The distinct function of Tep2 and Tep6 in the immune defense of Drosophila melanogaster against the pathogen Photorhabdus

Upasana Shokal, Hannah Kopydlowski, and Ioannis Eleftherianos

Insect Infection and Immunity Lab, Department of Biological Sciences, Institute for Biomedical Sciences, The George Washington University, Washington DC, USA

ABSTRACT

Previous and recent investigations on the innate immune response of Drosophila have identified certain mechanisms that promote pathogen elimination. However, the function of Thioester-containing proteins (TEPs) in the fly still remains elusive. Recently we have shown the contribution of TEP4 in the antibacterial immune defense of Drosophila against non-pathogenic E. coli, and the pathogens Photorhabdus luminescens and P. asymbiotica. Here we studied the function of Tep genes in both humoral and cellular immunity upon E. coli and Photorhabdus infection. We found that while Tep2 is induced after Photorhabdus and E. coli infection; Tep6 is induced by P. asymbiotica only. Moreover, functional ablation of hemocytes results in significantly low transcript levels of Tep2 and Tep6 in response to Photorhabdus. We show that Tep2 and Tep6 loss-of-function mutants have prolonged survival against P. asymbiotica, Tep6 mutants survive better the infection of P. luminescens, and both tep mutants are resistant to E. coli and Photorhabdus. We also find a distinct pattern of immune signaling pathway induction in E. coli or Photorhabdus infected Tep2 and Tep6 mutants. We further show that Tep2 and Tep6 participate in the activation of hemocytes in Drosophila responding to Photorhabdus. Finally, inactivation of Tep2 or Tep6 affects phagocytosis and melanization in flies infected with Photorhabdus. Our results indicate that distinct Tep genes might be involved in different yet crucial functions in the Drosophila antibacterial immune response.

INTRODUCTION

Drosophila melanogaster has served as an excellent model system to study innate immune defense mechanisms against microbial infections. To detect different types of pathogens, the fly uses specific pattern recognition receptors such as peptidoglycan recognition receptors, Gram-negative binding proteins, scavenger receptors or Thioester-containing proteins (TEPs). Most TEPs contain a highly reactive thioester motif that covalently binds to the microbial surfaces and leads to their elimination from the host. Although a vast amount of information is available on various pattern recognition receptors in Drosophila, the specific function of TEPs is still not entirely understood. However, their immune role is widely studied in the mosquitoes Anopheles gambiae and Aedes aegypti, and in vertebrates. The Anopheles TEP1 is involved in the process of phagocytosis of Escherichia coli and Staphylococcus aureus, as well as in the melanization of Plasmodium parasites. Similarly, A. aegypti macroglobulin complement-related (MCR) factor participates in fighting off flavivirus infection.

Previous studies have shown that Drosophila Tep1, Tep2, Tep3, and Tep4 genes are induced upon certain bacterial, fungal, parasitoid and parasitic challenges. Moreover, an in-vitro study has shown that phagocytosis of E. coli and S. aureus bacteria is regulated by TEP2, TEP3 and phagocytosis of Candida albicans spores by TEP6. Recently we have shown that Tep4 modulates the activation of Toll and IMD immune signaling in Drosophila flies responding to 2 species of the potent pathogen Photorhabdus. We further reported that inactivation of Tep4 leads to increased phenoloxidase and melanization activity upon Photorhabdus bacteria, and these effects alter the survival response of the flies to these pathogens.

The Photorhabdus genus contains bacteria that are highly virulent insect or human pathogens, which live in a mutualistic relationship with Heterorhabditid nematodes.
bacteria use distinct defense strategies that allow them to surpass the host immune responses. For example, a toxin secreted by *P. luminescens* has been shown to target a large number of insect hemolymph proteins encoding molecules that are involved in immune recognition, immune signaling and regulation of the coagulation cascade.\textsuperscript{14} In addition, *Photorhabdus* can subvert cellular immune responses by secreting toxins or virulence factors that induce freezing or apoptosis of insect hemocytes.\textsuperscript{15,16} Other studies have also revealed that *Photorhabdus* is able to interfere with the insect prophenoloxidase cascade.\textsuperscript{17-19} *Photorhabdus* bacteria are closely related to many mammalian pathogens such as *Yersenia pestis, E. coli* and *Salmonella*.\textsuperscript{20} Hence, results from studies on the pathogenicity of *Photorhabdus* in the context of host immune activity can be extrapolated to other pathogens of agricultural or medical importance.

To further our understanding on the immune role of *Drosophila* TEPs in the host defense against the pathogen *Photorhabdus*, here we have investigated the participation of *Tep* genes in the fly humoral and cellular antibacterial immune response. Using *tep* mutant flies together with gene expression assays and functional immune tests, we have shown that *Tep2* and *Tep6* are probably involved and act distinctly in the activation and regulation of immune signaling pathways, phagocytosis and phenoloxidase responses in the fly against the pathogen *Photorhabdus*.

### Results

**Tep genes are induced in *Drosophila* upon *Photorhabdus* challenge**

Previously we have shown that *Tep4* is transcriptionally activated upon *E. coli* or *Photorhabdus* infection. Therefore, we first examined whether other *Tep* genes (*Tep1–3* and *Tep6*) have altered expression in the background fly strain (w\textsuperscript{1118}) upon infection with these bacteria. We found that certain *Tep* genes (*Tep1, Tep2* and *Tep6*) were upregulated at 6 and 18 hpi by mostly *P. luminescens* and *P. asymbiotica* infection (Fig. 1). Only *Tep2* was induced in flies infected with non-pathogenic *E. coli* bacteria (Fig. 1A) at 6 hpi. In particular, there was a significant induction of *Tep1* and *Tep2* genes in *P. luminescens* infected flies at 6 hpi (Fig. 1A), and *Tep1, Tep2* and *Tep6* genes in *P. asymbiotica* infected flies at 18 hpi (Fig. 1A–B). While *Tep2* was mainly upregulated by *P. luminescens* and *P. asymbiotica* at both time points, *Tep1* was significantly induced at higher levels by *P. luminescens* only at the 6 h time-point (Fig. 1A–B). These results show that infection of *D. melanogaster* with the insect-specific pathogen *P. luminescens* and the related human pathogen *P. asymbiotica* as well as *E. coli* results in significant induction of certain TEP coding genes in the adult fly.

**Drosophila tep mutants have increased survival during the early and mid stages of *Photorhabdus* infection**

To examine the function of the *Tep* induced genes in the immune response of *Drosophila*, we first performed survival analysis of the infected mutant flies. We tested the survival response of *Tep2* and *Tep6* loss-of-function mutants and their background control to infection by the 2 *Photorhabdus* pathogens and the non-pathogenic *E. coli*. We excluded *tep1* mutants because *Tep1* and *Tep2* mRNAs were expressed at similar levels (Fig. 1A–B) and according to a previous phylogenetic analysis, *Tep1* and *Tep2* are likely to act redundantly (Bou Aoun...
et al., 2011). Moreover, we omitted tep3 mutants as we did not observe any changes in the mRNA levels of Tep3 (Fig. 1). To ascertain that we used loss-of-function tep mutants, we estimated the mRNA levels of Tep2 and Tep6 in the tep2 and Tep6 mutant flies injected with PBS, E. coli or Photobacterium bacteria (Fig. S1 A-B).

