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The roles of JAK/STAT signaling in *Drosophila* immune responses

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Summary: Innate immune responses are mediated by the activation of various signaling processes. Here, we describe our current knowledge on Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signaling in the *Drosophila* immune response. First, we briefly introduce the main effectors involved in the humoral and cellular responses, such as anti-bacterial peptides and hemocytes. Second, we describe the canonical JAK/STAT-signaling pathway, as established from extensive studies in mammalian systems, and we introduce the *Drosophila* components of the JAK/STAT pathway, as discovered from studies on embryonic development. Third, we describe the various roles of JAK/STAT signaling in both humoral and cellular responses. We present the JAK/STAT-dependent humoral factors, such as the thioester-containing proteins and the Tot peptides, produced by the fat body in response to septic injury. We also discuss the possible involvement of the JAK/STAT pathway in cellular responses, including hemocyte proliferation and differentiation. Finally, we present how cytokines, such as Upd3, might contribute to the integration of the immune responses at the organism level by orchestrating the response of various immune cells and organs, such as fat body, hemocytes, and lymph glands.

Introduction

Multicellular organisms are continuously exposed to potentially dreadful pathogenic microorganisms and have evolved various defense mechanisms that protect them from infection (1). *Drosophila*, as other insects, is particularly resistant to microbial infection (2). The first mechanism of protection of insects against pathogens is the external cuticle that constitutes an effective physical barrier, preventing the access of microbes to the hemolymph (the insect blood). Breakage of this barrier immediately induces a series of host reactions that seal the wound and prevent the spreading of microbes. Two types of immune responses, humoral and cellular, have been distinguished based on the nature of the effectors involved. The humoral immune response depends on hemolymph components and results in hemolymph coagulation, melanization, and synthesis of anti-microbial peptides. The cellular immune response is mediated by the hemocytes and leads to phagocytosis

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and encapsulation of the pathogens. Together, these responses constitute an effective protection against microbial infections and are referred to as innate immune responses, because they involve microbial pattern-recognition molecules encoded by the germ line (1). This is in contrast to the acquired immune responses present in mammals that involve molecules, such as immunoglobulins and T-cell receptors, which are generated by somatic DNA rearrangements.

Innate immunity responses are triggered by the immune challenge and therefore involve signaling processes. Most of our knowledge of the signaling pathways that are activated during the immune response is derived from studies on the expression of anti-bacterial peptide genes that are switched on following invasion of microbes. A large body of work in *Drosophila* has now well documented that, as is also the case for many genes involved in the mammalian immune response, the expression of anti-microbial peptide genes is regulated by two distinct nuclear factor-κB (NF-κB)-signaling pathways (3). In contrast, less is known about the role of other evolutionary conserved signaling pathways, such as the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) and c-Jun N-terminal kinase (JNK) pathways, which have been widely implicated in mammalian immunity. The roles of NF-κB pathway in innate immunity are reviewed elsewhere in this volume, Reichhart p. 59. Here, we describe our current knowledge on the role of JAK/STAT signaling in *Drosophila* immune response. First, we briefly introduce the main effectors involved in the humoral and cellular responses. Second, we describe the components involved in the *Drosophila* JAK/STAT pathway. Third, we describe the various roles of JAK/STAT signaling in both humoral and cellular responses. Finally, we present how cytokine signaling might contribute to the integration of the immune responses at the organism level.

Effectors of the *Drosophila* immune response

Humoral effectors

It is well established that non-self recognition is mediated through the activation of pattern-recognition receptors that specifically bind to the cell wall components of microbes, such as lipopolysaccharide and β-glucan. These events trigger the activation of proteolytic cascades that ultimately mediate biological responses including hemolymph coagulation, melanization, and synthesis of anti-microbial peptides. In addition to the coagulation reaction, breakage of the cuticle induces the formation of darkly pigmented area at the site of injury. At these sites, melanin is deposited as a result of the catalytic activity of phenoloxidase, an enzyme that catalyzes the oxidation of phenols to quinones, which subsequently polymerize to melanin (4). Apart from the site of injury, melanization is also observed at the surface of foreign objects, such as parasitoid eggs, and during the process of encapsulation. The reactive oxygen and nitrogen species formed, as well as melanin itself, are thought to be toxic to microorganisms (4).

A hallmark of the humoral response is the secretion of a battery of anti-microbial peptides by the fat body, an organ functionally equivalent to the liver in humans. To date, seven different classes of anti-microbial peptides, attacin, cecropin, defensin, diptericin, drosocin, drosomycin, and metchnikowin, have been identified in *Drosophila*, and their expression is controlled at the transcriptional level in response to immune challenge (5). Two signaling pathways are implicated in this process: the Toll and the Imd pathways, which lead to the activation of the NF-κB-like transcriptional regulator Dif and Relish, respectively (6, 7). While the Toll pathway is activated by gram-positive bacteria and fungi and controls the expression of anti-fungal peptide such as drosomycin, activation of the Imd pathway is mediated by Gram-negative bacteria and leads to the expression of anti-bacterial peptides, such as diptericin (8–10). Recently, the receptors involved in the specific recognition of Gram-positive and Gram-negative bacteria and leading to the activation of the Toll and Imd pathways have been identified (11–14). These molecules belong to the peptidoglycan-recognition protein (PGRP) family and are thought to mediate NF-κB signaling in the fat body by direct binding to the cell wall components of microorganisms upon infection.

