

Participation of the p38 pathway in *Drosophila* host defense against pathogenic bacteria and fungi

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The signaling network of innate immunity in *Drosophila* is constructed by multiple evolutionarily conserved pathways, including the Toll- or Imd-regulated NF- κ B and JNK pathways. The p38 MAPK pathway is evolutionarily conserved in stress responses, but its role in *Drosophila* host defense is not fully understood. Here we show that the p38 pathway also participates in *Drosophila* host defense. In comparison with wild-type flies, the sensitivity to microbial infection was slightly higher in the *p38a* mutant, significantly higher in the *p38b* mutant, but unchanged in the *p38c* mutant. The *p38b*; *p38a* double-mutant flies were hypersensitive to septic injury. The immunodeficiency of *p38b*; *p38a* mutant flies was also demonstrated by hindgut melanization and larvae stage lethality that were induced by microbes naturally presented in fly food. A canonical MAP3K-MKK cascade was found to mediate p38 activation in response to infection in flies. However, neither Toll nor Imd was required for microbe-induced p38 activation. We found that p38-activated heat-shock factor and suppressed JNK collectively contributed to host defense against infection. Together, our data demonstrate that the p38 pathway-mediated stress response contribute to *Drosophila* host defense against microbial infection.

Innate immunity is essential for multicellular organisms to defend themselves against microbial infection. Studies in *Drosophila melanogaster* revealed several signaling pathways that play essential roles in innate immunity (1). The Toll pathway mediates resistance to pathogenic fungi and Gram-positive bacteria by activating two NF- κ B proteins, Dorsal and Dorsal-related immunity factor (1, 2). The Imd pathway activates Relish, the third NF- κ B protein, in response to Gram-negative bacteria (3, 4). The NF- κ B family of transcription factors regulates the expression of a distinct set of genes coding for antimicrobial peptides (AMP) against bacteria and fungi (5). Recently the JNK and JAK-STAT pathways were shown to control additional targets induced by microbial agents or damage (6–10). Because the above pathways are also important in mammalian host defense, the innate immune responses appear to be largely regulated by evolutionarily conserved signaling pathways.

The p38 MAPK pathway is an evolutionarily conserved signaling pathway that responds to a variety of stresses (11). The p38 pathway can be activated by pathogens in animals, and is known to play a pivotal role in inflammatory responses in mammals (12). Mpk1, a p38 ortholog in *Caenorhabditis elegans*, is essential for innate immunity by participating in regulating antimicrobial peptides (13). The *D. melanogaster* genome encodes three p38 orthologs, designated as p38a (CG5475), p38b (CG7393, also known as mpk2), and p38c (CG3338) (14). Orthologs p38a and p38b have almost the same level of sequence similarity with mammalian p38 α or p38 β , thus whether p38a or p38b corresponds to p38 α cannot be determined. The ortholog p38c is unique for *Drosophila* because it does not contain dual regulation sites, the hallmark of MAPK members. Both p38a and p38b can be activated by bacterial stimuli in cultured cells (14). However, *p38a* mutant flies do not show a significant change in their susceptibility to bacterial infection (15). Likely because of the redundant functions of these three p38 orthologs in flies, whether the p38 pathway has any role in fly innate immunity still needs further investigation.

In the present study, we generated fly mutants for all three p38 orthologs and studied the role of the p38 pathway in host defense. We found that *p38a* and *p38b*, but not *p38c*, were involved in host defense, and *p38b* was functionally more important than *p38a*. Activation of p38 in flies is mediated by a MAP3K-MKK cascade, but not controlled by the Toll and Imd pathways. In addition, *p38a* and *p38b* suppress JNK activity, which contributes to the host-defense function of p38. Activation of heat-shock factor (Hsf) by p38 is an important part of antimicrobial reactions in flies. Taken together, our data indicate that the p38 pathway-mediated stress response is part of the innate immunity of flies.

Results

Generation of Mutants for p38 MAPKs. We obtained several *Drosophila* lines with *P*-element insertions that are closely located in the loci of p38 MAPKs from the Gene Disrupt Project database (<http://flypush.imgen.bcm.tmc.edu/pscreen/>) (Fig. S1). *P*-element-mediated imprecise excisions were carried out to obtain mutants for the all three p38 orthologs, following the method as described previously (16) (Fig. S1). Flies with single mutations of *p38a* and *p38c* or double mutations of *p38a* and *p38c* did not have any visible morphological or developmental abnormalities. The *p38b* mutant flies appeared to be morphologically normal, but compared with wild-type they required approximately two additional days to develop from egg deposition to eclosion at 22 °C. The *p38b*; *p38a* double-mutant flies typically died at larval and pupal stages under standard culture conditions, with only a few escapers. The mutants lacking all three p38 orthologs (*p38b*; *p38a*; *p38c*) died at early larval stages and rarely proceeded to third instar larvae. The above results indicate that the p38 homologs could compensate for each other to some extent.

