesion and phagocytic activity, and demonstrates that the JNK pathway is a positive regulator of periostial immune responses.

Discussion

Previous microarray and RNAseq analyses revealed genes and signaling pathways that are active in hemocytes (Bartholomay et al., 2004; Baton, Robertson, Warr, Strand, & Dimopoulos, 2009; Choi, Fuchs, Mayhew, Yu, & Christensen, 2012; H Kwon & Smith, 2019; Pinto et al., 2009; Rani et al., 2021; Thomas et al., 2016), including those that are activated in specific subpopulations of hemocytes (H. Kwon, Mohammed, Franzén, Ankarklev, & Smith, 2021; Raddi et al., 2020; Severo et al., 2018). However, these studies only focused on circulating hemocytes, and therefore, failed to capture the biology of sessile hemocytes. Yet, one quarter of hemocytes are sessile (King & Hillyer, 2013), and they play significant roles in hematopoiesis (Leitão & Sucena, 2015; Márkus et al., 2009), wound healing (Babcock et al., 2008), and pathogen killing (Horn, Leips, & Starz-Gaiano, 2014; Kocks et al., 2005; League & Hillyer, 2016). Therefore, extrapolating the molecular signatures of circulating hemocytes to those of sessile hemocytes likely misses the essential factors that make sessile hemocytes conduct their specific immune activities. Periostial hemocytes are a

subpopulation of sessile hemocytes that reside on the heart, where they sequester and kill pathogens in areas of high hemolymph flow (King & Hillyer, 2012; Sigle & Hillyer, 2016). To better understand how an infection drives the migration of hemocytes to the heart and how these immune cells kill pathogens at the periostial regions, we sequenced the periostial hemocyte transcriptome and discovered that the IMD/JNK pathway drives periostial immune responses (Figure 5).

Figure 5. The IMD and JNK pathways, together with TEP proteins (TEP1, TEP3 and TEP4) and Nimrod proteins (Draper and Eater), regulate heart-associated immune responses in mosquitoes.

The IMD pathway controls the production of antimicrobial peptides (Hillyer, 2016; Myllymäki et al., 2014), but here we show that the IMD pathway also regulates a cellular immune response: the transition of hemocytes from a circulating to a sessile state on

the heart. Specifically, knockdown of the positive regulator of the IMD pathway, *rel2*, decreases infection-induced peristial hemocyte aggregation, whereas knockdown of the negative regulator of the IMD pathway, *caspar*, increases the number of peristial hemocytes. We hypothesize that the IMD pathway drives peristial hemocyte aggregation via two cascades that are also driving phagocytosis: the TEP1-TEP3-LRP1-CED6L and TEP4-BINT2-CED2L-CED5L pathways (Moita et al., 2005). The IMD pathway induces the expression of *TEP1* and *TEP4* (Dong et al., 2011; Garver et al., 2009; Luna et al., 2006), and both of these genes positively regulate peristial hemocyte aggregation (Yan & Hillyer, 2019). Moreover, two downstream molecules – LRP1 and BINT2 – are transmembrane proteins that in mammals and insects have overlapping functions in phagocytosis and adhesion (Mantuano, Jo, Gonias, & Campana, 2010; Moita, Vriend, Mahairaki, Louis, & Kafatos, 2006; Rabiej et al., 2016), and therefore, likely facilitate the adhesion of hemocytes to the heart. In this study, we also confirmed the positive role that REL2 plays in pathogen killing (Dekmak, Yang, Zu Dohna, Buchon, & Osta, 2021; Dong et al., 2011; Dong, Simões, & Dimopoulos, 2020; Frolet et al., 2006; Kambris et al., 2010; Khan, Liew, Leong, & Lau, 2016; Meister et al., 2009; Meister et al., 2005; Mitri et al., 2009; Schnitger, Kafatos, & Osta, 2007). When we systemically knocked down the expression of *rel2*, bacteria proliferated uncontrollably during the later stages of infection. We hypothesize that knocking down *rel2* initially suppresses peristial hemocyte aggregation, but as the infection progresses and the intensity increases, the necessity of peristial immune responses also increases, and this pressure recruits hemocytes to the heart and enhances their collective phagocytic activity. Overall, our data show that the IMD pathway drives

periostial hemocyte aggregation during the early stages of infection and limits systemic bacterial intensity during the later stages of infection.

