**Signaling Role of Hemocytes in *Drosophila*:**

**JAK/STAT-Dependent Response to Septic Injury**

Hervé Agaisse,1,2,* Ulla-Maja Petersen,1,3
Michael Boutros,1 Bernard Mathey-Prevot,2
and Norbert Perrimon1,2

1Department of Genetics
Harvard Medical School
2Howard Hughes Medical Institute
200 Longwood Avenue
Boston, Massachusetts 02115
3Dana-Farber Cancer Institute
Division of Pediatric Oncology and Hematology
44 Binney Street
Boston, Massachusetts 02115

**Summary**

To characterize the features of JAK/STAT signaling in *Drosophila* immune response, we have identified *totA* as a gene that is regulated by the JAK/STAT pathway in response to septic injury. We show that septic injury triggers the hemocyte-specific expression of *upd3*, a gene encoding a novel Upd-like cytokine that is necessary for the JAK/STAT-dependent activation of *totA* in the *Drosophila* counterpart of the mammalian liver, the fat body. In addition, we demonstrate that *totA* activation also requires the NF-KB-like Relish pathway, indicating that fat body cells integrate the activity of NF-KB and JAK/STAT signaling pathways upon immune response. This study reveals that, in addition to the pattern recognition receptor-mediated NF-KB-dependent immune response, *Drosophila* undergoes a complex systemic response that is mediated by the production of cytokines in blood cells, a process that is similar to the acute phase response in mammals.

**Introduction**

In mammals, local disturbances of physiological homeostasis such as trauma, injury, or bacterial infection lead to a systemic response known as the acute phase response (Baumann and Gauldie, 1994). A major early step in acute phase response is the local release of signaling molecules, such as cytokines, by activated macrophages, fibroblasts, and endothelial cells at the site of damage. Upon systemic release, these cytokines act distally from the site of damage and induce changes in behavior and biosynthetic capacities of a variety of target cells and organs in the whole organism. For instance, the liver is a central organ in acute phase response, and changes in hepatocyte gene expression profiles during acute phase response result in dramatic changes in serum concentrations of acute phase proteins, such as C-reactive protein (CRP) and serum amyloid A and P components (Steel and Whitehead, 1994). The cytokine IL-6 has been identified as a major inducer of the metabolic changes that occur in liver during the acute phase response (Fattori et al., 1994; Kopf et al., 1994). IL-6 is produced at the site of damage in response to primary cytokines, such as IL1 or TNF (Sparacio et al., 1992; Zhang et al., 1990), or in response to the presence of bacterial cell wall compounds, such as lipopolysaccharide (LPS) (Zhang et al., 1994). Upon systemic release, IL-6 triggers JAK/STAT signaling in hepatocytes through activation of the cytokine class 1 receptor gp130 and the JAK kinases. This process ultimately leads to translocation of STAT dimers to the nucleus where they activate the transcription of target genes encoding acute phase proteins, such as C-reactive protein and LPS binding protein (LBP) (Alonzi et al., 2001; Schumann et al., 1996; Zhang et al., 1996).

In *Drosophila*, septic injury leads to the secretion of a battery of antimicrobial peptides by the fat body, an organ functionally equivalent to the liver in humans (Hoffmann et al., 1999). Two NF-KB-like signaling pathways are implicated in this process: the Toll and the Imd pathways (Lemaitre et al., 1995, 1996), which lead to the activation of the NF-KB-like transcriptional regulators Dif and Relish, respectively (Rutschmann et al., 2000; Stöven et al., 2000). While the Toll pathway is activated by gram-positive bacteria and fungi, activation of the Imd pathway is mediated by gram-negative bacteria (Lemaitre et al., 1997). Recently, the receptors involved in the specific recognition of gram-positive and gram-negative bacteria and leading to the activation of the Toll pathway and the Imd pathway, respectively, have been identified (Choe et al., 2002; Gottar et al., 2002; Michel et al., 2001; Ramet et al., 2002). These molecules belong to the peptidoglycan recognition protein (PGRP) family and are thought to mediate signaling in fat body by direct binding to the cell wall components of microorganisms upon infection.