Both Tep2 and Tep6 strains died within 36 h after P. luminescens infection and within 48 h after P. asymbiotica infection. We also observed that Tep2 flies died similarly to their background controls when infected with P. luminescens but survived significantly longer when infected with P. asymbiotica (Fig. 2A–B). We found that at 36 hpi with P. asymbiotica, 48% of Tep2 were alive compared with their background controls (~1%). However, there were 75% of Tep6 mutant flies alive compared with 14% of controls at 24 hpi with P. luminescens whereas 47% of Tep6 mutants were alive at 36 h post P. asymbiotica infection compared with controls (~2%) (Fig. 2B). We further found that injection with non-pathogenic E. coli or sterile PBS did not affect the survival of tep mutant flies and their background controls (Fig. S3A-B). These results indicate that loss-of-function mutations in Tep6 provide a survival advantage to D. melanogaster in response to infection with P. luminescens, while loss-of-function mutations in Tep2 and Tep6 promote the survival of flies against infection with P. asymbiotica.

![Figure 2](image-url)

**Figure 2.** Survival and bacterial load analysis for tep2 and tep6 mutants after Photobacterium infection. Survival curves for loss-of-function (A) tep2 mutants and (B) tep6 mutants with w1118 (background control flies) are shown. Flies (n = 20) were injected in the thorax by microinjection with 1XPBS (septic injury control), P. luminescens (Pl) or P. asymbiotica (Pa). Survival was monitored at 6 h intervals for 48 h. The black dotted line represents 50% survival. Colony forming units (CFU) of (C) P. luminescens and (D) P. asymbiotica are shown in tep2, tep6 and control flies (n = 5 per experimental condition) after 6 and 18 hpi. CFU were quantified through quantitative PCR of makes caterpillars floppy (mcf-1) in P. luminescens and the insecticidal toxin complex protein gene (tccC3) in P. asymbiotica. Significant differences are indicated with asterisks (* p < 0.05, ** p < 0.01, *** p < 0.001). The means from 3 independent experiments are shown and error bars represent standard errors (survival) and standard deviation (bacterial load).
To understand the basis for the increased survival of the *tep* infected mutant flies, we investigated the bacterial persistence at 2 time-points post infection. To estimate bacterial load, we evaluated the number of colony forming units (CFU) at 6 and 18 hpi. We noticed that although there were no significant differences in survival between *Tep2* mutants and their controls, there were 3-times fewer *P. luminescens* CFU in the mutant flies than in *w*1118 individuals at 18 hpi (Fig. 2C). Similarly, *Tep6* mutants had 18-times and 22-times less *P. luminescens* CFU than the control flies at 6 and 18 hpi, respectively (Fig. 2C). In the case of *P. asymbiotica* infections, there were 3.5-times and 8-times fewer CFU in *Tep2* and *Tep6* mutants compared with *w*1118 flies at 6 h only (Fig. 2D). Additionally, infections with non-pathogenic *E. coli* resulted in significantly lower numbers of CFU in *Tep2* and *Tep6* mutants compared with *w*1118 controls at both time-points post infection (Fig. S2C). Interestingly, *Tep2* mutants contained 20-times more *E. coli* cells than *Tep6* flies (Fig. S2C). These results show that deficiencies in *Tep2* and *Tep6* genes confer resistance to *P. luminescens*, *P. asymbiotica* and *E. coli*.

**Function of Tep2 and Tep6 genes is essential for immune signaling pathway regulation in Drosophila**

To explore the increased resistance of *Tep2* and *Tep6* mutants toward *Photorhabdus* and *E. coli*, we examined the transcriptional activation of Toll, Imd, JAK/STAT and JNK immune pathways in loss-of-function *Tep2* and *Tep6* mutant flies infected with these bacteria. We first tested at the activation of Toll pathway by evaluating the transcript levels of the AMP *Defensin*, which is a bacterial specific AMP. In addition, we have recently found low to moderate transcript levels of *Defensin* in wild-type flies infected with *Photorhabdus*. Here we asked whether flies with inactivated *Tep2* or *Tep6* have altered *Defensin* transcript levels upon infection with the pathogens. We found that *Defensin* was strongly induced in *w*1118 flies by either *Photorhabdus* species at 6 hpi but only by *P. luminescens* at 18 hpi (Fig. 3A–B). We further found that *Defensin* mRNA levels were significantly higher in *Tep6* mutant flies compared with their background controls at 6 hpi with *P. luminescens*, and at both 6 and 18 hpi upon infection with *P. asymbiotica* (Fig. 3A–B). We observed significant upregulation in the mRNA levels of *Defensin* in *Tep2* mutants at 6 and 18 hpi with *P. luminescens* compared with *Tep2* mutant flies injected with PBS (Fig. 3A–B). This indicates that *Tep6* but not *Tep2* gene activity is required in the induction of Toll pathway.

To evaluate Imd pathway activation, we estimated the transcript levels of the AMP-encoding gene *Diptericin* in infected flies (Fig. 3C–D). We observed that *Diptericin* mRNA levels were significantly induced in the *w*1118 flies by *Photorhabdus* and *E. coli* bacteria at 6 and 18 hpi, as well as in PBS injected flies at 6 hpi (Fig. 3C–D). Moreover, upregulation of *Diptericin* was significantly higher in *w*1118 flies infected with *P. asymbiotica* compared with *E. coli* infected flies of the same strain at 18 hpi (Fig. 3D). Interestingly, we found that transcript levels of *Diptericin* were consistently lower in *Tep2* mutants than in *w*1118 flies at both time points (Fig. 3C–D). In addition, there were no differences in *Diptericin* mRNA levels between *Tep6* mutants and *w*1118 background controls (Fig 3C–D). These results indicate that *Tep2* regulates Imd signaling in *D. melanogaster* adult flies in the context of *Photorhabdus* infection or response to wounding.