The JNK pathway modulates mosquito longevity, regulates oviposition, and limits infection with malaria parasites and viruses (Chowdhury et al., 2020; Garver et al., 2013; Nsango et al., 2013; Peirce et al., 2020; Ramphul, Garver, Molina-Cruz, Canepa, & Barillas-Mury, 2015; Souvannaseng et al., 2018). Here, we found that overexpressing the JNK pathway by knocking down the negative regulator, *puc*, increases the number of periostial and adjacent sessile hemocytes. This suggests that the JNK pathway positively regulates hemocyte adhesion. Indeed, the *Drosophila* ortholog of mosquito *JNK1* and *JNK3*, called *basket*, is involved in the formation of actin-rich and focal adhesion kinase-rich placodes in hemocytes (Williams, Wiklund, Wikman, & Hultmark, 2006). The JNK pathway also induces hemocyte differentiation in *Drosophila*, producing large lamellocytes that adhere and encapsulate parasitoid eggs (Zettervall et al., 2004). We found that overactivating the JNK pathway does not alter infection intensity in mosquitoes, but it increases the accumulation of *E. coli* and melanin on the heart, suggesting that the JNK pathway positively regulates the phagocytosis and melanization responses. Indeed, *JNK1* positively regulates melanization in mosquitoes that are refractory to malaria (Garver et al., 2013), and overexpressing the JNK pathway in aphids increases the melanin-producing activity of phenoloxidase and the phagocytic activity of hemocytes (Ma et al., 2020). We hypothesize that the JNK pathway regulates phagocytosis by periostial hemocytes in a manner that involves two proteins already known to be involved in periostial hemocyte aggregation: TEP1 and draper (Sigle & Hillyer, 2018a; Yan & Hillyer, 2019). The JNK pathway activates the expression of both

of these genes (Macdonald, Doherty, Hackett, & Freeman, 2013; Peirce et al., 2020; Souvannaseng et al., 2018), and TEP1 opsonizes pathogens for phagocytosis whereas draper activates phagocytic processes (Evans, Rodrigues, Armitage, & Wood, 2015; Levashina et al., 2001; Manaka et al., 2004; Moita et al., 2005; Sigle & Hillyer, 2018a; Yan & Hillyer, 2019). In our study, we could not distinguish between the roles that *JNK1* and *JNK3* play in peristial hemocyte aggregation. However, simultaneous knockdown of both resulted in phenotypes that were opposite of *puc* knockdown, strongly suggesting that JNK1 or JNK3 – or both – positively regulates peristial hemocyte aggregation.

Because both the IMD and JNK pathways induce the production of *TEP1* (Dong et al., 2011; Garver et al., 2009; Peirce et al., 2020; Souvannaseng et al., 2018), we hypothesize that they share the TEP1-TEP3-LRP1-CED6L phagocytosis cascade that leads to hemocyte aggregation. However, we also hypothesize that the IMD and JNK pathways use additional, independent mechanisms to regulate peristial hemocyte aggregation for three reasons. First, even though the two pathways share upstream signaling molecules, they bifurcate and activate their own set of effector genes (Silverman et al., 2003; Zhuang et al., 2006). Second, knocking down the components of the IMD and JNK pathways resulted in different phenotypes, especially for peristial and non-peristial sessile hemocytes. Third, in *Drosophila*, draper and the BINT2 ortholog (Integrin β_v) function independently (Nagaosa et al., 2011), and in mosquitoes, draper is regulated by the JNK pathway whereas BINT2 is involved in the IMD-regulated and TEP4-mediated phagocytosis cascade (Luna et al., 2006; Macdonald et al., 2013; Moita et al., 2006; Moita et al., 2005).

Bacteria, malaria parasites, and fungal components all induce periostial hemocyte aggregation, and this process is structurally conserved across the entire insect lineage (King & Hillyer, 2012; Sigle & Hillyer, 2016; Yan & Hillyer, 2020). Therefore, it is likely that other proteins contribute to this process. Indeed, infection also induces the upregulation of *Upd3* by periostial hemocytes, which is a ligand for the receptor of the JAK/STAT pathway (Chakrabarti et al., 2016). The TOLL pathway also induces hemocyte proliferation (Chiu et al., 2005; Qiu, Pan, & Govind, 1998) and the expression of *TEP1* (Frolet et al., 2006), which positively regulates periostial hemocyte aggregation (Yan & Hillyer, 2019). Beyond immune pathways, periostial hemocyte aggregation may also be under the influence of neuronal and hormonal control. For example, injecting mosquitoes with the neuropeptide, allatotropin, increases the number of periostial and non-periostial sessile hemocytes (Hernández-Martínez et al., 2017). Therefore, the regulation of periostial hemocyte aggregation is not expected to be limited to the IMD and JNK pathways.

To date, we have tested the involvement of several hemocyte-produced factors on periostial hemocyte aggregation. However, the heart itself may produce complementary factors that drive this immune response. For example, a fibroblast growth factor is highly regulated in the heart and periostial hemocytes. Its ortholog in *Drosophila*, called *Branchless*, is expressed in the heart and pericardial cells, and regulates hemocyte differentiation in the lymph glands of the larval heart (Destalminil-Letourneau, Morin-Poulard, Tian, Vanzo, & Crozatier, 2021; Dragojlovic-Munther & Martinez-Agosto, 2013). Moreover, reducing the expression of the *Drosophila* cardiac extracellular matrix (ECM) proteins, *Pericardin* and *Laminin A*, disrupts the formation of

the cardiac ECM and lowers the number of hemocytes on the heart (Cevik, Acker, Michalski, & Jacobs, 2019; Ghosh, Singh, Mandal, & Mandal, 2015). These data suggest that the heart and its associated structures facilitate the binding of circulating hemocytes to the periostial regions. Our RNAseq experiment also revealed that an infection upregulates transglutaminases in the heart and periostial hemocytes, which are known for crosslinking Rel2 and other proteins (Shibata et al., 2013), and thus, they may also play a role in periostial hemocyte aggregation.

