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# Injury-induced inflammatory signaling and hematopoiesis in *Drosophila*

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Inflammatory response in *Drosophila* to sterile (axenic) injury in embryos and adults has received some attention in recent years, and most concentrate on the events at the injury site. Here we focus on the effect sterile injury has on the hematopoietic organ, the lymph gland, and the circulating blood cells in the larva, the developmental stage at which major events of hematopoiesis are evident. In mammals, injury activates Toll-like receptor/NF- $\kappa$ B signaling in macrophages, which then express and secrete secondary, proinflammatory cytokines. In *Drosophila* larvae, distal puncture injury of the body wall epidermis causes a rapid activation of Toll and Jun kinase (JNK) signaling throughout the hematopoietic system and the differentiation of a unique blood cell type, the lamellocyte. Furthermore, we find that Toll and JNK signaling are coupled in their activation. Secondary to this Toll/JNK response, a cytokine, Upd3, is induced as a Toll pathway transcriptional target, which then promotes JAK/STAT signaling within the blood cells. Toll and JAK/STAT signaling are required for the emergence of the injury-induced lamellocytes. This is akin to the derivation of specialized macrophages in mammalian systems. Upstream, at the injury site, a Duox- and peroxide-dependent signal causes the activation of the proteases Grass and SPE, needed for the activation of the Toll-ligand Spz, but microbial sensors or the proteases most closely associated with them during septic injury are not involved in the axenic inflammatory response.

Toll/TLR | inflammation | blood | cytokine | wound

Breach of the *Drosophila* larval epidermis by septic injury elicits innate immune responses that have been studied in great detail in adult stages where it is systemically mediated, almost entirely, by the fat body via the Toll and Imd pathways (1–4). Such immune responses are less well-studied in the larval stages (4). However, an extensive literature describes parasitization by wasps, which is a uniquely larval response that involves several blood cell types, including the newly induced lamellocytes. Rarely seen in healthy larvae, these are relatively large, flattened cells that function in barrier formation around the parasitoid wasp eggs (5). The mature blood cell types that dominate the larva during homeostasis are the macrophage-like plasmatocytes (95%) that secrete antimicrobial peptides, remove cellular debris, and remodel tissue and their extracellular matrix. The third cell type is crystal cells that provide enzymes important for blood clotting and melanization and aid in injury resolution and antimicrobial responses. As in all invertebrates, *Drosophila* blood cells are functionally similar to those from the vertebrate myeloid lineage. Early genetic studies have shown that constitutive activation of Toll signaling causes blood cell proliferation, precocious lamellocyte differentiation, and melanization responses in larvae (6, 7).

Hemocytes (blood cells) that arise from the head mesoderm during embryonic development constitute a circulating and a sessile population of cells underneath the larval cuticle. Additionally, the major site of hematopoiesis in the larva is the specialized blood-forming organ, the lymph gland, which also originates in the embryo but matures during the larval stages, serving as a hematopoietic reservoir for the pupal and adult stages (8). At the onset of metamorphosis, the lymph gland disintegrates, releasing blood cells into circulation. Both larval circulating cells and lymph gland-derived cells comprise the adult fly hemocyte population (9).

This report probes the effects of axenic or sterile injury to the larvae and the cascade of events that follow, affecting the lymph gland and the circulating hemocytes. This is not the first study on sterile injury in *Drosophila*, but it is designed to probe a different aspect of the larger problem and thereby fulfills an important gap in our knowledge. First, other studies involve embryos or adults (10–15), and not the larval stages, where the majority of active hematopoiesis that will populate the pupal and adult stages occurs. Second, the emphasis in other studies is on events at the injury site and in its wound healing (10–22). Here, we concentrate on the molecular events that mediate the long-distance effect of remote sterile injury on the blood cells and the developing

## Significance

We explore mechanisms by which stress caused by acute injury affects blood cell development and inflammatory response in *Drosophila*. Similar to their mammalian myeloid counterparts, these cells are predisposed to sense and react to sterile injury at distant sites. Upon sterile injury, a breach of epidermis sets up a reactive oxygen species-based signal that bypasses the pathogen-sensing apparatus of septic immune challenge, but merges downstream to activate Toll. A number of autonomous and nonautonomous signaling pathways follow in a sequence and are mapped temporally by the appearance of their corresponding molecular phenotypes. A cell-type that fights deposited parasitic wasp eggs appears with sterile injury without the immune challenge, perhaps in anticipation, because in nature injury is usually followed by infection.

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The authors declare no competing interest.

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lymph gland, rather than how the macrophages and crystal cells help repair the injury site. The focus of previous studies in the larva—on melanization, lamellocyte formation, and transcriptomic analysis (23–28)—was not on the lymph gland or on the detailed mechanistic interaction between pathways that results in the hematopoietic response. The results presented here are consistent (but not redundant) with those seen in gut stem cells and inferred from survival, when adult flies are injured (15, 22). Altogether, it seems clear that although *Drosophila* has an open circulatory system, the responses to sterile injury are highly conserved and resemble those for vertebrate inflammatory pathways (29).

In mammals, tissue-resident macrophages and fast-responding neutrophils sense inducers of injury and inflammatory responses. They mediate local inflammation through the secretion of secondary proinflammatory signals (30, 31). In the case of microbial infection, inductive signals are referred to as pathogen-associated molecular patterns (PAMPs) whereas, for injury, they are the damage-associated molecular patterns (DAMPs) (32, 33). Blood cells utilize so-called pattern recognition receptors (PRRs) to sense both PAMPs and DAMPs. In mammals, a major class of PRRs is the Toll-like receptor (TLR) family, thus named for its shared homology with *Drosophila* Toll (34).