While the mechanisms involved in NF-KB activation upon immune challenge are well documented both in insects and mammals, little is known about activation of the JAK/STAT pathway in insects. DNA binding activity and translocation of STAT in fat body cells in response to bacterial challenge has been documented in *Anopheles gambiae* (Barillas-Mury et al., 1999). Moreover, the immune challenge-inducible expression of the *Drosophila* thiolester-containing protein TEP1 has been shown to be constitutive in larvae displaying a gain-of-function mutation in JAK and reduced in a corresponding loss of function (Lagueux et al., 2000). These studies indicate that, as in mammals, the JAK/STAT pathway is activated upon immune challenge in *Drosophila*.

Here, we investigate the mechanisms involved in the JAK/STAT-dependent immune response to septic injury in *Drosophila*. First, we have identified *totA* as a gene that is regulated by the JAK/STAT pathway in fat body in response to septic injury. Then, we show that *totA* activation is mediated by the *Drosophila* homolog of the vertebrate cytokine class I receptor Domeless (Dome) and requires the hemocyte-specific expression of *upd3*, a gene encoding an Unpaired (Upd)-like cytokine. Finally, we show that *totA* activation also requires activation of the *relish* pathway in fat body. Altogether, our
Figure 1. totA Expression Is JAK Kinase Dependent and Fat Body Specific

(A) Structure of the JAK/STAT pathway as required for embryonic development. The cytokine-like Upd binds the receptor Dome (Dome) resulting in receptor dimerization and activation of the receptor-associated JAK kinases Hopscotch (Hop) and tyrosine phosphorylation of the cytoplasmic domain of Dome. This creates docking sites for the SH2 domain of STAT. Upon recruitment to the active receptor complex, STATs become then phosphorylated by Hop and dimerize and translocate to the nucleus where STAT dimers activate transcription of target genes. The truncated version of Dome, Dome\textsubscript{ACYT}, lacks the intracellular region that is involved in signal transduction. This mutated receptor still contains the extracellular cytokine binding module and acts as a signaling antagonist, probably by titrating Upd.

(B) Transcriptional profiles of drosomycin, diptericin, and totA genes in wild-type and JAK mutant adult females. Northern blot analysis of total RNA extracted from adult flies 6 hr after infection with a mixture of gram-negative (E. coli) and gram-positive bacteria (M. luteus). Similar results were obtained for wild-type flies (OreR) grown at 24°C or 29°C. Flies displaying a JAK loss-of-function genotype (M38/msv1) were grown at 24°C. Flies displaying the thermosensitive JAK gain-of-function TumL were grown at 29°C.

(C) totA is expressed in fat body cells. totA expression was analyzed by in situ hybridization in abdominal adult fat body before (Control) and 6 hr after septic injury.

study indicates that Drosophila undergoes a complex pattern recognition-mediated and cytokine-mediated process in response to septic injury.

Results

totA Activation upon Septic Injury Relies on the JAK/STAT Pathway

In order to identify genes that are regulated by the JAK/STAT pathway in response to septic injury in adult flies, we screened for candidates that display an inducible expression upon immune challenge and that are constitutively expressed in flies carrying a gain-of-function mutation in the JAK/STAT pathway. To this end, we used custom-made cDNA microarrays (U.-M.P., unpublished data) to compare gene expression profiles of nonchallenged wild-type flies to gene expression profiles of challenged wild-type flies and to gene expression profiles of nonchallenged Tum\textsubscript{L} flies displaying a gain-of-function mutation in the Drosophila JAK kinase Hopscotch (Figure 1A) (Harrison et al., 1995; Luo et al., 1995). We identified MP1 as a gene that fulfilled both criteria for induction upon challenge and constitutive expression in a JAK/STAT gain-of-function mutation (Figure 1B). MP1 expression was not induced in challenged flies displaying loss-of-function mutation in hop (hop\textsuperscript{mt}/hop\textsuperscript{mtP}) [Perrimon and Mahowald, 1986], confirming the involvement of Drosophila JAK in MP1 expression (Figure 1B). As control experiments, we also analyzed the expression of drosomycin and diptericin, which are controlled by the NF-kB-like transcriptional regulators Dif and Relish, respectively. As expected, both loss-of-function and gain-of-function mutations in the JAK/STAT pathway did not affect drosomycin and diptericin expression in response to immune challenge, confirming the specific effects of JAK mutations on MP1 expression (Figure 1B).