Heart-associated immune responses occur in insects from at least 16 different taxonomic orders, including insects that are of agricultural, urban, and medical importance (Yan & Hillyer, 2020). Understanding the genetic factors that drive periostial hemocyte aggregation in *A. gambiae* sheds light on how this medically important insect and other insects of societal importance fight the pathogens that invade them. Indeed, the TEP gene family, the Nimrod gene family (Eater, Nimrod and Draper) and the JNK pathway are conserved amongst insects (Blandin & Levashina, 2004; Bou Aoun et al., 2011; Horton et al., 2011; Somogyi et al., 2008), and although some insects in the order Hemiptera lack components of the IMD pathway, all insects queried to date still have a functional IMD-based immune response (Gerardo et al., 2010; Mesquita et al., 2015; Nishide et al., 2019; Salcedo-Porras, Guarneri, Oliveira, & Lowenberger, 2019). Therefore, we conclude that the IMD and JNK pathways, together with TEP and Nimrod proteins, are primary regulators of the heart-associated immune responses in insects.

Materials and Methods

Mosquitoes, bacteria, infection, and replication

Anopheles gambiae, Giles sensu stricto (G3; Diptera: Culicidae), were maintained at 27 °C, 75% relative humidity, and a 12 hr:12 hr light:dark photoperiod (Glenn, King, & Hillyer, 2010). Experiments were done using female adults fed 10% sucrose. Tetracycline resistant, GFP-expressing *E. coli* (modified DH5 α ; GFP-*E. coli*) and *S. aureus* (RN6390) were grown in Luria-Bertani (LB) and tryptic soy broth, respectively, at 37 °C in a shaking incubator. Dilutions of the bacterial cultures were injected at the thoracic anepisternal cleft using a Nanoject III (Drummond Scientific Company, Broomall, PA). The infection dose was determined by plating the cultures and counting the colony forming units (CFUs).

In this study, a biological replicate is an independent experiment that utilizes mosquitoes from an independent egg batch. A technical replicate is an experimental resampling of a biological replicate. All data collected were included in the analysis; no data were excluded.

RNAseq: treatment, tissue collection, RNA isolation and library preparation

Six-day-old mosquitoes were randomly divided into four groups: (i) naïve (unmanipulated), (ii) injured by injecting 69 nL of sterile LB, (iii) infected by injecting 69 nL of GFP-*E. coli* (~75,779 CFUs), and (iv) infected by injecting 69 nL of *S. aureus* (~39,451 CFUs). Four hr after treatment, three tissues were isolated: (i) the heart with perioistial hemocytes, (ii) hemolymph with circulating hemocytes, and (iii) the entire abdomen. To isolate the heart, mosquitoes were bisected along the coronal plane in RNase-free PBS, and the heart with the perioistial hemocytes was resected by severing the alary muscles and detaching it from the cuticle (Sigle & Hillyer, 2018b). To isolate

circulating hemocytes, hemolymph was perfused by making an incision in the last abdominal segment, injecting RNase-free PBS into the hemocoel through the cervical membrane, and collecting the first 2 drops that exited the abdomen (Hillyer & Christensen, 2002). The abdomens were isolated by bisecting mosquitoes on the transverse plane along the thoraco-abdominal junction.

To isolate RNA from hearts or abdomens, samples were homogenized in TRIzol Reagent (Invitrogen, Carlsbad, CA), extracted following the TRIzol protocol, and resuspended in Buffer RLT (Qiagen, Hilden, Germany) with 2% 2-mercaptoethanol. The RNA was further purified using the RNeasy Micro Kit (Qiagen), DNase treated, and eluted in RNase-free water. To isolate RNA from circulating hemocytes, perfused hemolymph was collected in Buffer RLT and 2% 2-mercaptoethanol, and RNA was isolated using the RNeasy Micro Kit as above.

No a priori statistical method was used to pre-determine sample sizes. The number of RNAseq samples and biological replicates was determined prior to the initiation of the study, and was based on our determination that three biological replicates is a conservative approach to detect statistical differences (if any) in gene expression. Three biological replicates were conducted; each heart sample contained 72 hearts, each hemolymph sample contained perfusate from 108 mosquitoes, and each abdomen sample contained 36 abdomens.

The integrity and quantity of RNA was assessed on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) using an RNA 6000 Series II Nano kit for abdomen samples and a Pico kit for the heart and hemolymph samples. The library for sequencing was prepared using 1 µg of RNA and the NEBNext Ultra kit (New England

BioLabs, Ipswich, MA), according to the protocol for low-input samples. Library quality and concentration were assessed using a DNA 1000 Series II kit on the 2100 Bioanalyzer.