In *Drosophila*, PAMPs (such as  $\beta$ -glucan and peptidoglycan) are sensed by soluble PRRs (such as GNBPI and GNBPI3) in the hemolymph, and they initiate a proteolytic signaling cascade (including Spatzle processing enzyme and Grass) that eventually activates a cytokine-like protein ligand, Spatzle (Spz). Activated Spz binds Toll causing a translocation of the NF- $\kappa$ B-like proteins Dorsal and Dorsal-related immunity factor (Dif) to the nucleus (35, 36). The Dorsal/Dif transcription factors up-regulate the expression of many downstream genes, including antimicrobial peptides. While much is understood about the humoral function of Toll in the fat body, relatively little is known about Toll signaling in *Drosophila* blood cells. We report here the order and timing of events that link injury to Toll activation in blood cells and its downstream consequences on secondary signals and hematopoiesis.

## Results

**Sterile Injury Activates Toll Signaling in the Hematopoietic System.** Injury caused by postero-lateral body wall puncture (distal to gut and lymph gland) causes robust melanization of the wound area by 3 h postinjury (3 hpi) (Fig. 1A). To explore Toll pathway signaling, we used the reporters *Drosomycin* (*Drs-GFP*) and *D4-lacZ* containing four Dorsal binding sites from the *zen* enhancer (34, 37, 38) and found that injury with a sterilized needle is sufficient to activate these reporters in both circulating and lymph gland hemocytes by 3 hpi (Fig. 1B–K and *SI Appendix*, Fig. S1A and B). Importantly, Toll pathway activation by a sterile needle remains unchanged when the injured larvae are raised under axenic conditions (*Materials and Methods* and Fig. 1F–J). Taking these data together, we conclude that the Toll/DI pathway is an inflammatory response that is activated without pathogenic infection.

The expression patterns of *Drs-GFP* and *D4-lacZ* also accurately reflect the subcellular localization of Dorsal. In uninjured animals, Dorsal protein shows robust cytoplasmic localization in both circulating and lymph gland cells (*SI Appendix*, Fig. S1C and E). Interestingly, in the lymph gland, this cytoplasmic localization is largely seen in the progenitors of the medullary zone (*SI Appendix*, Fig. S1E and F). Following sterile injury,

Dorsal protein is now found in the nucleus of a great majority of these cells by 3 hpi (*SI Appendix*, Fig. S1D and F). The activation of *D4-lacZ* in response to injury is completely obliterated in a genetic background that is deficient for both *Dorsal* and *Dif* gene products (*SI Appendix*, Fig. S1G and H), or upon the loss of the intracellular Toll signaling adapter protein Myd88 (*SI Appendix*, Fig. S1I and J). Thus, the Toll pathway and its downstream NF- $\kappa$ B-like proteins control the sterile inflammatory response of the larval hemocytes. Given that Toll is autonomously required, the wound site must communicate with the blood cells by a systemic mechanism.

**Injury-Induced Toll Signaling in the Blood Requires Spz.** Spz is the only known ligand in *Drosophila* for the Toll receptor (34, 39, 40). We find that injured heterozygous control larvae (*spz<sup>mm7</sup>/+*) exhibit normal *D4-lacZ* activation upon injury (Fig. 2A), which is completely suppressed in *spz<sup>mm7</sup>* mutants (Fig. 2B). Additionally, qRT-PCR analysis demonstrates that injury induces the expression of the endogenous *Drosomycin* (*Drs*) gene, and loss of *spz* function completely suppresses this response (*SI Appendix*, Fig. S2A). Consistent with these data, injured control larvae (*spz<sup>mm7</sup>/+*) accumulate Dorsal in the nucleus of blood cells in circulation and in the lymph gland by 3 hpi (*SI Appendix*, Fig. S2B, D, and F), whereas injured *spz<sup>mm7</sup>* mutant blood cells maintain Dorsal in the cytoplasm (*SI Appendix*, Fig. S2C, E, and G). These data establish that injury-induced Toll pathway signaling in blood cells requires the ligand Spz.

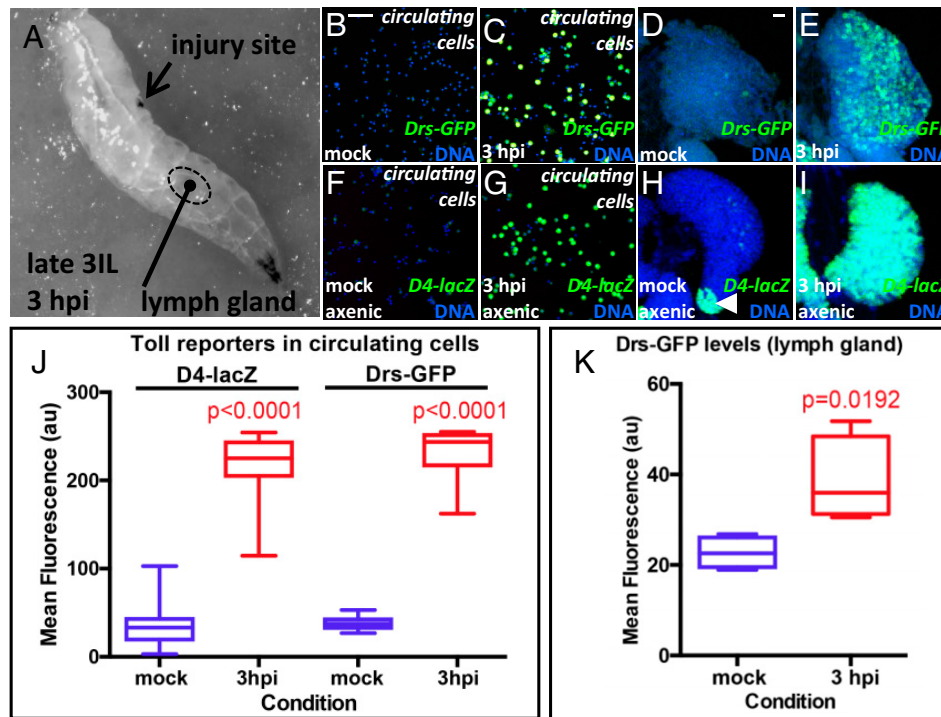
**The Toll-Mediated Injury Response in Blood Requires the Proteases Spz Processing Enzyme and Grass.** During infection, active Spz ligand is produced through proteolytic processing of a Spz propeptide (Pro-Spz) (41) by the Spz processing enzyme (SPE) (3). We find that hypomorphic loss of SPE activity also strongly suppresses *D4-lacZ* activation in blood cells in response to injury (Fig. 2C and D). Septic injury and the resulting infection leads to a sequential activation of the serine proteases: ModSP, Grass, and SPE (42–44). In the context of sterile injury, activation of Spz and downstream Toll signaling requires the two proteases SPE and Grass, that are immediately upstream of Spz, but not the further upstream protease ModSP (Fig. 2A–J).