Cellular effectors

The *Drosophila* hemocyte population displays three major cell types: plasmatocytes, lamellocytes, and crystal cells (15, 16). The plasmatocytes are small, rounded cells that display phagocytic activity. They represent 90% of the hemocyte population (three to five thousand per fly) and are devoted to removing dead cells (during embryogenesis and metamorphosis) and bacteria (during infection). The lamellocytes are the largest blood cells. They have a very flat shape, and their function is to encapsulate dead tissues during metamorphosis and foreign bodies that are too large to be phagocytosed, such as parasite eggs. Under normal conditions, lamellocytes represent less than 5% of the total larval blood cells. However, infestation by parasitoid wasp eggs in the larval hemolymph results in a massive differentiation of hemocytes present in the lymph glands into lamellocytes. It has been reported that septic injury also triggers lamellocyte differentiation, although to a

lesser extent as compared with wasp infestation (17). Crystal cells are slightly larger than plasmatocytes and represent about 5% of larval blood cells. The crystal cells contain the precursors to the prophenoloxidase cascade involved in the melanization process that is activated during wound healing or encapsulation.

In the embryo, hemocytes derive from the procephalic mesoderm and migrate to colonize the whole embryo and remove apoptotic cells (18–20). It has been reported recurrently that larval hemocytes originate from the lymph glands, a mesodermally derived organ that consists of four to six lobes closely associated with the dorsal blood vessel. However, recent embryonic transplantation analyses have revealed that most of the circulating hemocytes actually derive from embryonic hemocytes (21). The number of these hemocytes is less than 200 per fly in the first instar larvae and increases to 5000 in the third instar larvae, probably as a result of hemocyte proliferation (22). In late third instar larvae, just before the onset of pupariation, lymph gland-derived hemocytes are released into circulation (21). Thus, in pupae, the hemocyte population consists of a mixture of embryonic and larval hemocytes. No hematopoietic organ has yet been identified in adult flies. Transplantation analyses have demonstrated that adult hemocytes, primarily sessile cells attached to various organs (22), consist of a mixed population of cells of embryonic hemocytes and lymph gland-derived hemocytes (21). Although adult hemocytes, as larval hemocytes, clearly display phagocytic properties (23), they do not display mitosis figures and they do not differentiate into specialized cells, such as lamellocytes, upon immune challenge (22).

The JAK/STAT pathway

The JAK/STAT-signaling pathway in mammals

The JAK/STAT signal-transduction cascade was first identified in mammals and shown to transduce a variety of cytokines and growth factor signals (24). Extensive studies in mammalian systems have led to the development of a canonical pathway, in which the non-receptor tyrosine kinase JAK is associated with the intracellular portion of transmembrane cytokine receptors. Following ligand binding and receptor dimerization, two JAK molecules are brought into juxtaposition, where they transphosphorylate one another. JAKs activated in this manner phosphorylate tyrosine residues in their associated receptors, allowing normally cytosolic STAT molecules to dock onto the receptor complex via their SRC homology 2 (SH2) domains. The recruited STAT molecules are themselves activated by JAK-mediated phosphorylation of an invariant tyrosine residue in their C-terminal region and then either homo- or heterodimerize prior to nuclear translocation. Once in the nucleus, activated STAT dimers bind to consensus DNA target sites (consensus TTCNNNGAA), where they act as activators of transcription. In mammals, four *JAK* and seven *STAT* genes have been identified, and more than 30 cytokines and growth factors have been shown to activate specific combinations of JAK or STAT proteins (24).

Structure of the JAK/STAT pathway in flies

The JAK/STAT pathway in flies was originally identified through its role in embryonic segmentation (25). The four main components of this pathway are the ligand, unpaired (Upd), the receptor domeless (Dome), the JAK (Hopscotch/Hop), and the STAT (STAT92E/Marelle).

Upd is a secreted protein that bears strong homology to a gene called *Om1E* in the closely related fruit fly species *Drosophila ananassae*. However, no vertebrate homologs have been identified (26). The Upd protein has a signal sequence and several potential N-linked glycosylation sites. Although it has a predicted molecular weight of 47 kDa, Upd displays an apparent molecular weight of 65 kDa in electrophoretic analysis, presumably due to post-translational glycosylation. Tissue culture experiments have indicated that Upd associates tightly with the extracellular matrix, but it can be found in the supernatant after the addition of heparin to the media. When added to the fly imaginal disc Clone 8 cell line, recombinant Upd induces phosphorylation and activation of Hop (26). Taken together, these data indicate that Upd is a ligand that activates this pathway in flies. Recently, Blast search analysis revealed the presence of two other *upd*-like cytokine-encoding genes at the *upd* locus (27). *upd2* corresponds to *CG5988*, and *upd3* corresponds to *CG15062* (for the first and second exon) and *CG5963* (for the third exon) (28).