Illumina sequencing and differential gene expression analysis

All 36 samples – 4 treatments, 3 tissue types and 3 replicates – were sequenced across 3 lanes on an Illumina HiSeq 3000 (paired-end, 75 base pair read) at Vanderbilt University's Vantage facility. Reads were mapped to the *A. gambiae* genome (AgamP4.7) by STAR (Dobin et al., 2013; Giraldo-Calderón et al., 2015). The number of uniquely mapped reads per sample averaged 23,698,077 (range: 18,487,153 – 31,487,002), which represented 94.03% of the total reads in a sample. Differential expression was calculated based on reads per kilobase per million by DESeq2 (Love, Huber, & Anders, 2014). Genes were considered significantly regulated at $\log_2$ fold change ≥ 2 or $\log_2$ fold change ≤ -2 and the Benjamini–Hochberg adjusted $P < 0.05$. RNAseq results are presented in Dataset S1, and includes read counts and $\log_2$ fold changes. Using four-fold expression difference as the criterion, together with experimental replication and statistical validation, is a conservative approach that is proven to yield accurate and reliable results (Everaert et al., 2017). RNAseq data was deposited into NCBI (<https://www.ncbi.nlm.nih.gov>) as SRA data PRJNA730047.

Double-stranded RNA (dsRNA) synthesis and RNA interference (RNAi)

Double-stranded RNA was synthesized for *rel2*, *caspar*, *PGRP-LA*, *JNK1* and *JNK3*, and *puc*. *A. gambiae* cDNA was amplified by PCR using gene-specific primers

with T7 promoter tags (Figure 2–figure supplement 2). dsRNA was synthesis using the MEGAscript T7 Kit (Applied Biosystems) as described (Sigle & Hillyer, 2018a; Yan & Hillyer, 2019). As a negative control, dsRNA was synthesized for the non-mosquito gene, *bla*(*Ap^R*), using DNA from *E. coli* BL21(DE3) containing the pET-46 plasmid as template (EMD Chemicals, Gibbstown, NJ) (Sigle & Hillyer, 2018a; Yan & Hillyer, 2019).

Two- or three-day-old mosquitoes were intrathoracically injected 300 ng of dsRNA. Four days later, mosquitoes were divided into two groups for phenotypic analyses: (i) uninfected and (ii) infected with GFP-*E. coli* (~16,528 CFUs). Injured mosquitoes were not included in the experiments because injury does not induce periostial hemocyte aggregation (King & Hillyer, 2012, 2013; Sigle & Hillyer, 2016). RNAi efficiency of the targeted genes and mRNA abundance of the downstream effector genes *TEP1*, *cec3* and *def1* was determined by qPCR (Yan & Hillyer, 2019). Briefly, RNA was isolated using TRIzol from 10 whole bodies for each time and treatment, purified, and used for cDNA synthesis using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) as described (Sigle & Hillyer, 2018a; Yan & Hillyer, 2019). qPCR was conducted using gene-specific primers (Figure 2–figure supplement 2) and Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a Bio Rad CFX Connect Real-Time Detection System (Hercules, CA). Relative quantification was conducted using the $2^{-\Delta\Delta C_T}$ method, with *RpS7* as the reference and *RpS17* as a control (League, Estévez-Lao, Yan, Garcia-Lopez, & Hillyer, 2017). Two to three biological replicates were conducted per gene, and the value for each biological replicate is the average of two or three technical replicates.

Fluorescence labeling and mosquito dissection

Hemocytes were labeled with the Vybrant CM-Dil Cell-Labeling Solution (Invitrogen) as we described (King & Hillyer, 2012). Briefly, live mosquitoes were injected ~0.4 μ L of 67 μ M CM-Dil and 1.08 mM Hoechst 33342 (nuclear stain; Invitrogen) in PBS, incubated at 27 °C for 20 min, and injected 16% paraformaldehyde. Ten min later, abdomens were bisected along a coronal plane, and the dorsal portions containing the heart and periosial hemocytes were mounted on glass slides using Aqua-Poly/Mount (Polysciences; Warrington, PA).

Microscopy and image acquisition

Specimens were imaged on a Nikon Eclipse Ni-E compound microscope connected to a Nikon Digital Sight DS-Qi1 camera and Advanced Research NIS Elements software (Nikon, Tokyo, Japan). Z-stacks for bright field, red fluorescence (hemocytes), green fluorescence (GFP-*E. coli*) and blue fluorescence (nuclei) were acquired using a linear encoded Z-motor. Specific channels were selected and all images within a stack were combined into a two-dimensional image using the Extended Depth of Focus (EDF) function.

Quantification of hemocytes

Hemocytes, labeled with both CM-Dil and Hoechst 33342, were counted manually by examining all images within a Z-stack (Yan & Hillyer, 2019). A cell was a periosial hemocyte if adjacent to an ostium, and a non-periosial sessile hemocyte if attached to the abdominal wall outside of a periosial region (King & Hillyer, 2012,

2013). Periostial hemocytes were counted within abdominal segments 2-7 (all abdominal periostial regions) whereas non-periostial sessile hemocytes were only counted on the tergum of segments 4 and 5. Hemocytes were not counted on the aorta, the thoraco-abdominal ostia or the excurrent openings because few hemocytes are there, and infection does not induce aggregation at those locations (Sigle & Hillyer, 2018b, 2018c). Data were analyzed by two-way ANOVA, followed by Dunnett's multiple comparison test, with *dsb1a(Ap^R)* mosquitoes as the reference (Prism 9, GraphPad Software, San Diego, CA). Data presented for RNAi phenotypic experiments are for individual mosquitoes, which were sampled across three to four biological trials. The exception is experiments for PGRP-LA, where mosquitoes were sampled across two biological trials.