The activation of Spz/Toll in dorsal/ventral patterning of the embryo requires an entirely different set of proteases: Easter (Ea), Snake (Snk), Gastrulation defective (Gd), and Nudel (Ndl). Mutations in genes encoding these proteases do not affect injury-induced *D4-lacZ* expression (*SI Appendix*, Fig. S2H–O), and therefore the embryonic, developmental protease cascade is not involved in inflammatory response.

**Sterile Injury Response Is Independent of Microbe Sensors but Requires Injury-Site Production of Hydrogen Peroxide.**

The PRR GNPB1 is essential for sensing Lysine-type peptidoglycan (Lys-PGN) produced by gram-positive bacteria and assists in the downstream activation of ModSP and Grass (43–45). Sterile injury does not involve pathogens and accordingly, we detect no role for GNPB1 in this process (Fig. 2J and K). Additional PRRs that sense pathogens, such as GNBPI3 (46) and Persephone (Psh) (47), also do not affect postinjury induction of *D4-lacZ* (Fig. 2L–O). Thus, while Grass, SPE, and Spz are common to both injury- and infection-induced Toll signaling, the mechanism of injury sensation leading to Grass activation uses different components.

In both zebrafish and *Drosophila* embryos and adults, hydrogen peroxide produced at injury sites serves as a chemoattractant for migrating blood cells (15, 48, 49). To determine if injury site-



**Fig. 1.** Activation of NF- $\kappa$ B-like signaling in the blood system in response to injury. (A) Third-instar larva showing the location of a melanized puncture wound (arrow) relative to the location of the lymph gland (dotted circle). (B–I) Expression of *Drs-GFP* (B–E; green) and *D4-lacZ* (F–I; green), both of which are reporters for Toll signaling. DNA (blue) staining marks all cells. (B, C, F, and G) Circulating cells. (Scale bar, 50  $\mu$ m.) (D, E, H, and I) Lymph gland (primary lobes). (Scale bar, 20  $\mu$ m.) (C and E) Injury with a sterile needle. (G and I) Axenically reared larvae injured with a sterile needle. Expression of both reporters is low under mock (uninjured) conditions (B, D, F, and H) but are both induced 3 hpi in the circulating cells as well as in the lymph gland (C, E, G, and I). PSC cells constitutively express *D4-lacZ* (arrowhead in H). In *SI Appendix, Fig. S1 K–O*, we show that Dorsal is not expressed in PSC cells, but Dif is, and the expression of the reporter is lost in a *Dif* mutant even though the PSC remains intact. (J and K) Quantitation of data in B–G showing that the expression level of Toll reporters in circulating cells (J;  $n = 52$  cells) and lymph glands (K;  $n = 4$  lymph glands) is significantly higher after injury than in mock controls.