The receptor that activates the JAK/STAT pathway is encoded by *dome* (also known as *mom*) (29, 30). *dome* encodes a transmembrane protein that bears limited homology to the cytokine receptor, leukemia inhibitory factor receptor (29, 30). Dome signals through Hop and STAT92E. Hop is a 120-kDa protein that is most similar to human JAK2, with 27% identity overall and higher levels of homology in the kinase and kinase-like domains (25). No other JAK proteins are present in the fly genome (31). STAT92E is an 83-kDa protein that is most similar to human STAT5, with 37% overall identity. STAT92E contains an SH2 domain, a DNA-binding domain, and the single C-terminal tyrosine residue found in all STAT-like genes (32, 33). This residue is phosphorylated by *in vitro* activation of the pathway (33).

In addition to these four components, at least three classes of cytosolic proteins that modulate JAK/STAT signal transduction in mammals have homologs in flies. These include two negative regulator proteins, protein inhibitor of activated STAT and suppressor of cytokine signaling (34–36), and one positively acting protein signal-transducing adapter molecule (37). Interestingly, it was recently shown that two different primary transcripts originate from a dual promoter at the STAT92E locus. Some of the spliced forms encode a truncated form of STAT92E, such as ΔNSTAT92E that lacks the N-terminal 133 amino acids and acts as a dominant negative regulator of JAK/STAT signaling (38).

The JAK/STAT pathway and the humoral immune response

JAK/STAT activation in the fat body

The first evidence for the involvement of the JAK/STAT pathway in insect immune response was obtained from studies performed in the mosquito *Anopheles gambiae* (39). A hallmark of JAK/STAT pathway activation is the translocation of the activator of transcription STAT to the nucleus, where it activates target genes. This activation process was visualized by immunochemistry analysis of *Anopheles* STAT (aSTAT) cellular localization (39). In unchallenged mosquitoes, aSTAT protein was located both in the cytoplasm and in the nucleus. In challenged mosquitoes, STAT had substantially cleared the cytoplasm and accumulated in the nucleus. Similarly, STAT92E translocates into the nucleus of *Drosophila* fat body cells upon immune challenge (28). Furthermore, this translocation was abolished in animals with reduced JAK activity (hop^{M38}/hop^{msv1}) (28). Conversely, a very strong staining was detected both in the cytoplasm and in the nucleus of flies carrying a *Drosophila JAK* gain-of-function mutation [*Tumorous-lethal* (*Tum-l*)]. These results establish the existence of a JAK-dependent activation of STAT in *Drosophila* fat body cells in response to septic injury (28).

JAK/STAT-dependent expression of *tep1*

tep1 is part of a four-member family of genes that encode thioester-containing proteins (TEP) with significant similarities to members of the complement C3/α2-macroglobulin superfamily. *tep1* is expressed at low level in both larvae and adults and is strongly activated in fat body upon immune challenge (40). Interestingly, *tep1* was constitutively expressed in *Toll10B* flies that display a gain-of-function mutation in the Toll receptor, and *tep1* expression was also constitutively expressed in *Tum-l* flies. Accordingly, *tep1* activation was strongly decreased in larvae displaying a loss-of-function mutation in *hop*, confirming that *tep1* might be a target of the JAK/STAT pathway upon immune challenge (40). Interestingly, genetic interaction experiments revealed that *tep1* constitutive expression in *Toll10B* animals was suppressed by loss-of-function mutation in *hop*, suggesting that the Toll pathway might act upstream of the JAK/STAT pathway (40). Surprisingly, *tep1* was shown to be induced at a level comparable to wildtype in both *imd* and *Toll* mutants, suggesting that neither the imd pathway nor the Toll pathway is responsible for *tep1*-inducible expression (40). These results are in apparent conflict with the observed constitutive *tep1* expression in the *Toll10B* gain-of-function mutant. However, one possible explanation is that *tep1* constitutive expression in *Toll10B* animals might be an indirect consequence of the hemocyte overproliferation phenotype displayed in this genetic background (41). In *Toll10B* mutant, hemocytes possibly produce a ligand that activates the JAK/STAT pathway in the fat body, leading to concomitant *tep1* expression.

JAK/STAT-dependent expression of *totA*

In an effort to identify new genes involved in innate immunity that are controlled by the JAK/STAT pathway, gene expression profiles were analyzed in wildtype flies upon challenge and in unchallenged *Tum-l* flies (Petersen, unpublished results). Candidate JAK/STAT-dependent immune genes should display an inducible expression upon immune challenge as well as be constitutively expressed in flies carrying a gain-of-function mutation in the JAK/STAT pathway. These experiments identified *totA*, which had been previously identified by Ekengren and collaborators (42), as a gene coding for a polypeptide that is produced by the larval fat body and accumulates in hemolymph in response to various stress conditions, including immune challenge (28). Interestingly, *totA* expression was not induced in challenged adult flies displaying loss-of-function mutation in *hop* (hop^{M38}/hop^{msv1}), confirming the requirement for JAK activity in *totA* expression (28). Analysis of *totA* expression by *in situ* hybridization revealed that *totA* was mainly induced in adult fat body. Sequence analysis revealed the presence of at least four STAT-binding sites at the *totA* locus, suggesting that STAT might be directly involved in *totA* transcription. However, a detailed mutational analysis using *totA*-promoter region and reporter constructs in transgenic flies will be required to further confirm the functional importance of these motifs.

totA expression was used as a marker of JAK/STAT activation to further identify the components of the JAK/STAT pathway

involved in immune response in the fat body. In particular, the involvement of the receptor Dome in *totA* expression in adults was analyzed by taking advantage of the GAL4/upstream-activating signal (UAS) system. Dome ΔCYT, a dominant negative form of Dome that lacks the cytoplasmic part of the receptor, was expressed using the yolk-GAL4 driver that restricts expression to adult fat body. These experiments revealed that *totA* activation was totally abolished in the corresponding animals, showing that the *Drosophila* homolog of the vertebrate cytokine receptor, Dome, is involved in JAK/STAT signaling in the fat body upon immune challenge (28).