p38 MAPKs Protect *Drosophila* from Infections by Microbes in Food. The lethality of *p38b*; *p38a* and *p38b*; *p38a*; *p38c* mutant flies could be a result of developmental defects or impairment of host defense. We studied *p38b*; *p38a* double mutants to address this issue. Because *Drosophila* encounters many microorganisms when cultured with standard food in laboratories, we cultured *p38b*; *p38a* mutant flies in either near-sterile conditions (*Materials and Methods*) or with standard food containing antibiotics and observed a significant increase in survival rate under both culture conditions (Fig. 1A). Therefore, microbial infection was a major cause of lethality in *p38b*; *p38a* mutant flies cultured with standard methods. To confirm that microbial pathogens were the cause of lethality in *p38b*; *p38a* mutant flies, we introduced *Pseudomonas aeruginosa* into sterilized food and found an elevated death rate (Fig. 1B). Including nonpathogenic bacterium *Escherichia coli*

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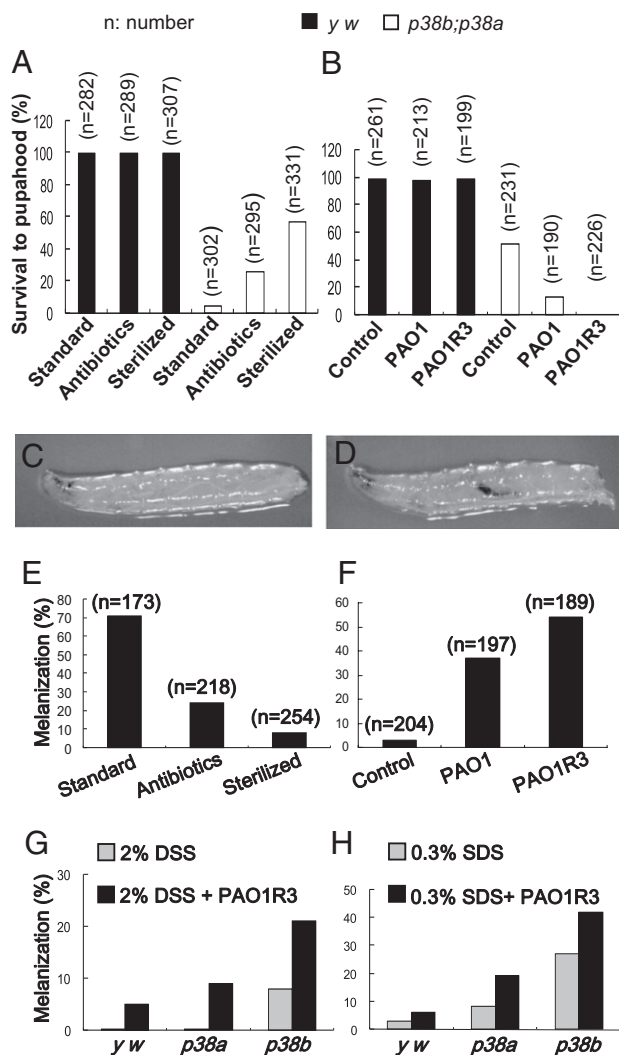


Fig. 1. The p38-deficient flies are more sensitive to microbial pathogens in food. (A) The survival rate to pupahood of the wild-type yellow white strain (*y w*) and *p38a*; *p38a* double-mutant (*p38a*¹³; *p38b*^{156A}) (Fig. S1) larvae cultured in standard food with or without a mixture of antibiotics (500 μ g/mL ampicillin, 50 μ g/mL tetracycline, and 200 μ g/mL rifamycin), or in sterilized food. (B) The survival rate to pupahood of the wild-type and *p38b*; *p38a* larvae cultured in sterilized food with or without adding 10^5 /mL *P. aeruginosa* (PAO1, wild-type strain; PAOR3, a more virulent mutant strain repressing quorum-sensing controlled genes). (C) Third instar larvae of *y w* as wild-type control. (D) Hindgut melanization in the *p38b*; *p38a* double mutant. (E) The melanization rate in the hindgut of the *p38b*; *p38a* larvae cultured in standard food with or without a mixture of antibiotics as in A, or in sterilized food. (F) The melanization rate in the hindgut of the *p38b*; *p38a* larvae cultured in sterilized food with or without adding 10^5 /mL *P. aeruginosa* as in B. (G and H) Melanization in the hindgut can be induced by dextran sodium sulfate (DSS) or SDS with the combination of PAO1R3.

or yeast *Saccharomyces cerevisiae* in sterilized food had no effect on survival, suggesting an increased immune susceptibility for pathogens like *P. aeruginosa*. The notion that microbes in the standard culture food could kill *p38b*; *p38a* double-mutant but not wild-type flies was further supported by the fact that over 30% of the *p38b*; *p38a* double mutants could reach adulthood, with a mean lifespan of almost 4 wk, under near-sterile conditions (Fig. S24). These results suggest that the immunity to microbial infection was weakened in *p38b*; *p38a* mutant flies compared with wild-type counterparts.