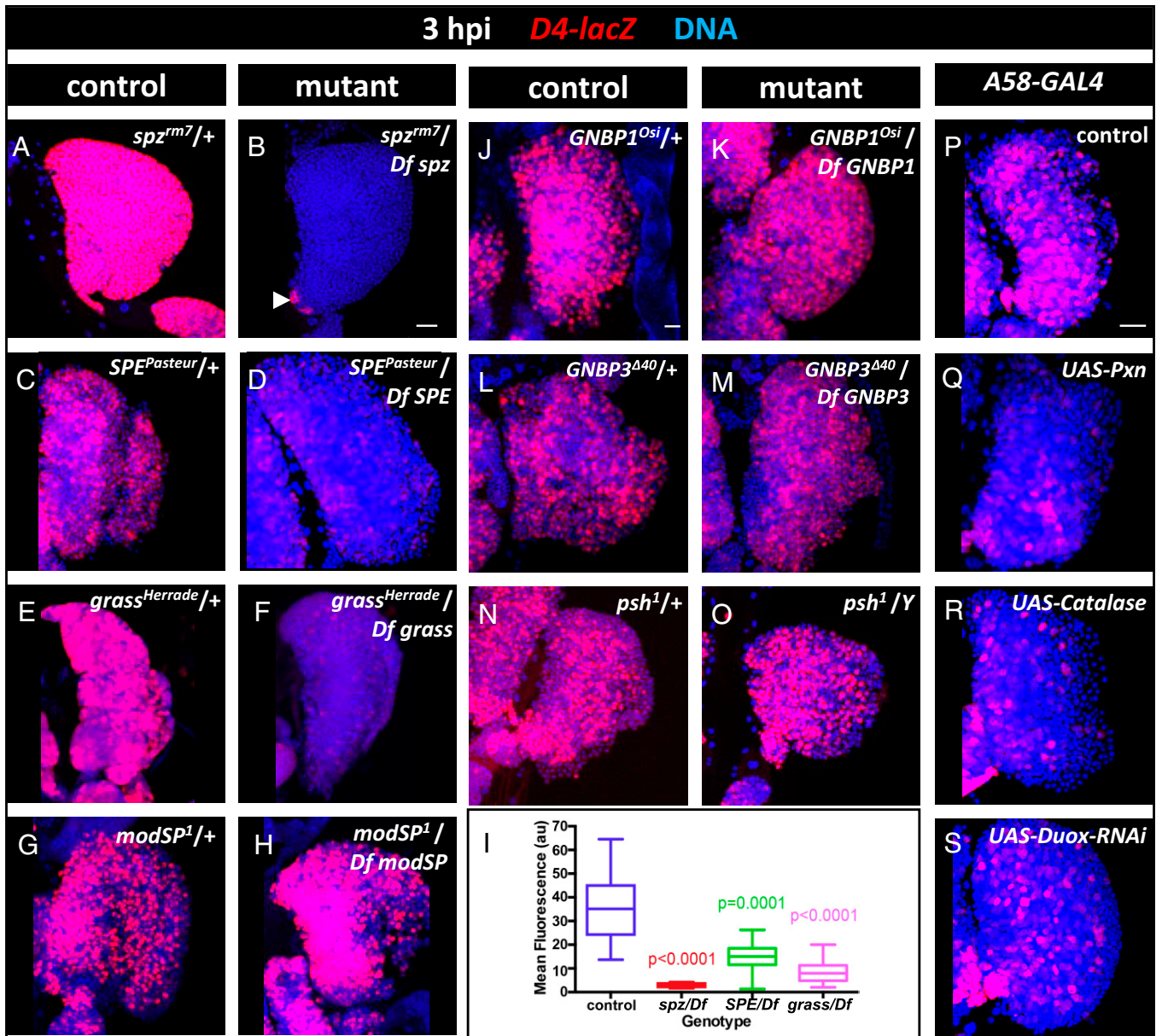
derived hydrogen peroxide also plays a role in Toll pathway activation in the blood, we specifically lowered peroxide levels in the epidermis by expressing the scavenging enzymes, *Drosophila* peroxidase (Fig. 2Q) or human catalase (Fig. 2R), and found that the injury-induced Toll signaling is markedly reduced in the blood cells (Fig. 2P–R and *SI Appendix, Fig. S2P*). In the *Drosophila* embryo and in vertebrates, immediately following injury, a flash of  $Ca^{2+}$  propagates outward from the wound site and activates the Dual oxidase (Duox) enzyme that generates hydrogen peroxide (48–53). Similar to the results on peroxide scavenging, we find that a specific knockdown of Duox activity in the epidermis also suppresses Toll signaling in blood cells induced by sterile epidermal injury (Fig. 2P and S and *SI Appendix, Fig. S2P*). Collectively, these data establish a causal role of Duox generated hydrogen peroxide in the systemic activation of Toll signaling in blood induced by sterile injury.

**Epidermal Injury Causes Toll-Dependent Hematopoietic Differentiation of Lamellocytes.** Hematopoietic differentiation of lamellocytes is closely associated with the cellular immune response to parasitoid wasp infestation of larvae (26, 27, 54, 55). Importantly, wasp oviposition also causes a puncture injury to the larval body wall as it delivers the wasp embryo and venom into the larval hemocoel. Subsequently, lamellocytes differentiate and, along with plasmatocytes and crystal cells, encapsulate the parasitic embryo (5). To our surprise, larvae injured with a sterile needle also cause lamellocytes to form in the lymph gland and in circulation by 24 h (Fig. 3); this phenotype is strongly suppressed upon loss of *spz* (Fig. 3E). The injury related lamellocytes have a similar size range (*SI Appendix, Fig. S3*) as fully mature lamellocytes induced by wasp infestation

(56) and they appear over a similar timescale (57). Thus, breach of epidermis alone is a sufficient trigger for initial lamellocyte induction even in the absence of a parasitic egg.

**Epidermal Injury Activates JNK Signaling in the Blood System in a Toll Pathway-Dependent Manner.** Genetic analysis has shown that lamellocyte differentiation relies on activation of the JNK pathway (58, 59), and we asked whether this is also true in the context of injury. A mild, but discernible, increase in the expression of the *puc-lacZ* reporter (60) is apparent in lymph gland cells by 3 hpi (Fig. 4A and B). Another JNK target encodes *Matrix metalloproteinase 1* (*Mmp1*), a secreted protease with developmental and extracellular matrix remodeling roles (61–64). In contrast to the weak *puc-lacZ* induction, *Mmp1* protein, mRNA, and reporter construct are robustly up-regulated at 3 hpi (Fig. 4C–F and H). *Drosophila* JNK (Basket; Bsk), is activated by the corresponding JNK kinase (JNKK) protein encoded by *hemipterous* (*hep*) (65), and in the genetic background of a null *hep*<sup>75</sup> mutant or targeted expression of a dominant-negative Bsk allele (*Hm<sup>A</sup>-GAL4 UAS-bsk<sup>DN</sup>*), expression of *Mmp1-lacZ* is no longer up-regulated postinjury (Fig. 4F–I). Thus, injury causes Dorsal nuclear localization and JNK activation as early as 3 hpi even though resulting lamellocytes take up to 24 h to form.