To analyze JAK/STAT and JNK signaling activation in *tep* mutants and control flies, we assessed the transcript levels of *Turandot-M* (*Tot-M*) and *Puckered* (*Puc*). We first observed that *Tot-M* was significantly upregulated in *w*1118 flies at 18 hpi with *P. luminescens* than flies injected with other bacteria or PBS (Fig. 3E–F). The *Tot-M* mRNA levels were significantly low in *w*1118 flies infected with *P. asymbiotica* than *P. luminescens* or PBS injected flies at 6 hpi (Fig. 3E). We found that *Tep2* mutants have significantly reduced *Tot-M* mRNA levels than the *w*1118 flies injected with any of the bacteria at both time points (Fig. 3E–F). We also observed that in *Tep2* mutant flies, *Tot-M* was slightly upregulated only at 6 hpi with *P. luminescens* compared with other treatments, but this induction was significantly lower compared with background flies infected by *P. luminescens* (Fig. 3E). We further noticed that *Tot-M* was significantly upregulated in *Tep6* mutants at 6 hpi with *Photorhabdus* bacteria and at 18 hpi with *E. coli* in relation to control flies (Fig 3E–F). We found that *Puc* mRNA levels were significantly lower in *tep* mutants compared with the control flies injected with *P. asymbiotica* at 18 hpi only (Fig. 3G–H). These results indicate that *Tep2* is required for full JAK/STAT pathway induction in the presence of certain bacterial infections of adult fruit flies. In addition, *Tep2* and *Tep6* gene activity is required for JNK signaling in *D. melanogaster* adult flies upon infection with *P. asymbiotica* during the late stages of infection.

**Functional hemocytes in Drosophila constitute a source of Tep2 and Tep6 transcription**

Because TEPs are secreted proteins and they are expressed in larval plasmatocytes, we examined whether changes in the function of hemocytes can affect
the upregulation of Tep2 and Tep6 in infected adult flies. For this, we pre-injected the \( w^{1118} \) flies with latex beads to ablate the function of hemocytes. A pre-injection with 1X PBS served as control. We found that Tep2 transcript levels were significantly higher in flies pre-injected with beads or PBS followed by any bacterial treatment at 6 hpi, but only by \textit{Photobacterium} challenge at 18 hpi (Fig. 4A–B). Moreover, we noticed significant upregulation of Tep2 in flies pre-injected with PBS compared with flies preinjected with beads followed by infection with \textit{P. luminescens} or \textit{E. coli} (Fig. 4A). There was also significant upregulation of Tep2 in flies pre-injected with
PBS compared with those treated with beads, at 18 hpi with *P. luminescens* (Fig. 4B). Furthermore, we found significant upregulation of *Tep6* in flies pre-injected with PBS than in those injected with beads at 18 hpi with either *Photorhabdus* species (Fig. 4C–D). These results indicate that functional hemocytes are one of the sources of *Tep2* and *Tep6* genes upregulation.

**Drosophila Tep2 and Tep6 mutants have differential number of hemocytes and fewer dead hemocytes against Photorhabdus infection**

To evaluate whether inactivation of *Tep2* and *Tep6* genes can affect activation of circulating hemocytes, we then investigated the cellular function of *Drosophila* against *Photorhabdus* and *E. coli* bacteria. We first looked at changes in the total number of hemocytes in infected and uninfected flies. Based on the hemocyte counting protocol, the total number of hemocytes was significantly higher in *w*¹¹¹⁸ as well as *Tep2* mutants infected with *P. asymbiotica*, *P. luminescens* or *E. coli* bacteria (Fig. 5A, Fig. S3A). Moreover, both *Tep2* [(1.24 ± 0.48) X10⁵] and *Tep6* mutants [(0.97 ± 0.35) X10⁵] had significantly fewer hemocytes than the *w*¹¹¹⁸ flies [(4.44 ± 1.98) X10⁵] after *P. asymbiotica* infection (Fig. 5B). However, we observed an increase in hemocyte numbers after *P. asymbiotica* infection in *Tep6* mutants compared with mutants injected with PBS (Fig. 5B). We also evaluated cell viability in *Tep2*, *Tep6* mutants and *w*¹¹¹⁸ flies after infection with *Photorhabdus* and *E. coli*. We observed reduced cell viability in all the strains after *Photorhabdus* and *E. coli* infection (Fig. 5C, Fig. S3B). We found that *tep* mutants contained significantly higher percentage of viable cells compared with *w*¹¹¹⁸ flies infected with *Photorhabdus* (Fig. 5C). These data suggest that *Tep2* and *Tep6*, plays an important role in the activation of hemocytes in *Drosophila* flies responding to infection with *Photorhabdus* or *E. coli* bacteria.

**Drosophila Tep2 and Tep6 are required for phagocytosis of Photorhabdus or E. coli bacteria**

To estimate whether inactivation of *Tep2* or *Tep6* affects the phagocytosis of bacteria in *Drosophila*, we injected opsonized inactive *E. coli* bioparticles in *tep* mutants and their control flies. We found that the
phagocytic activity was significantly reduced (~3 times lower) in the Tep2 and Tep6 mutants compared with w1118 flies at one hpi with E. coli (Fig. 6A–B). We also looked at the transcript levels of Eater gene, as a marker of phagocytosis, in flies injected with PBS, E. coli or Photobacterium bacteria at 6 and 18 hpi. The mRNA levels of Eater were significantly higher in control flies infected with E. coli at both time points but also with P. asymbiotica at 18 hpi (Fig. 6C). We found that Eater was significantly upregulated at 6 hpi with E. coli, P. luminescens or PBS in w1118 flies compared with the tep mutants (Fig. 6C). Additionally, we observed that Eater mRNA levels were significantly lower in both tep mutants compared with control flies at 18 hpi following bacterial or buffer injection (Fig. 6D). In particular, Eater mRNA levels were significantly lower in Tep6 mutants infected with P. asymbiotica compared with those injected with PBS at 18 hpi (Fig. 6D). Our data suggest that inactivation of Tep2 and Tep6 severely prevents the phagocytic activity in flies against certain bacterial infections.

**Tep2 participates in the drosophila melanization and phenoloxidase response against Photobacterium infection**

To examine whether inactivation of Tep2 or Tep6 influence the in vivo melanization response in *D. melanogaster*, we visually inspected the wound site at 3 hpi of mutant and control flies with *Photobacterium*, E. coli or PBS. We observed that w1118 flies and Tep2 mutants exhibited strong melanization response against all injection treatments (Fig. 7A, Fig. S4A). Melanin spots developed in Tep6 flies following injection with PBS or E. coli only (Fig. 7A). We also estimated the phenoloxidase (PO) enzyme activity in the hemolymph plasma of tep mutant and control flies injected with the different bacteria. We noticed that the PO activity was significantly reduced in control flies infected with *Photobacterium* bacteria (Fig. 7B). We found no significant changes in PO activity between Tep2 or Tep6 mutant flies and w1118 controls injected with PBS or E. coli (Fig. 7B, Fig. S4B). Furthermore, Tep2 mutant flies infected with *P. luminescens* or *P. asymbiotica* had substantially higher...
PO activity than Tep6 and w1118 flies infected with the pathogens. Interestingly, we found that upon P. asymbiotica infection, Tep6 mutants displayed significantly lower levels of PO activity than w1118 flies (Fig. 7B). These results suggest that the absence of functional TEP2 in D. melanogaster adult flies promotes phenoloxidase activity against infection with pathogenic Photorhabdus.