Microbes in Culture Food Causes Hindgut Melanization in *p38a/b* Mutants. We found melanization in the hindgut of *p38b*; *p38a* double-mutant larvae but not in wild-type, *p38a*, *p38b*, *p38c*, or *p38a*; *p38c* mutant larvae (Fig. 1 C and D, and Fig. S2B). Because the *Drosophila* hindgut encounters many microorganisms when cultured with standard methods in laboratories, and melanization is known to participate in host defense in insects, we sought to determine whether the melanization observed in *p38b*; *p38a* double mutant flies is related to microbial infections. A significant reduction in melanization was observed when *p38b*; *p38a* mutant flies were cultured in a near-sterile conditions or with standard food containing antibiotics (Fig. 1E), which is correlated with the increase in survival rate (Fig. 1A), indicating that microbial infection was a major cause of melanization in *p38b*; *p38a* mutants. In support of this conclusion, introducing *P. aeruginosa* into sterilized food elevated melanization, correlating with increased death rates (Fig. 1B and F). Nonpathogenic *E. coli* or *S. cerevisiae* had no effect on the melanization of the hindgut. Collectively, our data demonstrated that infection was a cause of melanization in the hindgut of *p38b*; *p38a* mutant larvae, and the rate of melanization correlated with the death rate.

The wall of the *Drosophila* hindgut is composed of a monolayer of epithelial cells mounted outside with a thin layer of circular muscle fibers (17). The melanization in *p38b*; *p38a* mutants could be observed as early as in the second instar larvae; it gradually extended and could eventually occupy the whole hindgut (Fig. S3A). The adult flies of *p38b*; *p38a* double mutants also showed melanization in the hindgut (Fig. S3B). The histology of paraffin sections showed that the melanization starts inside epithelial layer of hindgut in *p38b*; *p38a* mutant larvae (Fig. S3C). Electron microscope analysis detected vacuoles in the epithelial cells in the melanized regions (Fig. S3D). Muscle disruption could also be found after severe melanization (Fig. S3E). Although there were no visible morphological abnormalities in the hindgut of *p38b*; *p38a* mutant larvae before melanization, the peritrophic membrane, which is secreted by epithelial cells to cover the surface of the gut lumen and protect the epithelium from digestion and invasion by pathogens, was thinner in the hindgut of *p38b*; *p38a* mutants in comparison with wild-type counterparts, as determined by Calcofluor staining (Fig. S3F). This finding suggested that developmental defects in the hindgut in *p38b*; *p38a* mutants contributed to the increased susceptibility to infection of gut microbial pathogens.

Dextran sodium sulfate (DSS)-induced colitis is one of the most widely used models of intestinal inflammation in mice. DSS is directly toxic to gut epithelial cells of the basal crypts and affects the integrity of the mucosal barrier. Because melanization of the hindgut in flies is associated with tissue damage, we sought to test whether DSS has an effect on the fly gut. We found that melanization in the fly hindgut could not be induced by oral feeding with DSS in wild-type larvae but was induced in larvae of *p38b* single mutants (Fig. 1G), suggesting that epithelial injury can facilitate melanization in the hindgut. *P. aeruginosa*, at an amount that does not cause melanization, significantly enhanced the rates of DSS-induced melanization in a *p38b* and *p38a* flies (Fig. 1G), confirming that melanization was associated with infection. In addition, we observed that SDS was more potent than DSS in triggering melanization in the fly hindgut (Fig. 1H). Thus, similar to that in mammals, deficiency of p38 in gut epithelia could promote gut injury (18, 19).

p38 MAPKs Protect of *Drosophila* from Septic Injury. To further evaluate the role of p38a, p38b, and p38c in fly immunity, we examined adult flies that were either pricked with bacteria (*Enterobacter cloacae* and *Listeria monocytogenes*) or rolled with fungal spores (*Beauveria bassiana*). The *p38a* and *p38b* mutants, but not the *p38c* mutant, showed some increase in susceptibility to these microbial infections, with the *p38b* mutant being the most

susceptible (Fig. 2 A–C). The adult *p38b;p38a* double-mutant flies were more sensitive to infection than that of *p38a* or *p38b* single-mutant flies (Fig. 2 D–F). The *p38b;p38a* double-mutant flies were also challenged by septic injury with *E. coli*, *P. aeruginosa*, *Staphylococcus aureus*, and *Aspergillus fumigatus*, respectively, and compared with *y w* control, the mutant flies were hypersensitive to *P. aeruginosa*, *S. aureus*, and *A. fumigatus*, but were not sensitive to *E. coli* (Fig. S4). A rescue of *p38b* expression in *p38b;p38a* mutants by overexpression of UAS-*p38b* with da-Gal4 increased the survival rate of infected flies to a level close to wild-type (Fig. 2 D–F). Hence, the p38 pathway protects *Drosophila* from septic injury-induced death from a broad spectrum of pathogenic microbes, and p38b plays a major role in the p38 pathway-mediated host defense.