Sequence analysis of MP1 cDNA revealed that MP1 codes for TotA, a polypeptide that is produced by the larval fat body and accumulates in hemolymph in response to various stress conditions in flies (Ekengren et al., 2001; Ekengren and Hultmark, 2001). We next confirmed by in situ hybridization that totA expression was also mainly fat body specific in adult flies. As shown in Figure 1C, totA was weakly expressed in the fat body of unchallenged flies and strongly induced after septic injury.

STAT Is Activated in Fat Body Cells in Response to Septic Injury

Activation of the JAK/STAT pathway culminates in translocation of phosphorylated STAT dimers from the cytoplasm to the nucleus. In mosquitoes, it has been shown
that AgSTAT, the homolog of Drosophila STAT, translocates into the nucleus of fat body cells in response to bacterial infection (Barillas-Mury et al., 1999). To further confirm the activation of the JAK/STAT pathway in Drosophila fat body in response to septic injury, we next examined the subcellular location of STAT protein in fat body cells by immunostaining. In unchallenged flies, STAT protein was located both in the cytoplasm and nucleus (Figure 2). In challenged flies, STAT had substantially cleared the cytoplasm and had accumulated in the nucleus (Figure 2). In contrast, there was no STAT nuclear translocation in Drosophila JAK mutant adult flies (hop<sup>msv1</sup>/hop<sup>msv1</sup>) [Perrimon and Mahowald, 1986] (Figure 2). Conversely, a very strong staining was detected both in the cytoplasm and nucleus of flies carrying a Drosophila JAK gain-of-function mutation (TumL [Harrison et al., 1995; Luo et al., 1995]) (Figure 2). These results demonstrate that totA activation correlates with the JAK-dependent activation of STAT in Drosophila fat body cells in response to septic injury.

totA Expression Depends on <i>domeless</i> in Fat Body

Recently, a Drosophila homolog of the vertebrate cytokine class I receptor, Dome (a.k.a. Mom), has been identified (Brown et al., 2001; Chen et al., 2002). Mutations in <i>dome</i> result in embryonic defects similar to the embryonic phenotype associated with mutation in the JAK/STAT pathway components. A truncated version of Dome, Dome<sup>CYT</sup>, has been generated by deletion of the intracellular region that is involved in signal transduction (Brown et al., 2001). This mutated receptor still contains the extracellular cytokine binding module and acts as a signaling antagonist, probably by titrating the ligand (Figure 1A). Accordingly, Dome<sup>CYT</sup> overexpression during embryogenesis mimics the loss-of-function phenotype of <i>dome</i> mutants (Brown et al., 2001). To test whether Dome plays a role in totA expression, we used the GAL4/UAS system to express the dominant-negative form of Dome, Dome<sup>CYT</sup>, in adult fat body. Northern blot analysis revealed that totA expression upon immune challenge was totally abolished in the corresponding animals (Figure 3A). We conclude that totA activation in response to bacterial infection is the result of a signaling event that is transduced by the Drosophila homolog of the vertebrate cytokine receptor, Dome, in the fat body.