**Discussion**

Despite remarkable advances in the field of innate immunity, our understanding of the role of TEP molecules in the immune defense of Drosophila is mostly unexplored. Recently, we showed the participation of Tep4 in the humoral and phenoloxidase responses of the fruit fly against Photorhabdus infection. Here we investigated the role of other Tep genes in the antibacterial immune response of Drosophila. Previously, we observed induction of Tep4 in flies infected with 2 Photorhabdus species, therefore we hypothesized that presumably other Tep genes might also be activated upon infection with this pathogen.

Previous studies have reported upregulation of Tep1 and Tep2 genes but not Tep6 in adult flies infected with a mixture of E. coli and Micrococcus luteus.10,28 Our results are in accordance to the previous studies, as we observe an early upregulation of Tep2 by E. coli bacteria. Transcriptomic analysis has also shown that Tep1 and Tep2 are induced following P. luminescens, symbiotic Heterorhabditis nematodes (carrying Photorhabdus) or axenic nematodes (lacking Photorhabdus) infection.8,29 In accordance, upregulation of Tep1 and Tep2 in flies after Photorhabdus...
infection suggests their probable function in the immune response of the fly against the *Photorhabdus* bacteria. Although there are no reports of Tep3 induction by *Photorhabdus* or its symbiotic nematode partner, recent work has reported that tep3 loss-of-function mutants are sensitive to *Heterorhabditis* symbiotic nematode infections. No changes in the transcript levels of Tep3 could be due to its specificity only to nematodes. Also, induction of Tep6 in response to *P. asymbiotica* indicates a specific function of this molecule against this pathogen. However, as previously reported, we cannot exclude the possibility that the function of TEPs in the fly immune system might be redundant or that TEP molecules might act in combination with other factors to provide efficient levels of protection to the fly against certain pathogens.

Previously TEP molecules have been shown to be expressed in larval plasmatocytes, adult fat body of the head and digestive tract lining at basal levels. Reduced transcript levels of Tep2 and Tep6 in flies containing dysfunctional hemocytes upon *Photorhabdus* infection indicates that functional plasmatocytes are one of the major sources for Tep gene expression in the adult flies. However, other tissues such as gut or fat body may contribute toward Tep gene upregulation when hemocytes are inactive. This could explain the induction of Tep2 in flies containing non-functional hemocytes in response to *E. coli* or *Photorhabdus* infection.

**Figure 7.** Melanization response and PO activity are elevated in *D. melanogaster* tep2 mutants upon *Photorhabdus* infection. (A) Melanization of the wound site in tep2 and tep6 loss-of-function mutant flies and their background control strains (*w*1118) is shown at 10X magnification 3 h after injection with PBS, *P. luminescens* or *P. asymbiotica* bacteria. Arrows indicate the site of injury. (B) PO activity in the hemolymph plasma of tep2, tep6 mutants and control flies (*w*1118) at 3 hpi with PBS, *P. luminescens* (Pl) or *P. asymbiotica* (Pa) (n = 20 flies) as measured by the optical density at 492 nm after incubation with L-Dopa. Values represent the means from 3 biologic replicates and error bars represent standard deviations. Significant differences are indicated with asterisks (*p* < 0.05, **p** < 0.01, ***p*** < 0.001, ****p*** < 0.0001). The means from 3 independent experiments are shown and error bars represent standard deviation.
Inactivation of certain genes in *D. melanogaster* can alter the survival ability of the fly in response to microbial infections.\(^1\) A former study has shown that loss-of-function *Tep2* mutants are susceptible to *Porphyromonas gingivalis* infection.\(^30\) However, another study failed to identify changes in the survival of single, double or triple *tep* mutants in response to Gram-positive and Gram-negative bacterial pathogens as well as to fungal infection.\(^10\) Hence, prolonged survival of *Tep6* mutant flies against *P. luminescens* as well as of *Tep2* and *Tep6* mutants in response to *P. asymbiotica* infection suggests that the survival response is pathogen specific. In addition, the presence of significantly fewer *Photorhabdus* CFU in *Tep2* and *Tep6* mutants could explain their increased survival during the initial and intermediate stages of *Photorhabdus* infection. The finding that *Tep2* mutants are resistant to both *Photorhabdus* and *E. coli* could probably suggest that *Tep2* is evolving in relation to the different pathogen challenges *Drosophila* flies encounter in the wild. Interestingly, it has been previously proposed that *Drosophila* *Tep2* may have evolved under strong positive selection.\(^31\)

The modulation of immune pathways by *Tep4*\(^12\) and the effect of *Tep4* gene inactivation on the resistance of mutant flies to bacterial infection formed the basis for testing whether TEP2 and TEP6 can also play a central regulatory role in the *D. melanogaster* immune system. No effect on the Toll pathway activation and downregulation of IMD pathway in *Tep2* mutants suggests different mode of actions of *Tep2* and *Tep4* genes. Additionally, our results are in agreement with previous findings as JAK/STAT activation was severely impaired in *Tep2* mutants.\(^32\) Similar to *tep4*, here we find that Toll signaling activity is upregulated in *Tep6* mutants infected with *Photorhabdus*. Stimulation of JAK/STAT in *Tep6* mutants after *Photorhabdus* infection implies that *Tep6* may not participate in the activation of this pathway. Another explanation for the differential induction patterns of the immune signaling pathways in *Tep6* mutants could be the absence of a thioester motif in TEP6 that probably affects its function as an effector molecule. Nevertheless, increased activation of certain pathways in *tep* mutants compared with background controls injected with PBS could suggest that wounding initiates a response in these flies. However, this activation increases in the presence of the non-pathogenic bacteria *E. coli*, as seen in the case of *Tot-M* transcript levels in *Tep6* mutants. In contrast, *Photorhabdus* may interfere with the activation of these pathways by either increasing or suppressing them, as seen with the induction of *Tot-M* in *Tep6* mutants injected with *P. luminescens* or the reduction of *Puc* in *Tep6* mutants infected with *P. asymbiotica*, respectively. Altogether, the 3 TEP molecules-TEP2, TEP4 and TEP6, may modulate immune signaling pathways in discrete ways.

Complement proteins are known to activate mast and basophils in human lungs and blood as an inflammatory and allergic response.\(^33\) Additionally, in *Drosophila*, dramatic change in the number of circulating hemocytes is observed after pathogenic invasion.\(^34\) Nonetheless the function of TEPs in the recruitment and activation of hemocytes in *Drosophila* after bacterial infection is still undefined. The increase in the number of hemocytes in *Tep2* and *Tep6* mutants after *Photorhabdus* or *E. coli* infection indicates that *Tep2* and *Tep6* are not directly involved in the induction of hemocytes. However, inactivation of either *Tep2* or *Tep6* is not entirely insignificant, as we observed larger numbers of hemocytes in the control flies against *P. asymbiotica* infection. The increase in the number of hemocytes might also be a consequence of other activated TEP molecules, such as TEP4, as we have previously observed a significant decline in the number of hemocytes in the absence of TEP4 following *E. coli* or *Photorhabdus* infection (unpublished).