Other genes regulated by the JAK/STAT pathway

Because both *tep1* and *totA* are members of gene families (the *tep* and the *tot* families), it raises the question of whether other family members are also regulated by JAK/STAT signaling. No information is yet available about the other members of the *tep* family. However, in addition to *totA*, *totC* and *totM* were also found to be controlled by the JAK/STAT pathway upon septic injury, suggesting that this regulation is common to all the members of the *tot* gene family (Agaisse, unpublished results).

In addition to the *tot* family members, *CG11501* is also regulated by the JAK/STAT pathway (43). Computational analysis of gene expression profiles revealed that *CG11501* and *totM* (the only *tot* gene present on the Affymetrix microarray) displayed a similar temporal profile upon septic injury. This result suggested that these genes might share a similar mode of regulation. It was next confirmed in a JAK mutant that the integrity of the JAK/STAT pathway was required for *CG11501* expression (43). These experiments constitute a clear example of the predictive value of gene expression profiling and clustering analysis. However, genes that are regulated by a given pathway do not always display a similar expression profile. For example, although *tep1* and *totA* are both regulated by the JAK/STAT pathway, they display a different temporal expression profile. Therefore, further microarray experiments in various JAK/STAT mutant flies will be required to identify the whole set of immune genes that are regulated by the JAK/STAT pathway in *Drosophila*.

Function of the JAK/STAT-regulated humoral factors

The complement-like TEPs were identified by computational analysis of the *Drosophila* genome sequence as proteins displaying well-conserved motifs present in the members of the α2-macroglobulin/complement C3 family (40). Thus, TEP proteins were proposed to function either as opsonins that promote phagocytosis (like complement C3) or as protease inhibitor (like α2-macroglobulin). A TEP protein, aTEP1, was also identified in the mosquito *Anopheles gambiae*, where it was shown that *atep1* gene expression is regulated by immune challenge *in vivo* and that the aTEP1 protein is proteolytically processed in response to septic injury (44). Furthermore, studies in a mosquito blood cell line revealed that the efficient binding of aTEP1 to bacteria depends on the formation of a functional thioester bond. Furthermore, phagocytosis of gram-negative bacteria was reduced when aTEP1 was removed by RNA interference (RNAi) treatment in this mosquito blood cell line. Altogether, these data constitute the first demonstration that TEP proteins may at least promote phagocytosis in a complement-like manner in insects (44).

Apart from the *tot* family members themselves, the Tot proteins do not share similarities with any known proteins present in databases (45). Therefore, it is a difficult task to assign a function to the Tot proteins. Interestingly, while overexpression of a single anti-bacterial peptide can restore wildtype resistance to bacterial infection in otherwise highly susceptible NF-κB mutants (46), NF-κB mutants overexpressing the Tot peptides are still highly susceptible to bacterial infection, suggesting that Tot peptides do not display anti-bacterial activities (Agaisse, unpublished results). Interestingly, overexpression of *totA* confers a longer survival to flies subjected to heat stress (42). However, the exact mechanism of protection remains to be determined. It is noteworthy that, aside from septic injury, *tot* gene expression is also inducible by other stress conditions, suggesting a general role for Tot proteins in stress responses (42). Thus, it is possible that the Tot proteins are involved in physical protection/repair of damaged tissue. However, we cannot exclude a signaling role for the Tot proteins that might affect the physiology of flies upon stressful situations.

The JAK/STAT pathway and cellular immune response

Gain-of-function in the JAK/STAT pathway and hematopoiesis

One of the first indicators that the JAK/STAT pathway might be involved in the development of hemocytes came from the characterization of dominant gain-of-function alleles of *hop* (47, 48). Two temperature-sensitive mutations (*Tum-l* and *T42*) have been identified that result in proteins with constitutively hyperactivated kinase activity. The *Tum-l* allele is a point mutation (G431E) in a residue that is not conserved in other JAKs (49). The other mutation, *T42*, corresponds to a single amino acid substitution (E695K) present in the kinase-like domain and maps to a residue conserved in all known JAK homologs (50). *T42* mutation leads to a slightly more

pronounced effect than *Tum-l*, and an equivalent substitution to the T42 allele engineered into murine JAK2 results in similar overactive molecules (50). *Tum-l* individuals have five- to 20-fold more plasmatocytes when raised above the restrictive temperature, many of which prematurely differentiate into lamellocytes that contribute to black masses/melanotic tumors. When transplanted into a wildtype host, *Tum-l* hypertrophied larval lymph glands retain the ability to cause overproliferation of plasmatocytes and melanotic tumors, clearly indicating a cell-autonomous role of the JAK/STAT pathway (47–49).