The <i>upd/os</i> Locus Contains Three <i>upd</i>-like Genes

The involvement of <i>dome</i> in totA expression strongly suggested the existence of a cytokine-like molecule involved in the control of totA expression. <i>upd</i> has been characterized as a gene encoding the cytokine that activates the JAK/STAT pathway during Drosophila embryogenesis (Harrison et al., 1998). Strong alleles of <i>upd</i> are embryonic lethal, but weaker alleles, such as outstretched (<i>os</i>), give rise to adult flies that hold their wings at right angles and have small eyes. We found that totA activation was strongly decreased in <i>os</i> flies, suggesting that <i>upd</i> might be involved in totA expression (Figure 3B). However, totA activation was nearly wild-type in transheterozygous flies displaying the os mutation over a null mutation in <i>upd</i> (<i>upd</i><sup>YM55</sup>), suggesting that a defect in <i>upd</i> expression was not responsible for the lack of totA activation in the <i>os</i> genetic background (Figure 3B). Interestingly, totA activation was abolished in transheterozygous flies displaying the os mutation over a large deficiency (<i>os1A</i>) of the <i>upd</i> locus (Figure 3B). This suggests that the os mutation affects the expression of a gene involved in totA activation that maps to the <i>os</i>/<i>upd</i> locus, but that is not <i>upd</i>. Blast search analysis revealed the presence of two other <i>upd</i>-like cytokine-encoding genes at the <i>upd/os</i> locus (Figures 3C and 3D). <i>upd2</i> corresponds to CG5988 and maps 50 kb downstream from <i>upd</i> (Figure 3C). <i>upd3</i> corresponds to CG15062 (for the first and second exon) and CG5983 (for the third exon) and maps 25 kb downstream from <i>upd</i> (Figure 3C). These observations prompted us to hypothesize that <i>upd2</i> and/or <i>upd3</i> might be involved in totA expression (see below).

totA Expression in Fat Body Depends on <i>upd3</i>

Expression in Hemocytes

To further investigate the potential role of <i>upd2</i> and/or <i>upd3</i> in totA expression, we first analyzed whether the
Figure 3. Involvement of *dome* and the os Locus in *totA* Expression

(A) *totA* expression is *dome* dependent. Northern blot analysis of total RNA extracted from adult flies before challenge (0) or 6 hr after septic injury with a mixture of gram-negative and gram-positive bacteria. The effect of DomeΔCYT overexpression was tested in flies displaying the UAS construct P(UAS-domeΔCYT) and the enhancer trap P(C273-GAL4) driving GAL4 expression in fat body cells.

(B) *totA* is not activated in os flies. Northern blot analysis of total RNA extracted from OreR, os, os/updYM55 (a null upd allele), and os/os1A (a large deficiency covering the os locus) adult flies before (0) and 6 hr after septic injury.

(C) Map of the outstretched (os) locus. *upd* corresponds to CG5993, *upd2* corresponds to CG5988, and *upd3* corresponds to CG15062 (for the first and second exons) and CG5963 (for the third exon). os1A is a deficiency covering the entire os/upd locus.

(D) The Upd-like family members. Peptide sequence alignment of Upd, Om (1E) (from *Drosophila ananassae*), Upd2, and Upd3. SP indicates the signal peptide. Note that the N-terminal sequence of Upd3 is unknown. Asterisks indicate the position of introns. The four putative α helices are boxed.

expression of the *upd*-like genes was inducible upon septic injury. We failed to detect *upd2* expression in adult flies by using RT-PCR analysis. In contrast, we found that the level of *upd3* expression, which was very low in control animals, was significantly increased after septic injury (Figure 4A). We next analyzed the pattern of GFP expression in flies harboring a *upd3* promoter region-GAL4 fusion and a UAS-GFP reporter (see Experimental Procedures). We failed to detect any GFP production in fat body cells of control or challenged animals (Figure 4B). However, we noticed that GFP production was strongly increased in blood cells after challenge, indicating that hemocytes might be the main site of *upd3* expression (Figure 4C). To investigate the functional importance of *upd3* hemocyte-specific expression in *totA* expression, we designed an in vivo RNAi strategy to silence *upd3* expression in a tissue-specific manner (Lee and Carthew, 2002). We used the hemolectin-GAL4 and the yolk-GAL4 constructs to drive the expression of the UAS-iupd3 hairpin construct in hemocytes and fat body,
Altogether, these experiments suggest that upd3 activation in hemocytes subsequently leads to totA activation in the fat body.