One of the main evasion strategies of *Photorhabdus* involves targeting and attacking insect hemocytes. *Photorhabdus* can cause morphological changes to hemocytes by affecting the cytoskeletal components, such as actin, that can in turn disturb their normal functions.\(^35,36\) Moreover, *Photorhabdus* pathogens secrete several toxins that can induce apoptosis in the insect hemocytes.\(^16,37\) Increased hemocyte viability in *Tep2* and *Tep6* mutants indicates that inactivation of these *Tep* genes is advantageous for the hemocytes to respond against the *Photorhabdus* insult. This could further support the prolonged survival of the *Tep2* and *Tep6* mutants during the course of *Photorhabdus* infection.

An in vitro study has shown that *Tep2* and *Tep6* in *D. melanogaster* are involved in the phagocytosis of *E. coli* and *Candida albicans*, respectively.\(^11\) The decreased phagocytosis of inactive *E. coli* particles in the *Tep2* and *Tep6* mutants indicates the significance of TEPT2 and TEP6 in the phagocytosis process against *E. coli*. Moreover, the notably reduced transcript levels of *Eater* probably suggests a direct role of TEP2 and TEP6 in this process in response to *Photorhabdus* or *E. coli* infection. We propose that although *Tep2* and *Tep6* mutants contain high numbers of hemocytes after bacterial challenge, due to the inactivation of these 2 *Tep* genes, the phagocytosis function is substantially impaired in the mutants.

We also examined the effect of *Tep2* and *Tep6* on the melanization response, which forms an essential
and rapid cellular immunity process.\textsuperscript{38,39} The elevated melanization and phenoloxidase activity in Tep2 mutants against \textit{Photorhabdus} bacteria points out that TEP2 and TEP4 perform similar immune functions in response to \textit{Photorhabdus} infection in \textit{Drosophila}. This may also account for the reduced number of \textit{Photorhabdus} CFU in the Tep2 mutants. It could be possible that the growth of \textit{Photorhabdus} bacteria is restricted in the Tep2 mutants due to increased PO and melanization during the initial phase of infection. In contrast, inactivation of Tep6 leads to reduced PO and melanization in the flies after \textit{Photorhabdus} infection. The contrasting findings between Tep2 and Tep6 mutants may be best explained by the structural difference between the 2 proteins. TEP6, which lacks the thioester motif, regulates phenoloxidase activity and melanization in a different manner than TEP2 and TEP4 molecules, which contain the thioester motif.\textsuperscript{10}

In conclusion, we have extended our previous findings that TEPs serve an imperative function in the immune defense of \textit{Drosophila}. The experiments described herein were focused on critical immune responses of fruit defense of \textit{Drosophila}. We show that inactivation of Tep2 and Tep6 serve a protective and immunomodulatory role against certain insect pathogenic bacteria, such as \textit{Photorhabdus}. Furthermore, our data suggest that different TEP molecules act in a distinct manner in the \textit{Drosophila} antibacterial immune system. It has been shown that disruption of C5aR encoding the Complement protein 5a receptor results in increased resistance to acute Gram-negative bacterial infections in mice and this ultimately leads to reduced endotoxic shock.\textsuperscript{40} Similarly, we propose that the absence of TEP2 or TEP6 leads to lower levels of inflammation in the host following bacterial infection, which successively modulates their survival ability against potent entomopathogenic bacteria. We anticipate that such studies will lead to a better understanding of the complex mechanism of action of TEP molecules in the antibacterial immune response of the fruit fly. These findings could also be applied to insects of agricultural or medical importance.

**Materials and methods**

**Fly and bacterial strains**

The following \textit{D. melanogaster} strains were used in the study- \textit{w}\textsuperscript{118} (genetic background strain), Tep2 (f02756, Harvard), and Tep6 (f03851, Harvard). All strains were kept and amplified for experimentation with instant \textit{Drosophila} media (Carolina Biological Supply) with deionized water. All stocks were maintained at 25°C and a 12:12-hour light:dark photoperiod.

The bacterial strains used were \textit{Photorhabdus luminescens} subsp. laumondii (strain TT01), \textit{P. asymbiotica} subsp. asymbiotica (strain ATCC 43949) and \textit{Escherichia coli} (strain K12). Bacteria were cultured in sterile Luria–Bertani (LB) broth for approximately 18–22 h at 30°C on a rotary shaker at 220 rpm. The cultures were then pelleted down, washed and re-suspended in 1x sterile phosphate-buffered saline (PBS, Sigma Aldrich). For infections, bacterial concentrations were brought to an Optical Density (OD, 600 nm) of 0.1 for \textit{P. luminescens}, 0.25 for \textit{P. asymbiotica} and 0.015 for \textit{E. coli} using a spectrophotometer (NanoDropTM 2000c – Thermo Fisher Scientific).

**Infection assays and survival experiment**

All procedures were performed as described previously.\textsuperscript{12} In brief, 7–10 d old adult flies were anesthetized with CO\textsubscript{2} and then injected in the thorax with 18.4 nl (100–300 CFU) of each bacterial suspension (\textit{P. luminescens}, \textit{P. asymbiotica} or \textit{E. coli}) or sterile 1XPBS (septic injury control) using a Nanoject II apparatus (Drummond Scientific) equipped with glass capillaries prepared with a micropipette puller (Sutter Instruments). Two replicates of 10 flies each were used for each treatment and survival was recorded at 6-hour intervals and up to 48 hours. Each experiment was replicated at least 3 times.

**Bacterial load and gene transcription**

All procedures were performed as described previously.\textsuperscript{12} Briefly, 4-five adult flies were injected and subsequently frozen at 6 and 18 hours post infection (hpi). DNA was extracted from the frozen flies using DNeasy Blood and Tissue kit (Qiagen) using the manufacturer’s protocol. The DNA samples were adjusted to 500 ng for estimating bacterial load. Samples were run in technical duplicates and Quantitative PCRs were performed in twin-tech. semi-skirted 96 well plates on a Mastercycler\textsuperscript{®} ep realplex.\textsuperscript{3} Standard curves for each bacterium were used to estimate the bacterial load in infected flies.