Quantification of GFP-E. coli and melanin at the periostial regions

In NIS-Elements, each periostial region in an EDF image was delineated using the ROI tool. GFP-*E. coli* at the periostial regions was calculated by measuring the area of pixels with intensities above a threshold that distinguished GFP-*E. coli* from background fluorescence (Yan & Hillyer, 2019). Melanin was quantified by measuring the area of pixels with intensities below a threshold that distinguished dark melanized areas from non-melanized areas (Yan & Hillyer, 2019). For each mosquito, measurements from all ROIs were added. Data were analyzed by two-way ANOVA, followed by Dunnett's multiple comparison test, with *dsb1a(Ap^R)* mosquitoes as the reference.

Quantification of bacterial infection intensity

Mosquitoes that were infected with tetracycline resistant GFP-*E. coli* for 4 or 24 hr were homogenized individually in PBS. A dilution of the homogenate was spread on LB agar containing tetracycline, and plates were incubated overnight at 37 °C. The CFUs were counted and used to calculate infection intensity. Data were analyzed by two-way ANOVA, followed by Dunnett's multiple comparison test, with *dsbla*(*Ap^R*) mosquitoes as the reference. For *rel2* and *caspar* RNAi mosquitoes, data were first log₂ transformed to achieve normality, and then analyzed by two-way ANOVA.

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Figure supplements:

Figure 1–figure supplement 1. Upregulated genes in the heart with peristial hemocytes at 4 hr following both GFP-*E. coli* and *S. aureus* infection.

Gene ID	Gene name	Gene description (if annotated)	<i>E. coli</i> vs Injured		<i>S. aureus</i> vs Injured	
			Log2Foldchange	Adjusted P	Log2Foldchange	Adjusted P
AGAP000694	<i>cec3</i>	cecropin anti-microbial peptide	2.72	8E-14	3.20	2E-19
AGAP000786			2.88	4E-06	3.12	2E-07
AGAP001160			3.03	3E-21	4.46	1E-47
AGAP001212	<i>PGRPLB</i>	peptidoglycan recognition protein (long)	3.10	2E-25	4.21	3E-47
AGAP001508			3.19	4E-07	5.03	2E-17
AGAP001563		glycerol kinase	2.58	7E-12	2.70	3E-13
AGAP001597			2.14	9E-04	2.14	6E-04
AGAP001610			4.07	2E-21	4.13	2E-22
AGAP001634			3.52	1E-14	2.48	1E-07
AGAP001648	<i>CLIPB17</i>	CLIP-domain serine protease	2.24	4E-10	3.01	3E-18
AGAP001828			2.13	2E-16	2.38	9E-21
AGAP002064			2.05	7E-18	2.29	1E-22
AGAP002065		alpha-tocopherol transfer protein	2.14	1E-13	2.64	3E-21
AGAP002291		ebony	2.81	1E-30	3.62	2E-51
AGAP002323			2.82	2E-11	3.31	4E-16
AGAP002331	<i>esp</i>	sodium-independent sulfate anion transporter	3.15	8E-20	2.94	1E-17
AGAP002359		carbonic anhydrase	2.62	2E-02	4.62	4E-06
AGAP002536			2.48	3E-13	2.75	1E-16
AGAP002883			2.28	3E-24	2.69	5E-34
AGAP003488		nucleotide exchange factor SIL1	2.02	2E-06	2.49	1E-09
AGAP004170			2.18	4E-21	2.73	8E-34
AGAP004181		Fibroblast growth factor	2.53	1E-06	2.40	4E-06
AGAP004826			3.04	4E-09	4.30	2E-18
AGAP005205	<i>PGRPLA</i>	peptidoglycan recognition protein (long)	2.36	7E-47	2.66	1E-60
AGAP005610			2.44	1E-06	4.99	2E-27
AGAP005839		MFS transporter, FLVCR family	3.65	4E-41	3.97	3E-49
AGAP006426		cyanogenic beta-glucosidase	2.65	2E-03	5.58	2E-13
AGAP006670		gamma-glutamyl hydrolase	2.31	8E-24	2.90	4E-38
AGAP006671			3.18	6E-18	3.85	2E-27
AGAP006674		chymotrypsin-like protease (Precursor)	2.93	2E-02	7.46	1E-12
AGAP006745			2.35	4E-29	2.78	3E-41
AGAP006747	<i>rel2</i>	NF-kappaB Relish-like transcription factor	2.00	2E-33	2.21	3E-41
AGAP007659			2.61	4E-15	3.57	1E-28
AGAP008279	<i>d7l2</i>	D7 long form salivary protein	16.43	2E-05	13.73	3E-04
AGAP008851		E3 ubiquitin-protein ligase mind-bomb	2.66	2E-26	2.95	8E-33
AGAP009091	<i>ddc</i>	dopa decarboxylase	3.96	2E-50	4.81	3E-75
AGAP009099	<i>TGase3</i>	Transglutaminase	3.98	2E-17	4.05	1E-18
AGAP009100	<i>TGase1</i>	Transglutaminase	3.20	3E-37	3.76	1E-51
AGAP009145			2.12	1E-13	3.03	4E-28
AGAP009213	<i>SRPN16</i>	serine protease inhibitor (serpin) 16	2.61	8E-13	3.75	1E-26
AGAP009214	<i>CLIPB11</i>	CLIP-domain serine protease	2.13	1E-06	3.71	2E-19
AGAP009460	<i>JNK3</i>	c-Jun N-terminal kinase	2.22	1E-04	3.47	1E-10
AGAP009985		4-nitrophenylphosphatase	2.19	3E-08	3.16	4E-17
AGAP010197		SPRY domain-containing SOCS box protein 1	2.31	2E-20	2.85	2E-31
AGAP010398		Flavin-containing monooxygenase FMO GS-OX-like 1	2.78	8E-08	5.85	1E-33
AGAP010934			2.94	1E-30	3.17	3E-36
AGAP011294	<i>def1</i>	defensin anti-microbial peptide	2.13	2E-05	2.70	1E-08
AGAP011326	<i>IAP2</i>	inhibitor of apoptosis 2	3.21	2E-38	3.29	1E-40
AGAP011588			2.60	2E-29	3.33	4E-49
AGAP011654		membrane dipeptidase	2.13	5E-05	3.58	3E-13
AGAP011764			2.71	5E-63	2.87	4E-71
AGAP011992		lysosomal acid lipase	2.72	7E-05	3.01	4E-06
AGAP012166			3.70	2E-13	2.92	8E-09
AGAP013506	<i>upd3a</i>	JAK/STAT pathway cytokine unpaired 3 variant A	3.89	2E-12	4.05	8E-14
AGAP028615	<i>nat5</i>	nutrient amino acid transporter 5	2.54	4E-10	4.28	2E-28