The JAK/STAT pathway and the encapsulation response

A common defense reaction against parasites and foreign objects that invade the insect hemolymph is encapsulation. Parasitoid wasp females, such as *Leptopilina boulardi*, lay their eggs in the body cavity of *Drosophila* larvae. A massive differentiation of hemocytes into lamellocytes is triggered as a response to oviposition, and circulating lamellocytes can be detected a few hours after infestation (22, 51). Lamellocytes are very large and flat cells that form a multilayered capsule around the eggs (52). Within the capsule, which is eventually melanized, the parasite is killed by the production of toxic substances such as quinoid intermediates and reactive oxygen and nitrogen species (4). Until recently, it was generally admitted that lamellocytes differentiated in hemolymph from circulating plasmatocytes. However, a recent time course analysis using a specific marker of lamellocyte differentiation revealed that the lymph glands play a primary role in lamellocyte differentiation (22). The first signs of lamellocyte differentiation are observed in the anterior lobes of the lymph glands. About 10 h after infestation, the first lobes start dispersing, and lamellocytes are released in circulation. By 24 h, the first lobes are empty, and the second lobes are enlarged and contain numerous clusters of lamellocytes (22).

As previously discussed, *Tum-l* larvae contain numerous lamellocytes in the absence of any immune challenge. This observation strongly suggests that activation of the JAK/STAT pathway might be responsible for lamellocyte differentiation upon wasp infestation. A genetic analysis performed by M. Meister and colleagues recently confirmed this assumption (M. Meister, personal communication). They observed that larvae displaying a loss-of-function mutation in the JAK kinase Hop were not able to mount an encapsulation response upon wasp paratization. Therefore, in addition to its role in the production of humoral factors in fat body, the JAK/STAT pathway is also probably involved in the cellular immune response taking place in the lymph glands.

The JAK/STAT pathway and hemocyte proliferation

Immune challenge, such as septic injury with bacteria, does not usually trigger the proliferation of circulating hemocytes in *Drosophila*. However, it was shown that there is a significant increase in hemocyte count in lymph gland of third instar larvae upon wasp parasitization (51). Given the overproliferation phenotype in *Tum-l* larvae, this raises the question as to whether JAK/STAT signaling might also be involved in hemocyte proliferation in response to wasp infestation. To the best of our knowledge, this question has not been addressed in loss-of-function JAK/STAT mutants. However, the following evidence obtained from gain-of-function mutant analysis strongly suggests that there might be a link between JAK/STAT signaling and the involvement of the Ras/Raf/mitogen-activated protein kinase (MAPK)-signaling pathway in hemocyte proliferation. *Tum-l*-induced melanotic blood cell tumor phenotypes can be suppressed by the removal of a copy of the *STAT92E* gene (32). However, while STAT92E is obviously important in lamellocyte differentiation, it has been reported that the blood cell overproliferation phenotype is not affected by changes in STAT92E. It was therefore suggested that the pathway bifurcates downstream of Hop and that proliferation is a STAT92E-independent process (48). In agreement with this assumption, it was recently shown that a strong mutation in D-Raf, a component of the MAPK pathway, suppresses the overproliferation of *Tum-l* circulating blood cells (53). In addition, co-immunoprecipitation experiments performed on protein extracts from Sf9 insect cells overexpressing both Hop and D-Raf proteins revealed that Hop physically interacts with D-Raf *in vitro*. Altogether, these data suggest that the activated form of Hop interacts with the Ras/Raf/MAPK pathway, probably at the level of D-Raf, and leads to hemocyte overproliferation (53).

Finally, it has been shown that D-Raf is a direct transcriptional target for STAT activation and that the Raf promoter contains consensus STAT-binding sites (54). The functionality of these STAT-binding sites was demonstrated by analyzing the expression of wildtype and mutated version of D-Raf-lacZ fusions *in vivo*. Interestingly, it was shown that these STAT-binding sites are required for the activation of D-Raf expression in larval fat body upon septic injury. This observation might provide an additional control mechanism of Ras/Raf signaling by JAK/STAT signaling. However, whether this transcriptional regulation of D-Raf by STAT is also involved in the

control of hemocyte proliferation upon challenge remains to be tested.