**totA Expression Is Regulated by the Relish Pathway in Fat Body**

To further analyze the regulation of totA expression in fat body cells, we monitored totA expression in response to clean injury, septic injury with gram-negative bacteria (*E. coli*), or septic injury with gram-positive bacteria (*M. luteus*) (Figure 6A). Clean injury and septic injury with *M. luteus* resulted in a modest but significant induction of totA expression: 4-fold induction 6 hr after challenge and 7-fold induction 18 hr after challenge (Figure 6A). In sharp contrast, septic injury with *E. coli* resulted in a robust induction of totA expression: 25-fold induction at 6 hr and 35-fold induction at 18 hr (Figure 6A). Gram-negative bacteria therefore constitute the best inducer for totA expression. It is well established in flies that immune response to gram-negative bacteria is mediated by the Imd pathway through activation of TAK1 and the NF-KB-like transcription factor Relish (Hedengren et al., 1999; Lemaitre et al., 1995; Vidal et al., 2001). We therefore analyzed totA expression in TAK1 and in relish mutant flies. As shown in Figure 6B, totA activation after challenge was totally abolished in these mutants, indicating that, in addition to being JAK/STAT dependent, totA expression also requires the activity of the Relish pathway. We next analyzed whether the activity of the Relish pathway was specifically required in the fat body. To this end, we overexpressed relish dsRNA in fat body using the UAS-irel construct and the yolk-GAL4 driver. As shown in Figure 6C, dsRNA-mediated silencing of relish expression leads to a failure in totA activation, indicating that Relish activity is specifically required in the fat body. To this end, we overexpressed relish dsRNA in fat body using the UAS-irel construct and the yolk-GAL4 driver. As shown in Figure 6C, dsRNA-mediated silencing of relish expression leads to a failure in totA activation, indicating that Relish activity is specifically required in the fat body. Finally, we analyzed whether Relish activation in the fat body was sufficient to activate totA expression. It has been recently shown that overexpression of Imd in fat body cells leads to activation of Relish and therefore constitutive expression of the antimicrobial peptide genes, such as diptericin, in absence of immune challenge (Georgel et al., 2001) (Figure 6C). As shown in Figure 6C, totA was not constitutively expressed in the corresponding flies, indicating that Relish activation is required in fat body but is not sufficient to activate totA expression.

**Figure 4. upd3 Is Activated in Hemocytes in Response to Septic Injury**

(A) RT-PCR analysis of total RNA extracted from OreR adult flies before challenge (0), 3, 6, and 9 hr after septic injury. RT-PCR signals were quantified using a PhosphorImager and normalized to the corresponding signal level of rp49. Results are expressed in arbitrary units, 100 corresponding to the level of upd3 expression 3 hr after challenge.

(B and C) upd3 expression is hemocyte specific. Upd3 expression pattern was determined by analyzing GFP production (green) in fat body and hemocytes of upd3-GAL4/UAS-GFP flies before (Control) and after septic injury. Fat body tissue was visualized by DNA staining with propidium iodide (red), and hemocytes were identified by their ability to phagocytose fluorescent beads (red).

respectively (Figure 5A). While the production of upd3 dsRNA in fat body did not interfere with totA expression, upd3 dsRNA expression in hemocytes led to a strong decrease in totA activation upon septic injury (Figure 5B).
**Figure 5. upd3 Expression Is Required in Hemocytes for totA Fat Body Expression**

(A) Hemolectin (hml-GAL4) and yolk (yolk-GAL4) drivers are hemocyte and fat body specific, respectively. Expression specificity was assessed by analyzing GFP production (green) in hml-GAL4, UAS-GFP, and yolk-GAL4, UAS-GFP flies. Fat body tissue was visualized by DNA staining with propidium iodide (red), and hemocytes were visualized by actin staining with phalloidin (red).