Expression of a Variety of Immune- or Host Defense-related Genes Are Altered in *p38b* Mutants. We next carried out a gene-expression profiling of *p38b* mutants in comparison with wild-type (*y w*) flies. A genome-wide microarray analysis with 15,000 probes was performed on flies treated with or without *E. cloacae* or *L. monocytogenes* as described (20). The expression pattern of the genes was analyzed using Cluster and Treeview (Fig. S5). One-hundred ninety-seven genes were down-regulated and 112 genes were up-regulated in *p38b* (Fig. S5 and Dataset S1). Ninety-three of the 197 down-regulated genes and 20 of the 112 up-regulated genes were genes that responded to both *E. cloacae* and *L. monocytogenes* treatments (Fig. S5). Thirty-four of the down-regulated genes and 27 of the up-regulated genes in *p38b* mutants belong to *Drosophila* immune-regulated genes (21). In addition, 39 of the down-regulated genes and 18 of the up-regulated genes were also suggested to play a role in host defense based on annotation (Dataset S2). In *p38b* mutants, five AMP genes (*Def*, *DptB*, *Dro*, *Dros*, *Mtk*) were up-regulated, whereas three AMP genes (*AttA*, *AttC*, *CecB*) were down-regulated. Nine cytochrome P450 genes and six GSTs were down-regulated. However, three cytochrome P450 genes (*Cyp4d21*, *Cyp4e3*, *Cyp6a13*) were also up-regulated. All four *Tot* genes and two *Hsp* (*Hsp60D*, *Hsp70Bc*) genes were down-regulated (Dataset S2). We used qRT-PCR to validate the microarray results of several genes belonging to the AMP, cytochrome P450, tur-

andont (*Tot*), and *Hsp* families in *p38b* mutant and *p38b;p38a* double-mutant flies treated with or without septic injuries, and validated the microarray data (Fig. S6). The p38 pathway could both positively and negatively regulate a number of different immune- or host defense-related genes, and thus should affect host defense in both positive and negative ways. The increased susceptibility to infection in p38 mutants could be a result of the sum of the effects caused by the changes in gene expression.

Role of the p38 Pathway in Host Defense Against Microbial Infections Is Partially Mediated by Heat-Shock Factor. We then focused on heat shock proteins (HSPs) for further studies in addressing the host-defending role of the p38 pathway, because all genes in this group (*Hsp60D*, *Hsp70Bc*, *Hsp26*, and *Hsp27*) were down-regulated in *p38b* mutants and *p38b;p38a* double mutants (Fig. 3 A and B and Fig. S6). Hsf is a key transcription factor that regulates the expression of HSPs (22). *Drosophila* has a single Hsf, and the activation of Hsf by heat shock is known to be mediated by trimerization and localization of Hsf at specific sites on the polytene chromosome (22). We found a similar localization pattern of Hsf on the polytene chromosome 1 h after *E. cloacae* infection in wild-type flies (Fig. 3C). In contrast, we did not detect the localization of Hsf on polytene in *p38b;p38a* double-mutant flies under the same condition (Fig. 3D). Consistently, *E. cloacae* infection-induced trimerization of Hsf was delayed in *p38b;p38a* double-mutant flies, indicating that the p38 pathway is involved in regulating Hsf activity (Fig. 3E).

To assess whether the induction of Hsf and Hsps is a mechanism of host defense, we infected *Hsf^Δ*, a hypomorphic allele) and *Hsp27* mutants with *E. cloacae*, *L. monocytogenes*, and *B. bassiana*. Both *Hsf* and *Hsp27* mutants were more susceptible to pathogenic infections (Fig. 3 F–H). Introducing a copy of a wild-type *Hsf* transgene in a *Hsf^Δ* background or restoration of *Hsp27* activity (UAS-*hsp27* and da-Gal4) in a *Hsp27* mutant background largely rescued their phenotypic susceptibility to pathogenic infections (Fig. 3 F–H), indicating that Hsf activation and Hsf-mediated *Hsp27* expression are part of the *Drosophila* host-defense responses.