Given its rapid activation, we explored whether the JNK pathway is dependent on Toll signaling. Indeed, we found that loss of *spz* strongly suppresses *Mmp1* up-regulation in the lymph gland upon injury (Fig. 4J and K). Also, qRT-PCR analysis of circulating cells postinjury show an ~60% reduction of *Mmp1* mRNA expression in *spz* mutants (Fig. 4E). In Fig. 2C–F and R we show that injury-induced Toll signaling in the

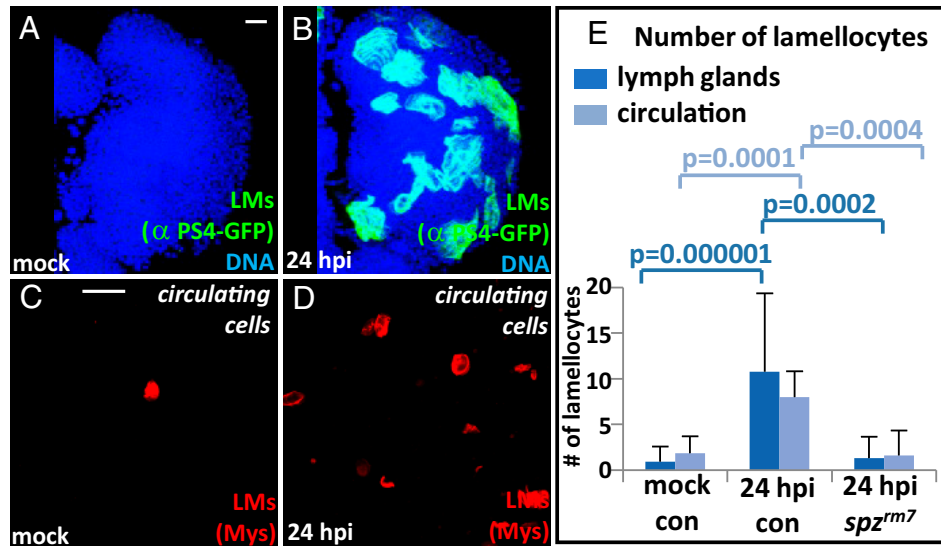


**Fig. 2.** The Toll pathway serine proteases Grass and SPE are required to activate Spz downstream of reactive oxygen species (ROS) for injury-induced signaling in blood cells. (A–S) *D4-lacZ* (Toll pathway reporter, red) in lymph glands 3 hpi; DNA (blue). (Scale bars, 20  $\mu$ m.) A minimum of seven lymph glands were used per genotype. (A–F) Toll reporter expression is robustly activated in the lymph gland by 3 hpi in heterozygous control animals (A, C, and E), but is completely suppressed in *spz*, *SPE*, or *grass* mutant lymph glands (B, D, and F). Note that *D4-lacZ* expression (arrowhead in B) is retained in PSC cells in this mutant background (compare with Fig. 1H). (I) Quantitation of injury-induced *D4-lacZ* levels in A–F shows that *spz*, *SPE*, or *grass* mutant lymph glands have significantly lower Toll activation than heterozygous controls. (G, H, J–O) Mutants in proteases *modSP* (H) and *psh* (O), or PRRs such as *GNBP1* (K) and *GNBP3* (M) that function upstream of Grass do not affect injury-induced *D4-lacZ* expression compared to heterozygous controls (G, J, L, and N). (P–R) Targeted expression in epidermal cells (*A58-GAL4*) of scavengers of ROS, *Pxn* (Q), or human catalase (R) suppresses injury-induced *D4-lacZ* expression in the lymph gland relative to control (P). A minimum of 12 lymph glands were used per genotype. (S) Specific knockdown of Duox function (*A58-GAL4, UAS-Duox<sup>RNAi</sup>*) also suppresses *D4-lacZ* activation in response to injury in the lymph gland relative to control (P). This establishes that Duox mediates ROS-dependent Toll signaling in blood cells.

blood requires the upstream function of SPE, Grass, and Duox. Consistent with these findings, global loss of *Grass* or *SPE*, or knockdown of *Duox* in the larval epidermis, suppresses injury-induced *Mmp1* up-regulation in the lymph gland (Fig. 4 J and L–M). This is true as well, with the global loss of function of the Toll adapter protein *Myd88* (Fig. 4 J and O). Thus, both injury-induced JNK signaling and the expression of its downstream target, *Mmp1*, in blood cells are Toll signal-dependent.

**Toll Pathway-Dependent Expression of Cytokine-Like Genes and JAK/STAT Activation.** In mammalian macrophage activation, the TLR/NF- $\kappa$ B signal increases the production of proinflammatory cytokines, such as interleukin (IL)-6, IL-12, and tumor necrosis

factor- $\alpha$  (TNF- $\alpha$ ) (30, 31). In *Drosophila*, the *unpaired* family of genes (*upd*, *upd2*, and *upd3*) encode cytokine-like ligands that mediate JAK/STAT signaling (66). In particular, *Upd3*, a four-helix-bundle ligand with homology to IL-6, has been linked to JAK/STAT signaling in blood cells (16, 67, 68). RNA-sequencing (RNA-seq) data show that under normal conditions the expression of *upd3* is much higher in blood cells than *upd2*, and *upd* was not detected (*SI Appendix, Fig. S4A*). *upd3* expression (*upd3-GAL4 UAS-GFP*) (67) is greatly up-regulated by injury throughout the lymph gland and in circulating blood cells by 24 hpi (Fig. 5 A–F), but not at 3 hpi when Toll and JNK signaling is already apparent. When analyzed by qPCR rather than indirectly through marker