For gene transcription studies, total RNA was isolated using the PrepEase RNA spin kit (Affymetrix USB), followed by cDNA synthesis and quantitative RT-PCR (qRT-PCR). \textit{ΔΔCt} method was used to perform analysis. Data are presented as the ratio between injected flies versus uninfected flies (baseline controls). All the experiments were performed at least 3 times. The list of primers used for the PCR assays are listed in Table 1.
**Hemolymph collection, hemocyte counts and viability**

Hemolymph was collected from adult female flies (n = 4) at 18 hpi with *P. luminescens*, *P. asymbiotica*, *E. coli* or 1X PBS injection; using a modified version of a previously published protocol. Briefly, flies were anesthetized using CO₂ and then injected into the thorax with 2–3 μL of incubation solution (60% Grace’s Medium (GM) supplemented with 10% of Fetal Bovine Serum (FBS) and 20% of Anticoagulant Buffer (98 mM NaOH, 186 mM NaCl, 1.7 mM EDTA and 41 mM citric acid, pH 4.5)) using a blunt end needle (16 gauge) fitted with a tubing connected to a 20 ml glass syringe. After 20 minutes of incubation on ice, the flies were kept on a petri dish and an incision was made between the 2nd and 3rd abdominal segments. Flies were again injected into the thorax with 5 μL of collection solution (90% of GM supplemented with 10% of FBS). Hemolymph was then collected in a 1.5 mL tube and used for further assays. Hemolymph samples (10 μL) were loaded on a hemocytometer and total numbers of cells as well as the different hemocyte types were estimated using 40X magnification of a compound microscope (Olympus CX21). For cell viability, Trypan blue exclusion assay was performed. All experiments were repeated at least 3 times.

**Functional ablation of hemocytes**

For ablating the function of hemocytes in *D. melanogaster*, flies were anesthetized with CO₂ and then injected with 69 nL of latex beads (0.3 um diameter, Molecular Probes, Invitrogen) into the thorax using a Nanoject II apparatus (Drummond Scientific) equipped with glass capillaries prepared with a micropipette puller (Sutter Instruments). Latex beads were prepared by washing them with sterile 1XPBS and used 4X concentrated in PBS (corresponding to 5–10% solids). 1XPBS served as control for the first round. After 18 h, the flies were injected again with each bacterial suspension (*P. luminescens*, *P. asymbiotica* or *E. coli*) or PBS (septic injury control) and used for further assays.

**Phagocytosis assay**

All procedures were performed as described previously. Briefly, Seven flies from each strain were injected with 50.4 nL of 1 mg/mL pHrodo labeled *E. coli* (Molecular Probes) and allowed to phagocytose at room temperature for 60 min. The flies were fixed ventrally on a glass slide using clear nail paint. Fluorescent images of the dorsal surface were obtained using Nikon ECLIPSE Ni microscope (10X magnification) fitted with Zyla (ANDOR) 5.5 camera. The images were analyzed using ImageJ software and analyzed. Each experiment was performed 3 times.

**Melanization and PO activity**

Melanization spots on the site of injury were observed at 3 hpi using a Nikon SMZ18 microscope with Zyla

---

**Table 1. List of primers used in the study.**

| Gene        | Accession number | Primer      | Primer Sequence                        | Tm (°C) |
|-------------|------------------|-------------|----------------------------------------|---------|
| Mcf-1       | BX571872         | Forward     | 5’-TTGGCGGGGTGTTAGTCG-3’                | 61      |
|             |                   | Reverse     | 5’-CAGTTGCTTCTTCTTCTA-3’                |         |
| 16s rRNA    | CP010445         | Forward     | 5’-GGAAAGCTGTCCTGTGTA-3’                | 61      |
|             |                   | Reverse     | 5’-AGCCGGGATGCAGTGAC-3’                 |         |
| TccC3       | FM162591         | Forward     | 5’-CGGAGGGAATAGTGAGG-3’                 | 61      |
|             |                   | Reverse     | 5’-TGATGGTCAAGAGGCAA-3’                 |         |
| RpL32       | CG7939           | Forward     | 5’-GGGACGAGACGCTGTTG-3’                 | 61      |
|             |                   | Reverse     | 5’-AGTCCTAAGAGCCGCTGA-3’                |         |
| Tep1        | CG18096          | Forward     | 5’-CACTCTGATCAAACGATTATG-3’             | 61      |
|             |                   | Reverse     | 5’-TGTTCTGAGCAAGCAGCAG-3’               |         |
| Tep2        | CG7052           | Forward     | 5’-CCGCGATCCATACGACGTC-3’               | 61      |
|             |                   | Reverse     | 5’-AAATCCAACGTTACCC-3’                  |         |
| Tep3        | CG7068           | Forward     | 5’-CAAAAGGTCATGATGTC-3’                 | 61      |
|             |                   | Reverse     | 5’-TAACTCCTCAACGCCCTCC-3’               |         |
| Defensin    | CG1385           | Forward     | 5’-CGATGACGCCCTTCC-3’                   | 57      |
|             |                   | Reverse     | 5’-ACCCGAGTCCATAC-3’                    |         |
| Diptericin  | CG10794          | Forward     | 5’-GTTTTCTCAACAGGTTTCAAACAGAT-3’        | 61      |
|             |                   | Reverse     | 5’-CTAAGGATGATGATGAT-3’                 |         |
| Turandot-M  | CG14027          | Forward     | 5’-AATAAACACTTTAACGCGTTAAATTG-3’        | 61      |
|             |                   | Reverse     | 5’-GGCTCATTGAAGCGGAGAAG-3’              |         |
| Puckered    | CG7850           | Forward     | 5’-GATTGCGAGCTTCAATGATG-3’              | 57      |
| Eater       | CG1624           | Forward     | 5’-ATACAGCTCATTCAATAC-3’                | 57      |
|             |                   | Reverse     | 5’-GATTGCGAGCTTCAATAC-3’                |         |
(ANDOR) 5.5 camera. Images were analyzed using Nikon Software Suite at 10X magnification. PO activity was measured as described previously (Shokal and Eleftherianos, 2016). Briefly, at 3 hpi the injected flies (n = 20) were placed on a spin column (Pierce, Thermo fisher) containing 2.5X protease inhibitor (Sigma) and covered with 5 4 mm glass beads (VWR). They were centrifuged at 4°C and 13,000 rpm for 20 min. Protein concentrations were then adjusted using a BCA test. A mixture of 15 μg of protein (diluted in 2.5x protease inhibitor) with 5 mM CaCl₂ was added to L-DOPA solution (15 mM in phosphate buffer, pH 6.6) making a final volume of 200 μL. The absorbance (OD 492 nm) for each sample was measured after 36 min incubation at 29°C in the dark against a blank control. Each experiment was performed in biologic duplicates and repeated 3 times.

**Statistical analysis**

All statistics were performed using the GraphPad Prism7 software. Analysis of survival experiments was conducted using a Log-rank (Mantel-Cox) and Chi-square tests. Unpaired 2-tailed t-test and 2-way analysis of variance (ANOVA) with a Tukey post-hoc test for multiple comparisons were used for analyzing bacterial load, gene expression data, hemocyte cell counts, cell viability and PO activity results. p values below 0.05 were considered statistically significant.

**Abbreviations**

Hpi hours post infection
PO Phenoloxidase
TEPs Thioester-containing proteins

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank members of the Department of Biological Sciences at GWU for critical reading of the manuscript. We also thank Dr. Mollie Manier for letting us use the fluorescence microscope and stereomicroscope in her laboratory.