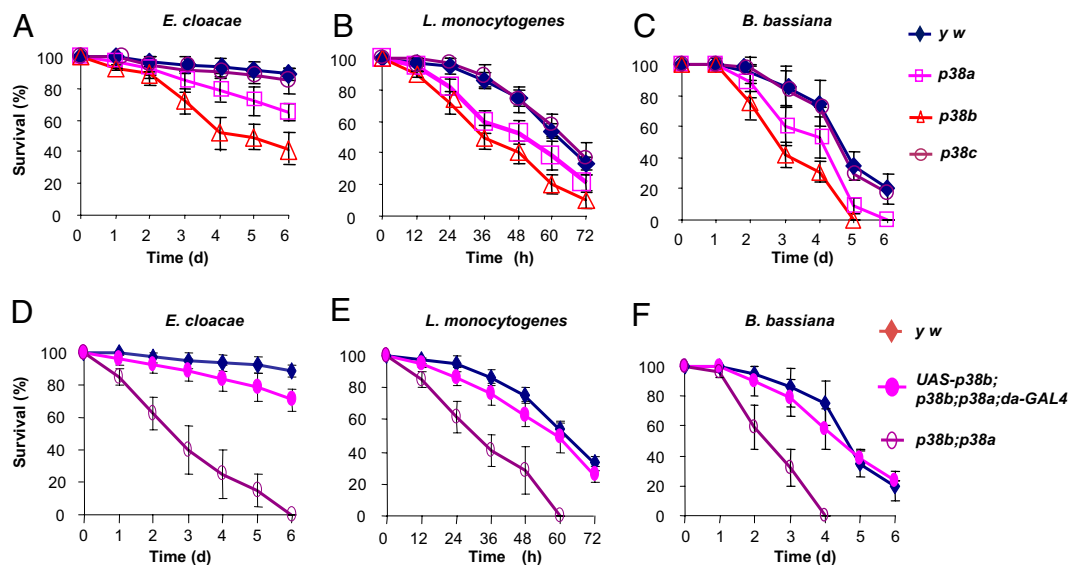


Fig. 2. The p38-deficient flies are more sensitive to septic injury. (A–C) The survival rates of infected adult flies of *p38a* (*p38a¹³*), *p38b* (*p38b^{156A}*), and *p38c* (*p38c⁷*) mutants, and wild-type flies (*y w*). (D–F) The survival rates of infected adult flies of *p38b;p38a* (*p38b^{156A};p38a¹³*) mutants, *p38b;p38a* mutants with overexpression of UAS-*p38b* driven by da-Gal4, and wild-type flies (*y w*). The data were obtained by using groups of 30 female or male adults, aged 2 to 4 d, pricked with *E. cloacae* or *L. monocytogenes* or rolled in *B. bassiana* spores. Six replicates were used for the determination of the survival rates. Error bars represent SEM in each group. *p38a* compared with *y w*, $P < 0.02$; *p38b* and *p38b;p38a* compared with *y w*, $P < 0.002$ and 0.0001, respectively.

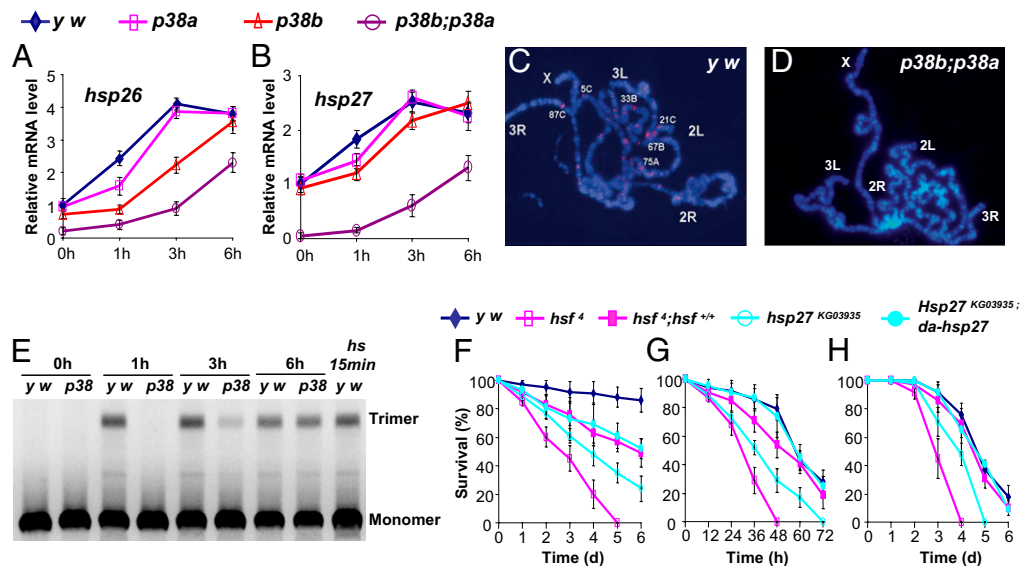


Fig. 3. Delayed induction of *Hsp* genes and Hsf activity in *p38* mutants. Relative mRNA levels of *Hsp26* (A) and *Hsp27* (B) in wild-type, *p38a*, *p38b*, and *p38b;p38a* mutants were shown with qRT-PCR normalized with the internal control *rp49*. Data from three independent experiments are expressed as mean \pm SEM; *p38b;p38a* compared with *y w*, $P < 0.005$. The third instar larval polytene chromosomes from wild-type (C) and *p38b;p38a* double mutants (D) were stained for DNA (DAPI, blue) and Hsf (red) 1 h after septic infection. (E) Hsf trimerization was shown with Western blotting after native gel electrophoresis. (F–H) *Hsf* (*hsf⁴*) and *Hsp27* (*hsp27^{KG03935}*) mutants are more susceptible to septic infection of *E. cloacae* (F) and *L. monocytogenes* (G), and natural infection of *B. bassiana* (H). The susceptibility of *Hsf* and *Hsp27* mutants can be rescued by introduction of a *Hsf* transgene (*hsf⁴;hsf^{+/+}*) and overexpression of *Hsp27* (*Hsp27^{KG03935};da > hsp27*), respectively. Forty males, aged 2 to 4 d were tested for each group as one experiment. Data from three independent experiments are expressed as mean \pm SEM; *Hsf* and *Hsp27* mutants compared with *y w*, $P < 0.0001$ and 0.001 , respectively.