(B) Transcriptional profiles of totA and diptericin genes in flies displaying the upd3-silencing construct (UAS-iupd3). Northern blot analysis of total RNA extracted from UAS-iupd3/+; TM3/+, UAS-iupd3/hml-GAL4, and UAS-iupd3/+; yolk-GAL4/ flies before (0) and 6 hr after challenge.

**TotA Is an Acute Phase Protein**

To analyze the role of the JAK/STAT pathway during septic injury, we first identified a target gene of the pathway, totA. We showed that totA expression (1) takes place in the fat body, which is the insect equivalent of the mammalian liver; (2) is strongly induced in response to septic injury; and (3) requires the Dome/JAK/STAT signaling pathway in fat body and the expression of upd3 in hemocytes. Similarly, the expression of the mammalian acute phase proteins (e.g., LBP and CRP) is modulated by activation of specific signaling pathways (e.g., the JAK/STAT pathway) in response to systemic release of cytokines (e.g., IL6) by activated macrophages (Schumann et al., 1996; Zhang et al., 1996). Altogether, our results demonstrate that TotA qualifies as a bona fide acute phase protein. In addition, totC and totM are also controlled by the JAK/STAT pathway upon septic injury, a characteristic that is probably shared by all the members of the tot family (H.A., unpublished data). Moreover, the tot family members are not the sole target of the JAK/STAT pathway upon septic injury. We have recently shown that expression of CG11501, a cysteine-rich polypeptide related to scorpion toxin, is also controlled by the JAK/STAT pathway (Boutros et al., 2002). These data indicate that the JAK/STAT pathway contributes to a global response upon immune challenge by controlling the expression of several acute phase proteins. As for most of their mammalian counterparts, the function of these Drosophila acute phase proteins is unclear. totA overexpression does not appear to protect NF-KB mutants, such as key, from gram-negative bacteria infection (H.A., unpublished data), indicating that TotA, unlike antimicrobial peptides such as Diptericin and Drosomycin, does not prevent bacterial growth. Altogether, these observations suggest that TotA is probably a general stress response factor involved in homeostasis of (damaged) tissues. Accordingly, it has been shown that TotA overexpression confers extended survival to flies subjected to heat stress, a treatment that certainly leads to disturbances of physiological homeostasis (Ekengren et al., 2001). Further in vivo characterization of acute phase protein function, such as TotA, using Drosophila as a model system will help us to understand the overall physiology of the acute phase response in insects and mammals.
that is produced in hemocytes in response to immune challenge. Although cytokine-like activities, such as IL1 and TNFα, have been previously reported as being produced by hemocytes from Lepidopteran larvae in response to LPS stimulation, none of these activities were shown to have a physiological function in vivo (Wittwer et al., 1999). To the best of our knowledge, upd3 is the first example of a gene coding for a cytokine that is expressed in hemocytes and is required for signaling in fat body. This study therefore constitutes (to the best of our knowledge) the first demonstration that sentinel cells, such as hemocytes, play a signaling role in the Drosophila immune response. The nature of the signals that are detected by hemocytes and the signaling pathway(s) that trigger upd3 activation in response to septic injury remain to be determined. Preliminary experiments indicated that upd3 expression was severely impaired in TAK1 flies after septic injury, suggesting that components of the Relish pathway (as defined in fat body cells) might be involved in upd3 activation in hemocytes in response to bacterial infection (H.A., unpublished data). However, further analysis in PGRP-LC and relish mutant backgrounds were not consistent with this hypothesis. Clearly, the mechanisms involved in upd3 regulation potentially constitute a new paradigm for studying the signals and the transduction machinery involved in the control of gene expression in activated hemocytes.