Cytokine signaling: integration of immune responses

Signaling from hemocytes to the fat body

The involvement of *dome* in the control of *totA* expression predicted the existence of a cytokine-like molecule involved in the control of *totA* expression. *upd* has been characterized as a gene encoding the cytokine that activates the JAK/STAT pathway during *Drosophila* embryogenesis (26). The potential involvement of *upd* in *totA* activation was tested in outstrechted flies that represent a weak allele of *upd* and give rise to viable adult flies (55). The *totA* activation was strongly decreased in outstrechted flies, suggesting that *upd* might be involved in *totA* expression (28). However, further genetic analysis in transheterozygous flies displaying the outstrechted mutation over a null mutation in *upd* revealed that *totA* activation was nearly wildtype in this genetic background. This finding suggested that a defect in *upd* expression was unlikely to be responsible for the lack of *totA* activation in the outstrechted genetic background. Further investigation on the potential role of the other two putative *upd* genes, *upd2* and/or *upd3*, in *totA* expression revealed that the level of *upd3* expression, which was very low in control animals, was significantly increased following septic injury. Moreover, *upd3* expression was not induced upon immune challenge in the outstrechted genetic background (Agaisse, unpublished results). Altogether, these data strongly suggested that *upd3* might be the JAK/STAT pathway ligand involved in *totA* expression. To further analyze *upd3* expression, the *upd3*-promoter region was fused to the *GAL4* gene, and the corresponding transgenic flies were crossed to UAS-green fluorescence protein (GFP) lines. Analysis of the pattern of GFP expression revealed that *upd3* was not expressed in fat body cells of control or challenged animals. However, GFP production was strongly increased in blood cells after challenge, thus indicating that hemocytes might be the main site of *upd3* expression (28). The functional importance of *upd3* hemocyte-specific expression in *totA* expression was next tested by RNAi silencing of *upd3* expression *in vivo*. Tissue-specific expression of the silencing construct was achieved through the use of the *hemolectin-GAL4* and the *yolk-GAL4*, which are hemocyte and fat body-specific Gal4 drivers, respectively. While the production of *upd3* dsRNA in the fat body did not interfere with *totA* expression, *upd3* dsRNA expression in hemocytes led to a strong decrease in *totA* activation upon septic injury. Altogether, these experiments constitute the first genetic demonstration that, in response to septic injury, the Upd3 cytokine is produced in hemocytes and subsequently activates *totA* expression in fat body (*Fig. 1*).

Signaling from hemocytes to lymph glands: is there a cytokine involved in lamellocyte differentiation?

As previously mentioned, wasp parasitization leads to a massive JAK/STAT-dependent lamellocyte differentiation in the lymph glands (22). Although we cannot formally exclude a role of the JAK/STAT pathway in lamellocyte precursor specification, it is likely that the JAK/STAT pathway is involved in signaling events that occur in response to the presence of wasp eggs in hemolymph. Interestingly, electron microscopy studies have revealed that in the early hours of infection, circulating plasmatocytes are the first cell type to attach to and spread on the surface of eggs (52). Then, several hours later, lamellocytes are deposited on plasmatocytes and on the surface of eggs. Lamellocytes accumulate in several layers and rapidly develop septate junctions between adjacent cells (52). Given this sequence of cellular events, it is tempting to speculate that early plasmatocytes somehow signal to the lymph glands to induce lamellocyte differentiation (*Fig. 1*). Although the nature of this signal is unknown, one obvious candidate might be Upd3. In this respect, it would be instructive to analyze Upd3 expression in the early plasmatocytes that attach to the egg surface using *upd3*-GFP reporter. Alternatively, another cytokine, such as Upd2 or a yet unidentified ligand of the JAK/STAT pathway, might be involved. It is also possible that JAK/STAT signaling occurs in the lymph gland in response to a primary signaling event that involves another signaling pathway in plasmatocytes. In this respect, it is noteworthy that several genetic interaction studies positioned the Toll pathway upstream from the JAK/STAT pathway. In particular, it has been shown that the activated form of the Toll-like receptor 18-Wheeler leads to lamellocyte differentiation, a phenotype that was suppressed in larvae displaying a loss-of-function mutation in *hop* (56). Unfortunately, the involvement of 18-Wheeler in lamellocyte differentiation has not been tested in a loss-of-function situation. Similar to activated form of 18-Wheeler, the same lamellocyte differentiation phenotype was also observed in *Toll10B* larvae displaying a gain-of-function allele of the Toll receptor (41). The lamellocyte differentiation process was found to be similar in Toll loss-of-function mutants and in wildtype flies (Sorentino and Govind, personal communication). Therefore, it is unlikely that the Toll receptor is involved in lamellocyte differentiation. However, we cannot exclude that one of the nine Toll-like receptors present in the fly genome, including 18-Wheeler,