Deficiency of *p38b* Results in Sustained JNK Activation. Gene profiling analysis also showed that the expression levels of *JNK* (*bsk*) are up-regulated in *p38b* mutants (Fig. S7A and Datasets S1 and S2). Using a phospho-JNK antibody, we also observed that phospho-JNK in *p38a*, *p38b*, and *p38b;p38a* mutants was higher than that in *y w* at both a resting stage and after septic injury with *E. cloacae* and *L. monocytogenes* (Fig. 4A and B). Although both *p38a* and *p38b* inhibit JNK expression and activation, the effect of *p38b* was stronger. The inhibition of JNK activation by *p38b* was at least partially mediated by *p38b*-mediated expression of a JNK phosphatase *puc*, because *puc* was not induced by *E. cloacae* and *L. monocytogenes* in *p38b* mutant flies (Fig. S7B and Datasets S1 and S2). Both the increased expression of *JNK* and decreased expression of *puc* might contribute to the sustained JNK activation in *p38* mutants.

To determine whether the sustained JNK activation contributes to the increased susceptibility of *p38* mutants to bacterial infection, we fed *p38* mutants with a JNK inhibitor and analyzed the rates of melanization in the hindgut and the viabilities of the mutants. Because spontaneous melanization in *p38a* and *p38b* single mutants was rare, we used SDS treatment in these experiments. We found that inhibition of JNK increased the survival rates of *p38b* mutants and decreased the melanization rates in their hindguts (Fig. 4C and D). These data suggest that sustained activation of JNK contributes to the melanization and lethality of *p38b* mutants.

Activation of *p38* by Microbes Is MAPK Cascade-Dependent but Toll- and Imd-Independent. Activation of *p38* family members is achieved by phosphorylation on the regulatory dual phosphorylation motif. Western blotting with phospho-*p38*-specific antibodies showed activation of *Drosophila* *p38* after either Gram-negative or Gram-positive injury (Fig. 5A). Because of the similar size of *Drosophila* *p38a* and *p38b* proteins, we were unable to determine whether phosphorylation occurs on *p38a*, *p38b*, or both. Nevertheless, Western blotting with phospho-*p38*-specific antibodies still could be used to evaluate the activation of fly *p38*.

Toll and Imd are the major pathways in flies that respond to different microbial pathogens. To determine the relationship between the *p38* pathway and the Toll pathway, we infected Toll mutant flies with *L. monocytogenes* and found that *p38* phosphorylation was not affected by a Toll loss-of-function mutation and a gain-of-function mutation *Toll^{lob}* (Fig. 5B). We infected *Imd* and *Imd;spz* double mutants with *E. cloacae* and found a similar level of *p38* activation when compared with wild-type flies (Fig. 5B). Knockdown of *licorne* (*lic*), the sole ortholog to mammalian *MKK3/6*, inhibited *L. monocytogenes*-induced *p38* phosphorylation (Fig. 5C and Fig. S7C). Knockdown of *hemipterous* (*hep*), an ortholog of mammalian *MKK4*, did not affect *p38* phosphorylation by infection. We further determined which MAP3K is involved in the activation of *p38* by infection. Knockdown of *slipper* (*slpr*), but not *Tak1*, *Ask1*, and *Mekk1* reduced *p38* activation (Fig. 5C). Therefore, our data reveal a *p38* MAPK cascade that participates in the host defense in *Drosophila*, and indicate that the activation of the *p38* pathway is mediated by an unidentified signaling mechanism.

Discussion

D. melanogaster has been widely used as a model to study innate immunity; however, the role of the *p38* pathway in a fly's host response to microbes remains largely unknown. This study analyzed all *p38* orthologs in flies and found that *p38a* and *p38b*, but not *p38c*, participate in the fly's defense against microbes. Although different *p38* isoforms can functionally compensate one another to a certain degree, *p38b* appears to be more important than *p38a* in host defense against pathogenic infections. Our data suggest that activation of Hsf and suppression of JNK are part of the mechanism used by the *p38* pathway in host defense. Although the *p38* kinase cascade is similar to that in mammals, the upstream signaling mediators (sensor and adaptors) that lead to the activation of the *p38* pathway in flies seems to be different from that of mammals and are currently unknown.