**Fig. 3.** Injury-induced lamellocyte differentiation requires Spz. Lamellocytes (LMs) differentiate in lymph glands by 24 hpi (A and B), identified by the specific expression of the  $\alpha$ PS4-GFP reporter (green). DNA (blue). (Scale bar, 20  $\mu$ m.) This is a maximum-intensity projection. Cells that appear clumped can be spatially resolved into individual lamellocytes, that is clearer in a three-dimensional (3D) rendering shown in *SI Appendix, Fig. S3A*. Lamellocytes also differentiate among circulating cells (C and D), identified by their high expression of Mys (red), also by 24 hpi. (Scale bar, 100  $\mu$ m.) (E) Quantitation of the number of lamellocytes per lobe (lymph glands) or per larvae (circulation) under mock conditions or at 24 hpi. Mutation in *spz* (*spz<sup>rm7</sup>/spz<sup>rm7</sup>*) completely blocks injury-induced lamellocyte differentiation.

expression, *upd3* transcripts exhibit a greater than 20-fold increase at 6 hpi (Fig. 5G). The expressions of *upd* and *upd2* also rise but to a lesser extent (Fig. 5G), consistent with the reported coregulation of these genes in many tissues (16).

Constitutive activation of Toll signaling (*Toll<sup>IOB</sup>* mutants, no injury) causes a robust increase in *upd3* reporter expression (*SI Appendix, Fig. S4 B and C*), suggesting that the Toll pathway functions upstream of Upd3. ModEncode chromatin immunoprecipitation-sequencing data (69) have identified multiple Dorsal binding regions (*SI Appendix, Fig. S4D*) within the *upd3* enhancer region (67). Importantly, upon injury, *spz*-null mutants do not up-regulate *upd3* or the other *upd* family ligands (Fig. 5G and *SI Appendix, Fig. S4 E–H*).

Involvement of *upd3* suggests a role for JAK/STAT signaling in sterile-injury response. To this end, we monitored the expression of an in vivo reporter that responds to nuclear STAT (*10XSTAT-GFP*) (70), as well as that of the JAK/STAT target genes *mysospheroid* (*mys*) (71) and *chronically inappropriate morphogenesis* (*chinmo*) (72). By 24 hpi, the *10XSTAT-GFP* reporter is elevated relative to cells from uninjured control larvae (Fig. 6 A and B). Likewise, the expression of *Mys* and *Chinmo* are up-regulated in circulating cells by 24 hpi (Fig. 6 D, E, G, and H). Importantly, qPCR assays show that RNA levels for downstream components *mys* and *chinmo* are up-regulated as early as 6 hpi (*SI Appendix, Fig. S4 I and J*). The increase in STAT targets is lost when *dome<sup>DN</sup>*, a dominant-negative version of the Upd3 cytokine receptor, Domeless, is expressed (Fig. 6 C, F, and I). Blocking receptor function in this manner also strongly reduces injury-induced lamellocyte differentiation (Fig. 6 J–M). We conclude that upon sterile injury the cytokine Upd3 is transcriptionally induced by the Toll pathway, and via the activation of JAK/STAT signaling, it promotes lamellocyte differentiation.