Integration of Signaling Pathway Activities in Fat Body
The NF-KB pathway is necessary and sufficient to activate the expression of antimicrobial peptides in response to septic injury in Drosophila. In contrast, totA activation in fat body requires both the JAK/STAT and the NF-KB pathways (Figure 7). Our study therefore reveals that fat body cells integrate various inputs, such as the presence of bacterial compounds (through NF-KB activation) and cytokines (through JAK/STAT activation), in response to septic injury (Figure 7). Interestingly, septic injury is not the only stimulus that activates totA expression. It has been shown that stress treatments, such as heat shock, dehydration, and mechanical pressure, also induce totA expression (Ekengren et al., 2001). Similarly, we have observed that injection of detergent solution into the hemolymph also triggers totA activation. Moreover, this response was strongly affected in both hop and relish mutants (H.A., unpublished data). Altogether, these observations indicate that totA regulation is achieved through detection of various stress stimuli and integration of the activity of at least two different signaling pathways, the JAK/STAT and the NF-KB pathways. We believe that such diversity in the mechanisms involved in the control of totA activation reflects the broad function of TotA as a general stress-response factor.

Concluding Remarks
In the past decade, Drosophila has emerged as a highly attractive model for the study of innate immunity. Remarkably, the mode of detection of microbial patterns through activation of pattern recognition receptors and NF-KB-like signaling pathways has been conserved throughout evolution. In addition to the detection of exogenous ligands such as LPS, the mammalian acute
phase response also involves the production of endogenous signaling molecules, such as cytokines. Our analysis indicates that a cytokine-mediated response also takes place in *Drosophila* in response to septic injury. This suggests that, very early in the evolution of multicellular organisms, a pattern recognition-mediated response was associated with a cytokine-mediated response. This association may reflect the need for integration at the organism level of a complex series of reactions that are executed by various organs not only to respond to exogenous stimuli, such as invading microorganisms, but also to orchestrate endogenous processes devoted to the maintenance of homeostasis.

### Experimental Procedures

#### Fly Strains and Procedures

The *hop* alleles used (*M38*, *msv1*, and *TumL*) have been previously described (Harrison et al., 1995; Perrimon and Mahowald, 1986). PGRP-LC flies were provided by P. Manfruelli (Ramet et al., 2002), *TAK1* flies were provided by B. Lemaitre (Vidal et al., 2001), *relish* flies were provided by D. Hultmark (Hedengren et al., 1999), P(*UAS-dome:CYT*) line was obtained from J. Castelli-Gair Hombria (Brown et al., 2001), P(*UAS-imd*) and the P(*yolk-GAL4*) lines were provided by S. Naitza and P. Georgel (Georgel et al., 2001). The P(*hml-GAL4*) line used for hemocyte-specific expression has been generated by A. Goto (Goto et al., 2001) and is available from the Bloomington Center.

### Infection Experiments

We used 1-week-old adult flies that were put on fresh medium and kept at room temperature 24 hr prior to challenge. Parameters such as temperature, humidity, food quality, population density, and age introduced considerable variability in *totA* expression. Septic injury was performed by pricking flies in the thorax using a thin needle previously dipped in a 10-fold concentrated overnight culture of *E. coli* and/or *M. luteus*.

### Immunostainings

The 2.2 kb DNA fragment encoding the DSTAT protein was PCR amplified using a 4.0 kb cDNA clone (Hou et al., 1996) as a template and the following oligonucleotides as primers: GAGAACATTCCTATGAGCTTTGGAAACGGCATGTC, GGAGGAATCTCAAAGTTCTCAAGTTGT. The fragment was then ligated to the pBSK after digestion with EcoRI and BamHI. The fragment was then digested with Ndel and BamHI and subcloned into the Ndel and BamHI restriction sites of pET16b (Novagen), giving pET-STAT. *E. coli* strain BL21 was used to overexpress the DSTAT protein, using the T7 expression system. DSTAT protein was purified as inclusion bodies and injected into rat to generate anti-DSTAT antibodies. For immunostainings, flies were dissected in PBS 0.1% tween 4% formaldehyde, the remaining abdominal carcasses were incubated for 15 min in fresh PBS 0.1% tween 4% formaldehyde, washed twice in PBS 0.1% tween, and incubated overnight with the primary anti-DSTAT antibody PBS 0.1% tween 2% BSA at 4°C. The samples were then washed three times in PBS 0.1% tween and incubated overnight with a FITC-conjugated anti-rabbit antibody (Jackson Lab.) in PBS 0.1% tween 2% BSA at 4°C. The samples were then treated with RNase A (400 µg/ml) and propidium iodide (20 µg/ml) for 30 min, washed three times, and mounted in 80% glycerol antifade buffer (Molecular Probes). Fluorescent images were captured with a confocal microscope (Leica, TCS-NT) and subsequently processed using NIH image and Adobe Photoshop software.