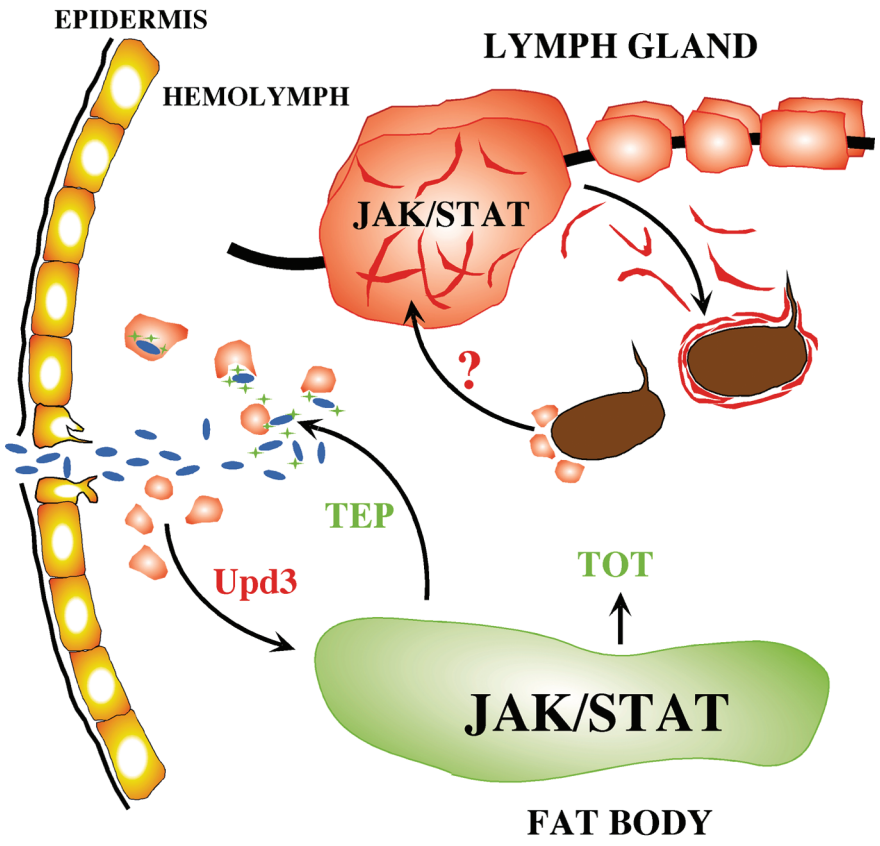

Fig. 1. Overview of the JAK/STAT-dependent *Drosophila* immune responses. Upon septic injury, breakage of the cuticle and epithelium barriers leads to the presence of bacteria in the hemolymph. These events lead to the activation of *upd3* expression in hemocytes. The Upd3 cytokine is presumably released in hemolymph and subsequently activates JAK/STAT signaling in fat body. These signaling events control the activation of genes coding for the thioester-containing proteins (TEP) and the TOT peptides. The TEP proteins presumably promote phagocytosis, whereas the function of the TOT peptides is unknown. Upon wasp parasitization, plasmatocytes spread on the surface of the eggs and are thought to send a yet uncharacterized signal to the lymph glands. This signaling event triggers massive JAK/STAT-dependent differentiation of lamellocytes in the lymph glands. Upon lymph gland dispersal, lamellocytes are released into hemolymph and encapsulate the wasp eggs.

might effectively be devoted to the primary detection of wasp egg by plasmatocytes that subsequently send a signal to the lymph glands to activate JAK/STAT signaling.

Perspectives on JAK/STAT signaling in *Drosophila* immune response

Function of JAK/STAT-regulated humoral factors

It is now well established that the NF-κB pathways controlled the expression of anti-microbial peptides involved in microbial killing upon infection. As a consequence, NF-κB mutant flies are highly susceptible to infection. Unlike NF-κB mutant animals, preliminary experiments indicate that JAK/STAT mutants are not more susceptible to immune challenge. This suggests that the JAK/STAT-regulated humoral factors probably do not display anti-microbial activities (Agaisse, unpublished results). Given that JAK/STAT signaling in the fat body relies on hemocytes, one might hypothesize that the overall function of the JAK/STAT-regulated humoral factors secreted into hemolymph by the fat body is to assist hemocytes in their immune functions. In agreement with this hypothesis, the aTEP1 protein, whose *Drosophila* homolog (TEP1) expression is regulated by the JAK/STAT pathway, promotes the phagocytic activity of a hemocyte-like cell line in mosquitoes (44). Similarly, the Tot peptides might also be involved in promoting phagocytosis in *Drosophila* hemocytes. Alternatively, it is well known that *Drosophila* immune response leads to the production of highly reactive species by hemocytes (4). It is therefore possible that the JAK/STAT-dependent peptides, such as the Tot family peptides, protect the fly from the deleterious effect of these components.

Analyzing the role of the JAK/STAT pathway in *Drosophila* immune response remains a challenging task. It is important to note that the NF-κB-dependent anti-microbial peptide response is so efficient in *Drosophila* that some aspects of the immune response have long been ignored as they were apparently not required for survival. There is a need for defining immune response-related assays that are not simply based on survival experiments. These new assays will help to investigate the respective role of signaling pathways that are activated in various tissues during *Drosophila* immune response, such as the JAK/STAT and JNK pathways.

Lamellocyte differentiation

Lamellocyte differentiation is the hallmark of JAK/STAT signaling during the *Drosophila* cellular immune response in larvae. The exact sequence of events leading to differentiation remains

to be determined. First, the nature of the hemolymph signal leading to differentiation of these cells in the lymph glands remains unknown. Second, the genes involved in the differentiation process remain to be characterized. The availability of hemocyte-specific GAL4 drivers will certainly help in these studies. For instance, it is possible to trigger lamellocyte differentiation in a manner similar to wasp infestation by driving UAS-*Tum-l* with these hemocyte-specific drivers (Agaisse, unpublished results). Thus, it might be possible to purify hemocytes by fluorescence-activated cell sorting and compare the expression profiles of various subpopulations, including plasmatocytes and lamellocytes. Such studies should provide markers that will help to further analyze the differentiation process *in vivo*.