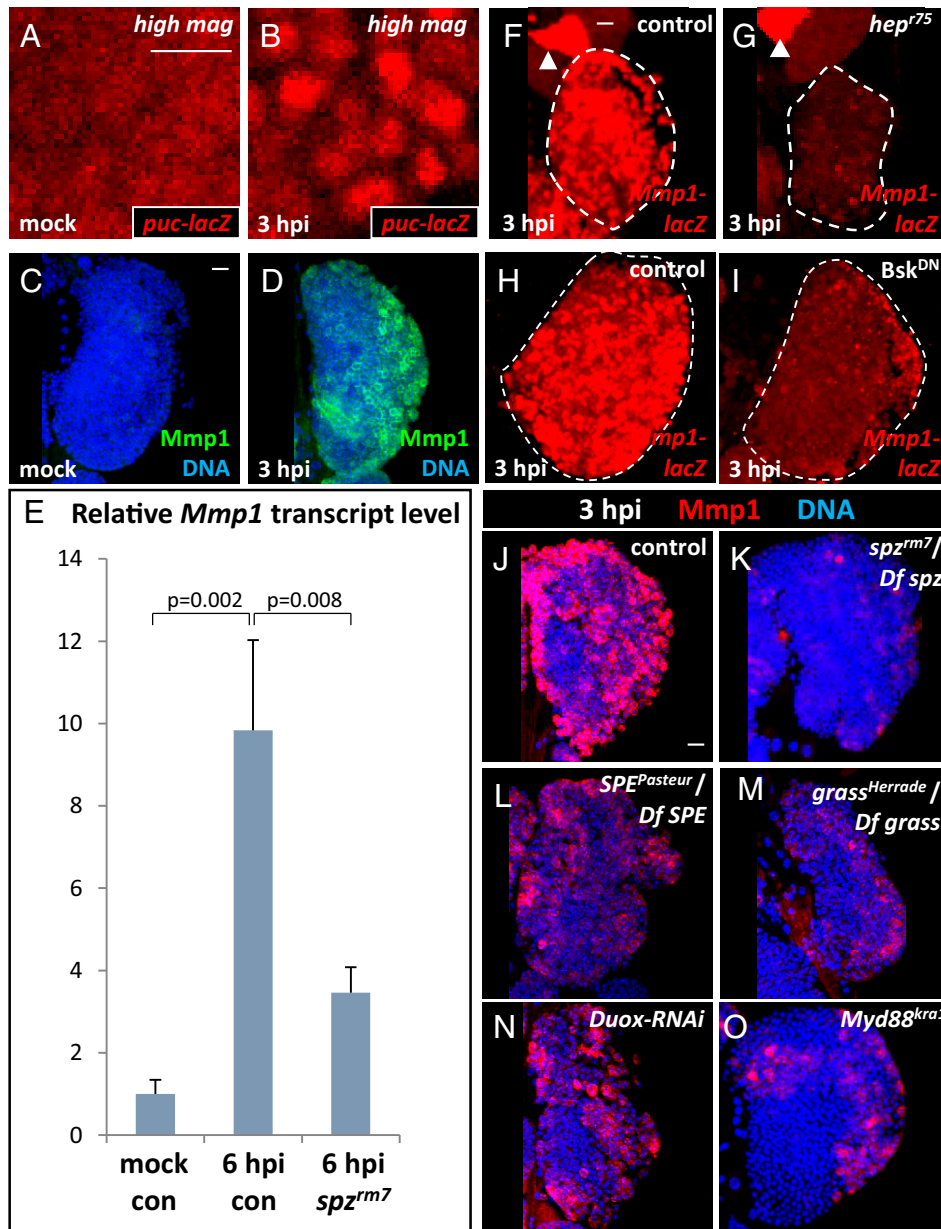
## Discussion

Past studies of the response to mechanical injury in *Drosophila* have largely focused on deciphering the repair and resolution mechanisms associated with the injury site and, in some cases, how blood cells interact with and facilitate these processes (10, 13,

21, 22, 51, 73–75). Only recently have we started to get some understanding of how wounds change the blood cells themselves in the context of the embryo and the adult (15, 28). The majority of hematopoietic events occur in larval stages, and the larval cuticle is the most susceptible to injury. The work presented here is therefore focused on two questions: 1) How do *Drosophila* blood cells in the hematopoietic organ (lymph gland) sense and respond to epidermal injury at a distance? 2) What is the sequence of the signaling pathways within the blood cells that explains the changes they experience upon sterile injury?

The Toll pathway and the events that lead up to the activation of its ligand Spz have been described in significant detail for septic injury associated with microbial infection, but how the hematopoietic system responds to sterile injury was not fully clear. Using axenic culture conditions and sterile injury methods, we show that injury alone can lead to activation of Spz by proteolytic cleavage. This pathway utilizes none of the embryonic dorsal/ventral patterning enzymes that activate Spz. Immunity-related proteases that function close to the Spz-activation cascade (e.g., Grass and SPE) function in the context of sterile injury, but the further upstream pathogen-sensing components, including GGBP1 and GGBP3, and proteases such as ModSP and Psh, which are required during infection, are not involved in the case of sterile injury. In retrospect, this is to be expected since sterile injury does not involve the sensing of an invading particle. We surmise that since both kinds of injuries involve breach of the epidermis, septic injury activates two pathways, one from the epidermis and the other originating from the microbe. The two pathways intersect at the level of proximal proteases, such as Grass, and then follow a common pathway toward Toll activation. The timescales of the two pathways are different, with a fast-priming injury response followed by a delayed, but much larger response if a microbe is detected.

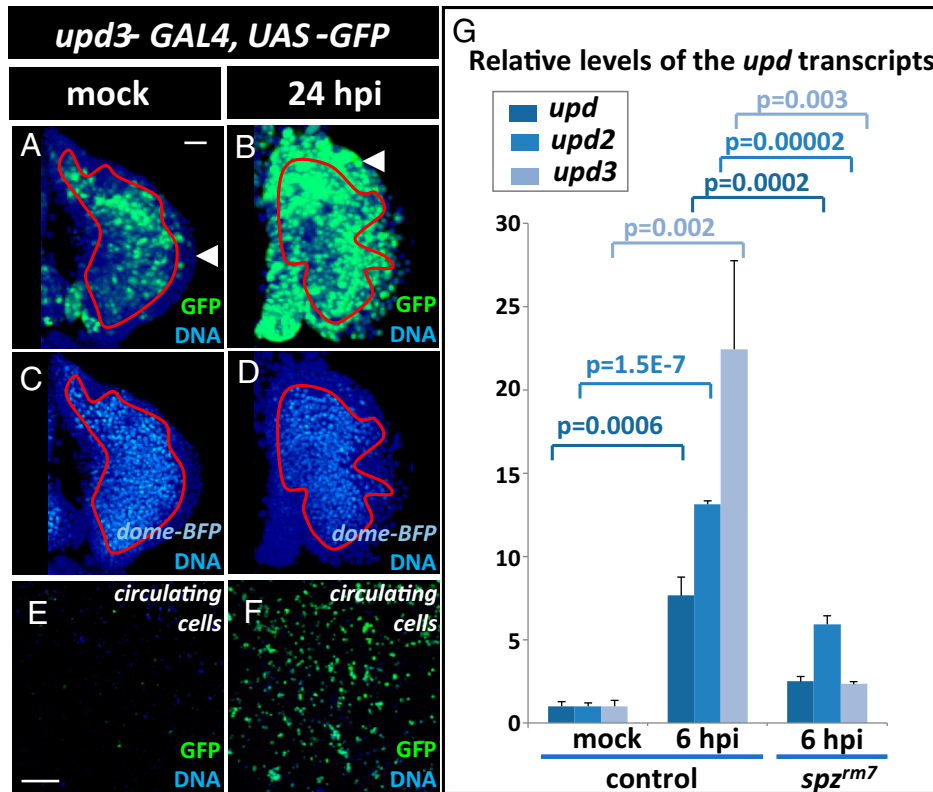
How epidermal injury activates Grass is not yet fully understood, but our data agree with previous observations in the *Drosophila* embryo and in zebrafish that Duox-mediated hydrogen peroxide ( $H_2O_2$ ) produced at the injury site is a critical mediator of this process (15, 49, 53). A key finding of this work is that this initial event dictates the activation of Toll in distant hemocytes, and their precursors in the hematopoietic organ.