In mammals, the Toll and Imd (RIP) proteins are linked in a signaling cascade, but in flies they are found in independent

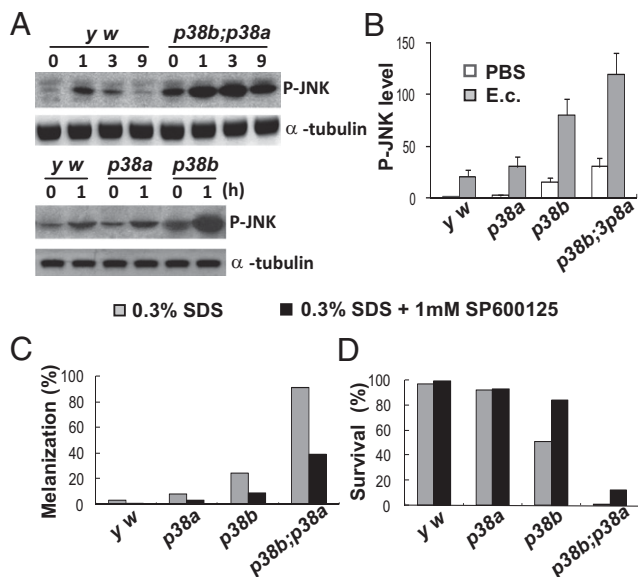


Fig. 4. p38 attenuates JNK expression and activity. (A) Wild-type, *p38a*, *p38b*, and *p38b;p38a* mutant flies were infected with *E. cloacae* for a time period, as indicated. JNK activity determined by immunoblotting with phospho-JNK antibodies. (B) The relative intensity of phospho-JNK (P-JNK) in wild-type, *p38a*, *p38b*, and *p38b;p38a* mutants was measured by Western blotting bands of three independent experiments, with pixel information obtained using Kodak Molecular Imaging Software Version 4.0. (C) The rate of hindgut melanization and (D) rate of survival to adult of the wild-type, *p38a*, *p38b*, and *p38b;p38a* mutants cultured in standard food containing 0.3% SDS with or without the JNK inhibitor SP600125 ($n > 200$). Data in B represent mean (\pm SEM) fold changes of P-JNK compared with *y w* injured with PBS alone; *p38b* versus *y w*, $P < 0.01$; *p38b;p38a* versus *y w*, $P < 0.001$.

pathways. Both p38 and NF- κ B are often activated simultaneously by signaling from the same receptor in mammals, but here we found that these two pathways are likely activated by different upstream receptors or sensors in flies. Mammalian p38 primarily participates in host defense by regulating cytokine expression, but fly p38 seems to have a minimal role in the specific antimicrobial response, such as AMP expression. We show here that activation of Hsf, a key transcription factor in stress responses, was impaired in p38 mutants, providing unique evidence that the p38 pathway directly or indirectly regulates this transcription factor. Hsf activates the transcription of a large number of genes that regulate protein homeostasis, including the molecular chaperones Hsps. Our data suggest that the p38 pathway regulates Hsf before or during its trimerization. Because of the conservation of Hsf, it is likely that p38 also regulates Hsf in mammals. Because of the role of Hsf in general stress, the anti-infection function of the p38 pathway in flies is at least in part caused by its general stress responses. However, the regulation of Hsf by the p38 pathway in *Drosophila* appears to be selective for bacterial pathogens, because heat shock-induced activation of Hsf is normal in *p38b;p38a* mutants.

Recently *p38a* was shown to be involved in dual oxidase (Duox) induction in *Drosophila*, and Duox-dependent reactive oxygen species is known to serve on the front line microbicidal function in fly gut (23). Although *p38a* was shown to play a minor role in the host defense against microorganisms in our study, its role in Duox induction may contribute to the phenotype observed in *p38b;p38a* mutants. Because Duox plays a role in peritrophic matrix cross-linking in mosquitoes (24), it is possible that the control of Duox expression by the p38 pathway is an underlying mechanism for the defect in peritrophic membrane observed in *p38b;p38a* flies. Furthermore, *p38b* was found to play