Figure 1–figure supplement 2. Upregulated genes in the circulating hemocytes at 4 hr following both GFP-*E. coli* and *S. aureus* infection.

Gene ID	Gene name	Gene description (if annotated)	<i>E. coli</i> vs Injured		<i>S. aureus</i> vs Injured	
			Log2Foldchange	Adjusted P	Log2Foldchange	Adjusted P
AGAP000899			3.31	3E-07	4.74	8E-16
AGAP001212	<i>PGRPLB</i>	peptidoglycan recognition protein (long)	2.68	2E-18	4.39	4E-51
AGAP001430			2.27	2E-18	3.39	7E-43
AGAP001508			2.06	3E-03	3.26	1E-07
AGAP002291		ebony	2.46	3E-23	3.73	2E-55
AGAP004115		cystinosin	2.07	5E-03	5.38	5E-18
AGAP005610			2.45	8E-08	4.09	7E-23
AGAP005639	<i>abcb2</i>	ATP-binding cassette transporter (ABC transporter) family B member 2	2.23	1E-12	3.47	1E-31
AGAP007651		growth arrest and DNA-damage-inducible protein	2.19	8E-11	3.30	2E-25
AGAP009091	<i>ddc</i>	dopa decarboxylase	2.06	2E-13	2.93	3E-28
AGAP010349			2.28	3E-05	3.50	1E-12
AGAP010387		alanine-glyoxylate aminotransferase	2.29	3E-03	3.54	3E-07
AGAP010571			2.43	5E-06	3.25	3E-11
AGAP010855		solute carrier family 45, member 1/2/4	3.24	7E-06	5.23	7E-16
AGAP011326	<i>IAP2</i>	inhibitor of apoptosis 2	2.26	2E-18	2.58	4E-25
AGAP011391			2.07	7E-07	3.62	8E-22
AGAP011588			2.43	7E-25	3.18	8E-45
AGAP011589			2.95	2E-02	3.46	3E-03
AGAP012565			3.58	7E-02	3.78	5E-02
AGAP013285	<i>ir7u</i>	ionotropic receptor IR7u	2.36	3E-03	3.46	9E-07
AGAP028615	<i>nat5</i>	nutrient amino acid transporter 5	2.27	9E-08	4.06	2E-25

Figure 1–figure supplement 3. Upregulated genes in the abdomen at 4 hr following both GFP-*E. coli* and *S. aureus* infection.

Gene ID	Gene name	Gene description (if annotated)	<i>E. coli</i> vs Injured		<i>S. aureus</i> vs Injured	
			Log2Foldchange	Adjusted P	Log2Foldchange	Adjusted P
AGAP001212	<i>PGRPLB</i>	peptidoglycan recognition protein (long)	3.22	4E-26	3.97	8E-41
AGAP001508			2.54	3E-04	4.63	5E-14
AGAP001610			3.33	1E-13	2.75	1E-09
AGAP001648	<i>CLIPB17</i>	CLIP-domain serine protease	2.04	8E-08	3.27	1E-20
AGAP002291		ebony	2.52	3E-24	2.47	1E-23
AGAP006670		gamma-glutamyl hydrolase	2.08	1E-18	2.33	9E-24
AGAP006745			2.06	8E-21	2.39	2E-28
AGAP007651		growth arrest and DNA-damage-inducible protein	2.17	2E-10	2.16	1E-10
AGAP007659			2.13	2E-09	2.45	7E-13
AGAP009145			2.18	2E-13	3.51	2E-36
AGAP010398		Flavin-containing monooxygenase FMO GS-OX-like 1	2.62	2E-06	5.93	3E-33
AGAP010934			2.59	6E-23	2.25	1E-17
AGAP011294	<i>def1</i>	defensin anti-microbial peptide	2.04	2E-04	2.39	3E-06
AGAP011326	<i>IAP2</i>	inhibitor of apoptosis 2	2.16	2E-16	2.17	8E-17
AGAP013348			2.40	4E-22	3.34	2E-43