Regulation of cytokine expression and hemocyte activation

The nature of the signal(s) and the signaling pathways involved in *upd3* regulation are unclear. Preliminary experiments indicate that *upd3* expression is not affected in NF-κB mutants (Agaisse, unpublished results). Therefore, *upd3* constitutes a new paradigm for studying gene activation in response to septic injury in *Drosophila*. A candidate approach is currently being conducted to test the potential involvement of signaling pathways, such as the JNK and the p38 pathways, which have been shown to be involved in the regulation of cytokine expression in mammals. The nature of the signal(s) sensed by hemocytes upon septic injury constitutes a particularly important question. It remains to be determined whether hemocytes directly detect the presence of bacteria upon septic injury and whether pattern-recognition receptors, such as PGRPs, are involved in that process. Alternatively, it is possible that molecules released by damaged tissues lead to hemocyte activation upon septic injury. Further genetic investigations using *upd3* expression as a model system should lead to the identification of components involved in the early steps of hemocyte activation upon immune challenge.

Integration of signaling pathway activities

In addition to the JAK/STAT pathway, *totA* activation in fat body also requires the Relish pathway (28). Therefore, *totA* activation in fat body cells is achieved through the integration of various inputs such as the presence of bacterial compounds (through NF-κB activation) and cytokines (through JAK/STAT activation) in response to septic injury. The exact molecular mechanisms involved in such integration are not known. Numerous studies performed in mammalian cells indicate that the input of various signaling pathways, such as the JNK and the NF-κB pathways, is integrated at the level of cytokine promoters. Whether this is the case for *totA* regulation by the NF-κB and JAK/STAT pathways will require further functional analysis of *totA*-promoter region.

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 (42). Similarly, we have observed that injection of detergent solution into the hemolymph also triggers *totA* activation. This response was strongly affected in both *hop* and *relish* mutants (Agaisse, unpublished results). The signaling events leading to both JAK/STAT and NF-κB activation upon this kind of treatment remain to be determined. For instance, it will be of interest to determine whether *upd3* is involved in this response.

Cytokine-mediated signaling in insects and mammals

In the past decade, striking similarities have emerged between *Drosophila* and mammalian innate immunity. The mode of detection of microbial patterns through activation of pattern-recognition receptors and NF-κB-like signaling pathways has been conserved throughout evolution. The finding that, as in mammals, a cytokine-mediated response also takes place in *Drosophila* in response to septic injury raises several questions with respect to the corresponding studies in mammals. For instance, in addition to the JAK/STAT pathway, several other signaling pathways are involved in cytokine-mediated signaling in mammalian systems. The NF-κB-signaling pathway is not only activated by the Toll-like receptor-mediated recognition of pathogen motifs but also by interleukins (IL), such as IL1, which bind to specific receptors. Interestingly, the IL1 receptor and the Toll-like receptors belong to a family of transmembrane receptors that display the intracytoplasmic Toll-IL1 receptor (TIR) domain involved in signal transduction (57). However, while the extracellular part of Toll contains leucine-rich repeats, the extracellular part of the IL1 receptor displays immunoglobulin domains. Analysis of the *Drosophila* genome sequence revealed the presence of nine genes encoding TIR domain-containing proteins. All of these TIR domain-containing transmembrane receptors display leucine-rich repeats in their extracellular part and are therefore potential Toll-like receptors. As a consequence, there is probably no homolog of the IL1 receptor in flies. However, we cannot exclude the presence of a structurally unrelated system equivalent to the mammalian IL1/IL1 receptor and leading to NF-κB activation during *Drosophila* immune response. In addition to NF-κB activation, the innate immune response also leads to the activation of the tumor necrosis factor (TNF) pathway in mammals. Importantly, a homolog of the mammalian TNF

factor, Eiger, and its receptor, Wegen, have been recently identified in *Drosophila* (58, 59). However, the involvement of this system in *Drosophila* immune response remains to be tested.

The activation of *totA* in *Drosophila* fat body as a response to the release of Upd3 by hemocytes is very similar to some aspects of the mammalian acute-phase response. For example, the production of the mammalian acute-phase proteins by the liver (the functional equivalent of *Drosophila* fat body) is modulated by the JAK/STAT pathway in response to the systemic release of IL6 by activated macrophages (60). In addition to this type of communication between macrophages and liver, several other tissues talk to each other during the mammalian immune response. For instance, infected tissues, such as fibroblasts and endothelial cells, release cytokines, such as IL8, which are highly chemotactic to circulating neutrophils and monocytes (61). As a consequence, these blood cells leave the circulatory system and infiltrate the infected tissues. Although insects display an open circulatory system and hemocytes are therefore in contact with most of internal organs, we cannot exclude that local disturbance of tissue homeostasis leads to the release of factors altering the adhesion properties of circulating hemocytes. Accordingly, several peptides modifying the adhesion properties of hemocytes have been described in *Lepidopteran* species (62). Whether such peptides are also present in *Drosophila* remains to be investigated.

A fundamental aspect of the acute-phase response in mammals is the resolution process. The proinflammatory cytokines trigger anti-inflammatory reactions that generate molecules devoted to the control of proinflammatory cytokines. For example, the activity of IL1 is balanced by IL1 receptor antagonist, which is massively produced in the liver as an acute-phase protein in response to proinflammatory cytokines (63). Whether such a mechanism has been conserved throughout evolution is currently under investigation.

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