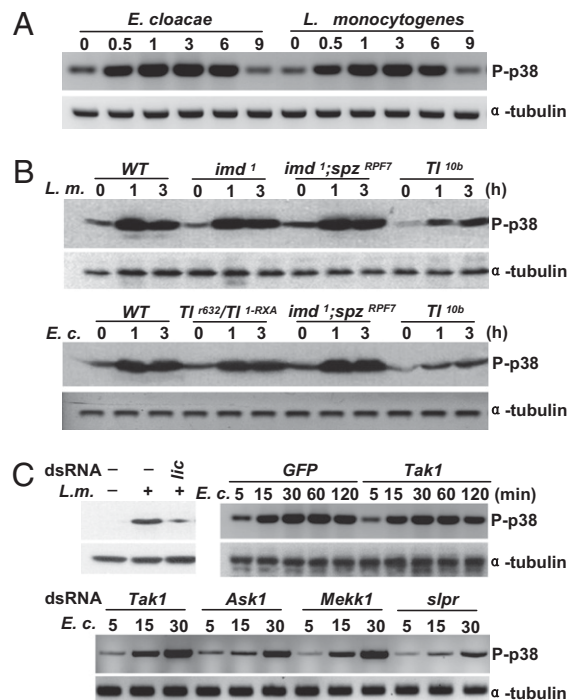


Fig. 5. A canonical MAP3K-MKK cascade is required for p38 activation by septic infection, but Toll and Imd are dispensable for this pathway. (A) Phospho-p38 in wild-type flies was detected at different time points after septic injury with *E. cloacae* and *L. monocytogenes*. (B) Phosphorylation of p38 is unchanged in *Imd*, *Tl*, and *Imd;spz* double mutants (loss-of-function) as well as in *Tl^{10b}* mutants (a gain-of-function mutation of *Tl*) following infection with both Gram-positive *L. monocytogenes* and Gram-negative *E. cloacae*. (C) *Toll* homologs or *licorne* (*lic*, a MKK3/6 homolog), *TAK1*, *ASK1*, *Mekk1*, and *Slpr* were knocked down with dsRNAs and the *L. monocytogenes*- or *E. cloacae*-induced phosphorylation of p38 in S2 cells are shown. Knockdown of *Tak1*, *ASK1*, and *Mekk1* had no significant effect on bacteria-induced p38 phosphorylation, and *lic* and *Slpr* are required for p38 activation. Phosphorylation was assayed by Western blotting using phospho-specific antibodies of p38. α -Tubulin is shown as a loading control. All Western blots have been repeated at least twice and all showed the same tendency.

a role in phagocytic encapsulation, which increase survival rate of intracellular bacterial infected *Drosophila* (25). Although intracellular infection is not a model we used in the present study, the defect in phagocytic encapsulation may contribute to the hypersusceptibility to microbial infection in *p38b* mutant flies. We showed that *p38c* had no role in protecting *Drosophila* from infection, but it may still have some functions during bacterial infection because it was reported that induction of decarboxylase gene by both Gram-positive and -negative septic injury is *p38c*-dependent (26).

The production and deposition of melanin pigments on invading pathogens and parasites represents a unique, innate immune response in insects (27). This immune response in flies also occurs at the sites of wounding, which may explain the enhancement of infection-induced melanization by DSS or SDS in our experiments. Although the production of melanin is a defense mechanism in insects, we show in this article that heavy melanization of the hindgut leads to fly death, which seems to mimic the consequences of overactive inflammatory signaling in mammals. The work described in this article suggests that melanization of the hindgut in *Drosophila* can be used as a model system to study host responses to microbial infection. The hindgut melanization may provide a model to study inflammatory bowel disease in flies,

as the gut is one of the most ancient organs in animals and the hindgut of flies is homologous to the bowels in mammals (17).

The increased susceptibility to infection in p38 mutants must result from multiple changes caused by p38 deficiency, as microarray analysis identified changes in the expression of a large number of genes. This study shows that the activation of Hsf and suppression of JNK are part of the mechanisms used by the p38 pathway in fly host defense. Like NF- κ B, JNK, and Jak-STAT, the evolutionarily conserved p38 pathway also participates in host defense in flies.

Materials and Methods

Fly Husbandry and Fly Stocks. Flies were raised at 25 °C on standard yeast-cornmeal-agar medium (JazzMix, Fisher Scientific A5153) except as otherwise mentioned. For sterile conditions, plastic vials, cotton balls, and media were autoclaved for 30 min. Media were divided into vials and plugged in a biosafety cabinet. The eggs were collected and surface-sterilized in White's solution for 20 min, as previously described (28). Antibiotic food was prepared as described by adding 1 mL of a 100 \times stock of antibiotics in 50% ethanol per 100 mL of liquefied food to a final concentration of 500 μ g/mL ampicillin, 50 μ g/mL tetracycline, and 200 μ g/mL rifamycin. Control antibiotic-free food was prepared by adding the same concentration of ethanol alone. The $y^1 w^{67c23}$, ry^{506} and related precise excision lines were used as wild-type controls in this study, because P-element insertion lines $p38b^{KG01337}$, $p38c^{KG05834}$, $dMK2^{EY11791}$, and $Hsp27^{KG03935}$ share the same genetic background. $UAS-p38b$, $UAS-Hsp27$, $da-Gal4$, Hsf^A , Hsf^+ , imd^1 , Tl^{632} , Tl^{1-RXA} , Tl^{10b} , and imd^1 ; spz^{RPF7} , have been described elsewhere (2, 22, 29). A full description of all stocks used in this study is included in the *SI Materials and Methods*.

Immune-Response Assays. Procedures for septic injury with Gram-negative or -positive bacteria and fungal infection were as described previously (2). See *SI Materials and Methods* for details.

Complementary DNA Microarray Analysis. Complementary DNA microarrays containing 15,000 known or predicted genes of the *D. melanogaster* genome were processed (20).

RT-PCR. See *SI Materials and Methods* for details on RT-PCR.

RNA Interference Experiments. RNA interference was performed as described in *SI Materials and Methods*.

SDS/PAGE and Immunoblotting. Standard methods were used. See *SI Materials and Methods* for details.

Light Microscopy, Imaging, and Transmission Electron Microscopy. Standard methods were used. See *SI Materials and Methods* for details.

Statistics. Digital image processing used IDL software (Research Systems) and custom-devised computer algorithms. Statistical data are expressed as mean \pm SEM. Student's *t* test and paired *t* test were applied when appropriate. A *P* value of less than 0.05 was considered statistically significant.

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