Figure 1–figure supplement 4. The IMD and interlinked JNK pathways are two of the major immune pathways in mosquitoes. The activation of PGRP-LC triggers the IMD signaling pathway, which includes IAP2, imd, fadd and dredd. This activation dissociates the transcription factor, rel2, from the inhibitor protein, caspar. Rel2 then translocates into the nucleus and induces the production of effectors such as cec3 and def1. Although PGRP-LC is the canonical activator of the IMD pathway, PGRP-LA has also been implicated as an upstream activator of this immune pathway. The IMD pathway bifurcates into the JNK pathway, which activates JNK1 and JNK3, and induces the expression of effector genes. The JNK pathway is negatively regulated by pucker.

Figure 1-figure supplement 5. IMD and JNK pathway genes are more highly upregulated after infection in the heart and periostial hemocytes than in the circulating hemocytes or the abdomen. Heat map showing that all six IMD and JNK pathway genes identified in our unbiased screen are more highly upregulated after *E. coli* and *S. aureus* infection in the heart and periostial hemocytes than in the other tissues.

Figure 2-figure supplement 1. RNA interference-mediated gene silencing of *rel2* and *caspar* reduces their mRNA abundance while altering the mRNA abundance of *TEP1*, *cec3* and *def1*. Relative mRNA abundance of *rel2* (A), *caspar* (B), *TEP1* (C), *cec3* (D), and *def1* (E) in mosquitoes injected *dsbla(Ap^R)*, *dsrel2* or *dscaspar*. mRNA abundance was measured in mosquitoes that were not infected or had been infected with GFP-*E. coli* for 4 or 24 hr. Horizontal line, mean; Circles, individual biological trials. Data are relative to mRNA abundance in the *dsbla(Ap^R)* group of that specific trial and treatment.

1043 **Figure 2–figure supplement 2. Gene names, gene IDs, and primers used.**

Application	Gene	dsRNA	VectorBase ID ^a	Primer sequence (forward and reverse) ^b
qPCR	<i>Rps7</i>		AGAP010592	GACGGATCCCAGCTGATAAA
qPCR				GTTCTCTGGAATTCTGAACG
qPCR	<i>Rps17</i>		AGAP004887	GACGAAACCACTGCGTAACA
qPCR				TGCTCCAGTGCTGAAACATC
qPCR	<i>rel2</i>		AGAP006747	TTCGCTGCTCGACTTTCACT
qPCR				CCAACCGAAGGCAGCAAATC
qPCR	<i>caspar</i>		AGAP006473	TCGTAGCCAATGCGAAGTGT
qPCR				TCGTTCGTTCTCTTCACGCA
qPCR	<i>PGRP-LA-RA</i>		AGAP005205	GGCAATGGCGGAAATACGAC
qPCR				GGTTCCGCTGAAGGTAGTCC
qPCR	<i>PGRP-LA-RB</i>		AGAP005205	GGTACGTTTGACGCGGATC
qPCR				GTCCGCGGTTCTGATTGTT
qPCR	<i>PGRP-LA-RC</i>		AGAP005205	GCAATGGCGGAAATACGAC
qPCR				ATGCCCGGCGAAGTATATG
qPCR	<i>JNK1</i>		AGAP029555 ^c	TGTTTCCGACCGACTCGAA
qPCR				CGTCCACGCTGTGATCGTA
qPCR	<i>JNK3</i>		AGAP009460	ACAGTCTTAAGCATCCCGGC
qPCR				GCGGCAAAACACACTCCTTT
qPCR	<i>puckered</i>		AGAP004353	ATTCACGGAGGTCGATTG
qPCR				AACTTTCTAGCCCCATCGT
qPCR	<i>TEP1</i>		AGAP010815	GACGTCCAATACGGATCTCA
qPCR				CTTTCAGGCATCACCCGTAT
qPCR	<i>cec3</i>		AGAP000694	TTCCCAGTGCACTGTGTCC
qPCR				GAGCTTTGTAGCCGCAATG
qPCR	<i>def1</i>		AGAP011294	GCAACGATCGTCTGTACCATT
qPCR				GCTTGGCCCGATAGTTCTC
RNAi	<i>bla(Ap^R)</i>	<i>dsbla(Ap^R)</i>	(Bacterial gene)	<u>TAATACGACTCACTATA</u> AGGGCCGAGCGCAGAAGTGGT
RNAi				<u>TAATACGACTCACTATA</u> AGGGAACCGGAGCTGAATGAA
RNAi	<i>rel2</i>	<i>dsrel2</i>	AGAP006747	<u>TAATACGACTCACTATA</u> CGGAGAAGTCAAGAAAACG
RNAi				<u>TAATACGACTCACTATA</u> CACAGGCACACCTGATTGAG
RNAi	<i>caspar</i>	<i>dscaspar</i>	AGAP006473	<u>TAATACGACTCACTATA</u> AGGGACAGAAGGCCGCTTT
RNAi				<u>TAATACGACTCACTATA</u> AGGGAGTGGTGGTGACAGATT
RNAi	<i>PGRP-LA</i>	<i>dsPGRP-LA-RARB^d</i>	AGAP005205	<u>TAATACGACTCACTATA</u> AGGGAGATACTGTTTCGACACCA
RNAi				<u>TAATACGACTCACTATA</u> AGGGCTGGCTGTGGTAGGTTT
RNAi	<i>PGRP-LA</i>	<i>dsPGRP-LA-RC</i>	AGAP005205	<u>TAATACGACTCACTATA</u> AGGGCACATGGTCATCGATC
RNAi				<u>TAATACGACTCACTATA</u> AGGGATCGATTTTGGGTATTC
RNAi	<i>JNK1/JNK3</i>	<i>dsJNK1/3^e</i>	AGAP0290555 /AGAP009460	<u>TAATACGACTCACTATA</u> AGGGGTACGCCTTCTACACCGTC
RNAi				<u>TAATACGACTCACTATA</u> AGGGCAGCCCAAAGTCGAGGATT
RNAi	<i>puc</i>	<i>dspuc</i>	AGAP004353	<u>TAATACGACTCACTATA</u> AGGGTGAAACATAAATCGCCCTGC
RNAi				<u>TAATACGACTCACTATA</u> AGGGGACAGACCCTTGTAGCGCAT