**Funding**

This research was supported by a start-up fund from the Columbian College of Arts and Sciences at GWU to I.E., the Harlan Summer Fellowships from the Department of Biological Sciences at GWU and a scholarship from the Cosmos Club Foundation (Washington, DC) to US. The I. E. laboratory is funded by the National Institutes of Health, (grant 1R01AI110675-01A1).

**References**

[1] Lemaitre B, Hoffmann J. The host defense of Drosophila melanogaster. Ann Rev Immunol 2007; 25:697-743; PMID:17201680; https://doi.org/10.1146/annurev.immunol.25.022106.14161510.1146/annurev.immunol.25.022106.141615

[2] Pal S, Wu LP. Pattern recognition receptors in the fly: lessons we can learn from the Drosophila melanogaster immune system. Fly (Austin) 2009; 3:321-9; PMID:19440043; https://doi.org/10.4161/fly.882710.4161/fly.8827

[3] Buresova V, Hajdusek O, Franta Z, Sojka D, Kopacek P. IrAM-An alpha2-macroglobulin from the hard tick Ixodes ricinus: characterization and function in phagocytosis of a potential pathogen Chryseobacterium indologenes. Dev Comp Immunol 2009; 33:489-98; PMID:18948134; https://doi.org/10.1016/j.dci.2008.09.011

[4] Blandin S, Shiao SH, Moita LF, Janse CJ, Waters AP, Kafatos FC, Levashina EA. Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector Anopheles gambiae. Cell 2004; 116:661-70; PMID:15006349; https://doi.org/10.1016/S0092-8674(04)00173-410.1016/S0092-8674(04)00173-4

[5] Xiao X, Liu Y, Zhang X, Wang J, Li Z, Pang X, Wang P, Cheng G. Complement-related proteins control the flavivirus infection of Aedes aegypti by inducing antimicrobial peptides. PLoS Pathog 2014; 10:e1004027; PMID:24722701; https://doi.org/10.1371/journal.ppat.1004027.10.1371/journal.ppat.1004027

[6] Ricklin D, Hajishengallis G, Yang K, Lambris JD. Complement: a key system for immune surveillance and homeostasis. Nat Immunol 2010; 11:785-97; PMID:20720586; https://doi.org/10.1038/nl.192310.138/nl.1923

[7] Blandin SA, Marois E, Levashina EA. Antimalarial responses in Anopheles gambiae: from a complement-like protein to a complement-like pathway. Cell Host Microbe 2008; 3:364-74; PMID:18541213; https://doi.org/10.1016/j.chom.2008.05.00710.1016/j.chom.2008.05.007

[8] Arefin B, Kucerova L, Dobes P, Markus R, Strnad H, Wang Z, Hyrsl P, Zurovec M, Theopold U. Genome-wide transcriptional analysis of Drosophila larvae infected by entomopathogenic nematodes shows involvement of complement, recognition and extracellular matrix proteins. J Innate Immun 2014; 6:192-204; PMID:23988573; https://doi.org/10.1159/00035373410.1159/000353734

[9] Wertheim B, Kraaijeveld AR, Schuster E, Blanc E, Kafatos FC, Levashina EA. Complement-like protein to a complement-like pathway. Cell Host Microbe 2008; 3:364-74; PMID:18541213; https://doi.org/10.1016/j.chom.2008.05.00710.1016/j.chom.2008.05.007