### RNA Preparation and Northern Blot Analysis

Adult flies were collected in eppendorf tubes, flash frozen in liquid nitrogen, and kept at –80°C. Total RNA extraction was performed with the RNA Trizol (GIBCO, BRL) method. Northern blot analysis was performed as previously described (Agaissa and Lereclus, 1996). The probes were generated by PCR amplification using genomic DNA as template and the following oligonucleotides as primers: diptericin, GGAATCGGGGATTTCTTCTTT, GACCAGAAGGGAATCTCCCGAT; drosomycin, ATCAAGATCTGTTGCCTCCCTTGCTGTC, ATATGCCTGAGTATGGGAGGTACAAAGC; mp1, TCCGCCCAAGCATAGGCGCCAGATCGT, TACTCTGTTCTACAGACGCACGCCG; andtotA (MPS): TACATCGGGTCTGCTGACCCGCAATAAACGAGGCA. The samples were then diluted 100-fold, and 1 µl of the diluted sample was subjected to PCR amplification using the following primers: *totA*: AGTCCATTGTTGCTTCCCTCTGCCTGTC, ATATGCCTGAGTATGGGAGGTACAAAGC. The samples were then treated with RNase A (400 µg/ml) and propidium iodide (20 µg/ml) for 30 min, washed three times, and mounted in 80% glycerol antifade buffer (Molecular Probes). Fluorescent images were captured with a confocal microscope (Leica, TCS-NT) and subsequently processed using NIH image and Adobe Photoshop software.

Figure 7. Model for *totA* Activation in Response to Septic Injury

Septic injury triggers the activation of a cytokine-like molecule in hemocytes (Upd3), which in turn is necessary for activation of the JAK/STAT-dependent expression of *totA* in fat body. In addition, the gram-negative bacteria-triggered activation of the NF-KB-like Relish pathway, which is necessary and sufficient to induce the expression of antibacterial peptide genes such as *totA*. The *totA* expression thus results from the integration of pattern recognition receptor (PGRP-LC)- and cytokine (Upd3)-mediated responses in fat body cells.
CGTCCCTGTGAGAACGCAAGCG. PCR cycles were performed as follows: 1 min at 94°C, then 30 s at 94°C, 30 s at 55°C, 1 min at 72°C for 20 cycles and, finally, 10 min at 72°C. Twenty microliters of a 100 μl PCR reaction were fractioned on a 1% agarose gel and then subjected to Southern blot analysis. In a typical experiment, as described in Figure 4, exposure time was 30 min at room temperature for rp49 and 24 hr at -70°C for upd3. Signals were quantified by PhosphorImager and normalized to the corresponding signals for rp49 expression. Results are expressed in arbitrary units; 100 corresponds to the maximum level of expression obtained for a given time course experiment.

Cloning and Transgene Constructs
The upd3 promoter region was PCR amplified using the following oligonucleotides as primers: GATCGCCTTGCACAGACTCTTACGCTGGTTCG; GCTCTAGACCGAC. The silencing constructs were generated by cloning sequentially into the Nhel and Avrl restriction sites of pWIZ (Lee and Carthew, 2002) a 500 bp DNA fragment that was PCR amplified using specific oligonucleotides (displaying XbaI restriction site tails) as primers and an RT reaction of total RNA from challenged adults as template. Specific oligonucleotides were as follows: UAS-upd3: GCTCTA GAATCGCCTTGCACAGACTCTTACGCTGGTTCG; GCTCTAGACCGAC.

Acknowledgments

References

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