- a Vectorbase IDs were obtained from the AgamP4 assembly in www.vectorbase.org (exception: *bla(Ap^R)*).
- b Underlined sequences are specific to the T7 RNA polymerase promoter sites needed for dsRNA synthesis.
- c Previous gene ID of JNK1 is AGAP009461.
- d *dsPGRP-LA-RARB* targets both the RA and RB splice variants of *PGRP-LA*.
- e *dsJNK1/3* targets both *JNK1* and *JNK3*.

Figure 2-figure supplement 3. The IMD pathway affects GFP-*E. coli* and melanin accumulation at the periostial regions. (A) Fluorescence images show the accumulation of GFP-*E. coli* (green) around a single pair of ostia (asterisks) on a segment of the heart (outlined by dotted lines) of *dsbla(Ap^R)*, *dsrel2* and *dscaspar* mosquitoes that had been infected with GFP-*E. coli* for 4 or 24 hr. (B) Bright field images show the accumulation of melanin (dark deposits) around a single pair of ostia (asterisks) on a segment of the heart (outlined by dotted lines) of *dsbla(Ap^R)*, *dsrel2* and *dscaspar* mosquitoes that were not infected or had been infected with GFP-*E. coli* for 4 or 24 hr. Anterior is on top. Quantification of all mosquitoes assayed is presented in figures 2D and 2E.

Figure 3-figure supplement 1. RNA interference-mediated gene silencing of *PGRP-LA* reduces the mRNA abundance of all splice variants. Relative mRNA abundance of *PGRP-LA-RA* (A), *PGRP-LA-RB* (B) and *PGRP-LA-RC* (C) in mosquitoes injected *dsbla(Ap^R)*, *dsPGRP-LA-RARB* or *dsPGRP-LA-RC*. mRNA abundance was measured in mosquitoes that were not infected or had been infected with GFP-*E. coli* for 4 or 24 hr. Horizontal line, mean; Circles, individual biological trials. Data are relative to mRNA abundance in the *dsbla(Ap^R)* group of that specific trial and treatment.

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Figure 4—figure supplement 1. RNA interference-mediated gene silencing of *JNK1*, *JNK3* and *puc* reduces their mRNA abundance while having a limited impact on the mRNA abundance of *TEP1*, *cec3* and *def1*. Relative mRNA abundance of *JNK1* (A), *JNK3* (B), *puc* (C), *TEP1* (D), *cec3* (E), and *def1* (F) in mosquitoes injected ds $bla(Ap^R)$, ds $JNK1/3$ or ds puc . mRNA abundance was measured in mosquitoes that were not infected or had been infected with GFP-*E. coli* for 4 or 24 hr. Horizontal line, mean; Circles, individual biological trials. Data are relative to mRNA abundance in the ds $bla(Ap^R)$ group of that specific trial and treatment.

Figure 4–figure supplement 2. The JNK pathway affects GFP-*E. coli* and melanin accumulation at peristial regions. (A) Fluorescence images show the accumulation of GFP-*E. coli* (green) around a single pair of ostia (asterisks) on a segment of the heart (outlined by dotted lines) of *dsbla(Ap^R)*, *dsJNK1/3* and *dspuc* mosquitoes that had been infected with GFP-*E. coli* for 4 or 24 hr. (B) Bright field images show the accumulation of melanin (dark deposits) around a single pair of ostia (asterisks) on a segment of the heart (outlined by dotted lines) of *dsbla(Ap^R)*, *dsJNK1/3* and *dspuc* mosquitoes that were not infected or had been infected with GFP-*E. coli* for 4 or 24 hr. Anterior is on top. Quantification of all mosquitoes assayed is presented in figures 4D and 4E.