[10] Bou Aoun R, Hetru C, Troxler L, Doucet D, Ferrandon D, Matt N. Analysis of thioester-containing proteins during the innate immune response of...
Drosophila melanogaster. J Innate Immun 2011; 3:52-64; PMID:21063077; https://doi.org/10.1159/000321554
10.1159/000321554
[11] Stroschein-Stevel SL, Foley E, O’Farrell PH, Johnson AD. Identification of Drosophila gene products required for phagocytosis of Candida albicans. PLoS Biol 2006; 4: e4; PMID:16336044; https://doi.org/10.1371/journal.pbio.0040004
[12] Shokal U, Eleftherianos I. Thioester-Containing Protein-4 Regulates the Drosophila Immune Signaling and Function against the Pathogen Photorhabdus. J Innate Immun 2017; 9:83-93; PMID:27771727; https://doi.org/10.1159/000450610.1159/000450610
[13] Joyce SA, Watson RJ, Clarke DJ. The regulation of pathogenicity and mutualism in Photorhabdus. Curr Opin Microbiol 2006; 9:127-32; PMID:16480919; https://doi.org/10.1016/j.mib.2006.01.004
[14] Felfoldi G, Marokhazi J, Kejpo M, Venekel I. Identification of natural target proteins indicates functions of a serralysin-type metalloprotease, PrtA, in antimicrobial mechanisms. Appl Environ Microbiol 2009; 75:3120-6; PMID:19304826; https://doi.org/10.1128/AEM.02271-08
[15] Vlisidou I, Dowling AJ, Evans IR, Waterfield N, ffrench-Constant RH, Wood W. Drosophila embryos as model systems for monitoring bacterial infection in real time. PLoS Pathog 2009; 5:e1000518; PMID:19609447; https://doi.org/10.1371/journal.ppat.1000518
[16] Daborn PJ, Waterfield N, Silva CP, Au CP, Sharma S, ffrench-Constant RH. A single Photorhabdus gene, makes caterpillars floppy (mcf), allows Escherichia coli to persist within and kill insects. Proc Natl Acad Sci U S A 2002; 99:10742-7; PMID:12136122; https://doi.org/10.1073/pnas.102068099
[17] Eleftherianos I, Boundy S, Joyce SA, Aslam S, Marshall JW, Cox RJ, Simpson TJ, Clarke DJ, ffrench-Constant RH, Reynolds SE. An antibiotic produced by an insect-pathogenic bacterium suppresses host defenses through phenoloxidase inhibition. Proc Natl Acad Sci U S A 2007; 104:2419-24; PMID:17284598; https://doi.org/10.1073/pnas.0610525104.10.1073/pnas.0610525104
[18] Eleftherianos I, Waterfield NR, Bone P, Boundy S, ffrench-Constant RH, Reynolds SE. A single locus from the entomopathogenic bacterium Photorhabdus luminescens inhibits activated Manduca sexta phenoloxidase. FEMS Microbiol Lett 2009; 293:170-6; PMID:19243439; https://doi.org/10.1111/j.1574-6968.2009.01523.x
[19] Ullah I, Khan AL, Ali L, Khan AR, Waqas M, Lee IJ, Shin JH. An insecticidal compound produced by an insect-pathogenic bacterium suppresses host defenses through phenoloxidase inhibition. Molecules 2014; 19:20913-28; PMID:25514230; https://doi.org/10.3390/molecules191220913.10.3390/molecules191220913
[20] Eleftherianos I, ffrench-Constant RH, Clarke DJ, Dowling AJ, Reynolds SE. Dissecting the immune response to the entomopathogen Photorhabdus. Trends Microbiol 2010; 18:552-60; PMID:2035345; https://doi.org/10.1016/j.tim.2010.09.006
[21] Imler JL, Bulet P. Antimicrobial peptides in Drosophila: structures, activities and gene regulation. Chem Immunol Allergy 2005; 86:1-21; PMID:15976485.
[22] Shokal U, Yadav S, Atri J, Accetta J, Kenney E, Banks K, Katakam A, Jaenike J, Eleftherianos I. Effects of co-occurring Wolbachia and Spiroplasma endosymbionts on the Drosophila immune response against insect pathogenic and non-pathogenic bacteria. BMC Microbiol 2016; 16:16; PMID:26862076; https://doi.org/10.1186/s12866-016-0634-6
[23] Castillo JC, Shokal U, Eleftherianos I. Immune gene transcription in Drosophila adult flies infected by entomopathogenic nematodes and their mutualistic bacteria. J Insect Physiol 2013; 59:179-85; PMID:22902989; https://doi.org/10.1016/j.jinsphys.2012.08.00310.1016/j.jinsphys.2012.08.003
[24] Wicker C, Reichhart JM, Hoffmann D, Hultmark D, Samakovlis C, Hoffmann JA. Insect immunity. Characterization of a Drosophila CDNA encoding a novel member of the dipterin family of immune peptides. J Biol Chem 1990; 265:22493-8.
[25] McEwen DG, Peifer M. Pucked, a Drosophila MAPK phosphatase, ensures cell viability by antagonizing JNK-induced apoptosis. Development 2005; 132:3935-46; PMID:16079158; https://doi.org/10.1242/dev.019491.10.1242/dev.01949
[26] Brun S, Vidal S, Spellman P, Takahashi K, Tricoire H, Lemaître B. The MAPKKK Mekk1 regulates the expression of Turandot stress genes in response to septic injury in Drosophila. Genes Cells 2006; 11:397-407; PMID:16611243; https://doi.org/10.1111/j.1365-2443.2006.00953.x
[27] Chung YS, Kocks C. Recognition of pathogenic microbes by the Drosophila phagocytic pattern recognition receptor Eater. J Biol Chem 2011; 286:26524-32; PMID:21613218; https://doi.org/10.1074/jbc.M110.214007.10.1074/jbc.M110.214007
[28] Irving P, Troxler L, Heuer TS, Belvin M, Kopczynski C, Wicker C, Reichhart JM, Hoffmann D, Hultmark D, Samakovlis C, Hoffmann JA. Insect immunity. Characterization of a Drosophila cDNA encoding a novel member of the dipterin family of immune peptides. J Biol Chem 1990; 265:22493-8.
[29] McEwen DG, Peifer M. Pucked, a Drosophila MAPK phosphatase, ensures cell viability by antagonizing JNK-induced apoptosis. Development 2005; 132:3935-46; PMID:16079158; https://doi.org/10.1242/dev.019491.10.1242/dev.01949
[30] Brun S, Vidal S, Spellman P, Takahashi K, Tricoire H, Lemaître B. The MAPKKK Mekk1 regulates the expression of Turandot stress genes in response to septic injury in Drosophila. Genes Cells 2006; 11:397-407; PMID:16611243; https://doi.org/10.1111/j.1365-2443.2006.00953.x
[31] Chung YS, Kocks C. Recognition of pathogenic microbes by the Drosophila phagocytic pattern recognition receptor Eater. J Biol Chem 2011; 286:26524-32; PMID:21613218; https://doi.org/10.1074/jbc.M110.214007.10.1074/jbc.M110.214007
[32] Irving P, Troxler L, Heuer TS, Belvin M, Kopczynski C, Wicker C, Reichhart JM, Hoffmann D, Hultmark D, Samakovlis C, Hoffmann JA. Insect immunity. Characterization of a Drosophila cDNA encoding a novel member of the dipterin family of immune peptides. J Biol Chem 1990; 265:22493-8.
[33] McEwen DG, Peifer M. Pucked, a Drosophila MAPK phosphatase, ensures cell viability by antagonizing JNK-induced apoptosis. Development 2005; 132:3935-46; PMID:16079158; https://doi.org/10.1242/dev.019491.10.1242/dev.01949
complement-like protein in toll and JAK gain-of-function mutants of Drosophila. Proc Natl Acad Sci U S A 2000; 97:11427-32; PMID:11027343; https://doi.org/10.1073/pnas.97.21.1142710.1073/pnas.97.21.11427

[33] Ali H. Regulation of human mast cell and basophil function by anaphylatoxins C3a and C5a. Immunol Lett 2010; 128:36-45; PMID:19895849; https://doi.org/10.1016/j.imlet.2009.10.007

[34] Vallet-Gely I, Lemaitre B, Boccard F. Bacterial strategies to overcome insect defences. Nat Rev Microbiol 2008; 6:302-13; PMID:18327270; https://doi.org/10.1038/nrmicro187010.1038/nrmicro1870

[35] Visschedyk DD, Perieteanu AA, Turgeon ZJ, Fieldhouse RJ, Dawson JF, Merrill AR. Photox, a novel actin-targeting mono-ADP-ribosyltransferase from Photorhabdus luminescens. J Biol Chem 2010; 285:13525-34; PMID:20181945; https://doi.org/10.1074/jbc.M109.077339

[36] Lang AE, Schmidt G, Schlosser A, Hey TD, Larrinua IM, Sheets JJ, Mannherz HG, Aktories K. Photorhabdus luminescens toxins ADP-ribosylate actin and RhoA to force actin clustering. Science 2010; 327:1139-42; PMID:20185726; https://doi.org/10.1126/science.118455710.1126/science.1184557

[37] Costa SC, Girard PA, Brehelin M, Zumbihl R. The emerging human pathogen Photorhabdus asymbiotica is a facultative intracellular bacterium and induces apoptosis of macrophage-like cells. Infect Immun 2009; 77:1022-30; PMID:19075024; https://doi.org/10.1128/I AI.01064-0810.1128/IAI.01064-08

[38] Tang H. Regulation and function of the melanization reaction in Drosophila. Fly (Austin) 2009; 3:105-11; PMID:19164947; https://doi.org/10.4161/fly.3.1.774710.4161/fly.3.1.7747

[39] Eleftherianos I, Revenis C. Role and importance of phenoloxidase in insect hemostasis. J Innate Immun 2011; 3:28-33; PMID:21051882; https://doi.org/10.1159/0003219310.1159/000321931

[40] Hollmann TJ, Mueller-Ortiz SL, Braun MC, Wetsel RA. Disruption of the C5a receptor gene increases resistance to acute Gram-negative bacteremia and endotoxic shock: opposing roles of C3a and C5a. Mol Immunol 2008; 45:1907-15; PMID:18063050; https://doi.org/10.1016/j.molimm.2007.10.037

[41] Castillo JC, Robertson AE, Strand MR. Characterization of hemocytes from the mosquitoes Anopheles gambiae and Aedes aegypti. Insect Biochem Mol Biol 2006; 36:891-903; PMID:17098164; https://doi.org/10.1016/j.ibmb.2006.08.010