**Fig. 4.** Injury rapidly activates JNK signaling in the lymph gland in a Toll- and epidermal Duox- dependent manner. (A and B) Compared with control (A), the JNK signaling reporter *puc-lacZ* (red) is up-regulated upon sterile injury at 3 hpi (B). (Scale bar, 20  $\mu$ m.) (C and D) The JNK pathway target protein Mmp1 is strongly induced by 3 hpi (D) compared with uninjured control (C). DNA (blue). (Scale bar, 20  $\mu$ m.) (E) qRT-PCR results showing that *Mmp1* mRNA transcript levels are robustly induced in circulating cells by 6 hpi, but are much lower in *spz* mutants ( $n = 3$ ). (F-I) Injury-induced up-regulation of *Mmp1-lacZ* (red) (F and H) is suppressed in a *hep<sup>75</sup>* mutant background (G) or by dominant-negative Bsk (*Hml<sup>Δ</sup>-GAL4 UAS-Bsk<sup>DN</sup>*; I), both indicating a suppression of the injury-induced JNK activation. The white arrowheads in F and G show that control *Mmp1-lacZ* expression in ring glands is not affected by the loss of JNK activation. (Scale bar, 20  $\mu$ m.) (J-O) Induction of Mmp1 protein (red) at 3 hpi. DNA (blue). (Scale bar, 20  $\mu$ m.) (J and K) Mmp1 protein is strongly suppressed by a mutation in *spz*. (L-O) As with loss of *spz*, loss of *SPE* (L) or Grass (M) suppresses injury-induced Mmp1 up-regulation. Knockdown of Duox in epidermal cells (*A58-GAL4 UAS-Duox-RNAi*; N) also suppresses injury-induced Mmp1 expression as does a mutation in *Myd88* (*Myd88<sup>kra1</sup>*; O), indicating that Toll pathway-mediated JNK activation in response to injury occurs downstream of the Toll/Myd88 complex.

Another important finding is that larval epidermal injury also causes rapid JNK pathway activation within the hematopoietic system, and that this JNK activity is Toll pathway-dependent. Mammalian TLR signaling also causes JNK activation (76, 77), and RNA-seq results in the context of *Drosophila* injury indicate rapid (by 45 min) up-regulation of the JNK activation signature (28). Nevertheless, previous evidence of such cross-talk in *Drosophila* is relatively scant (78). In the context of infection in adult flies, Boutros et al. (79) demonstrated the up-regulation of JNK pathway target genes, a subset of which were found to be also dependent upon Toll signaling.

Our work indicates that, in the case of injury and specifically in blood cells, Toll signaling activates the JNK pathway in a fairly direct manner. The two pathways are activated at the same time following the injury. Since reporters for both Toll and JNK pathways are expressed in a vast majority of blood cells ( $88 \pm 8\%$  of the cells for Toll targets and  $92 \pm 3\%$  for JNK), the two signals overlap extensively in the hematopoietic cellular compartments. Interestingly, loss of Myd88, a critical component of Toll signaling, eliminates JNK targets, such as MMP1, with an efficiency that parallels their loss in a JNK or JNKK loss-of-function backgrounds. This result parallels



**Fig. 5.** Sterile injury causes a Spz-dependent induction of *upd3* and other cytokine-related genes. (A–F) *upd3-GAL4, UAS-GFP* (A, B, E, F; green); *dome-BFP* (C, D; cyan); DNA (A–F; blue). Lymph gland (A–D) and circulating hemocytes (E and F) are assayed. Medullary zone boundaries (red outlines in A–D) are based on *dome-BFP* expression in the same gland (in C and D). White arrowheads (in A and B) point to the cortical zone. In mock (uninjured) lymph glands (A and C), *upd3-GAL4* is developmentally expressed in the medullary zone. Following sterile injury (B and D), by 24 hpi, *upd3-GAL4 UAS-GFP* expression is up-regulated in both the medullary zone and the cortical zone. Circulating hemocytes from uninjured larvae (E) do not express *upd3-GAL4*, but by 24 hpi (F), this reporter is strongly up-regulated in the circulating hemocytes. (Scale bars, 20  $\mu$ m in A–D and 100  $\mu$ m in E and F.) (G) Induction of endogenous *upd*, *upd2*, and *upd3* mRNAs measured by qRT-PCR. Each is up-regulated by 6 hpi, with *upd3* showing the strongest (>20-fold) increase in its mRNA. The injury-induced expression of genes encoding these cytokines is lost in homozygous *spz<sup>rm7</sup>* mutants.

findings in mammalian systems, where careful biochemical analysis has identified a protein called JIP3 (JNK interacting protein) (76), which functions as a scaffold that enables direct interaction between TLR4 and multiple kinases of the JNK (and MAPK) pathways. In that study, the authors demonstrate the formation of tripartite complexes of TLR4, JIP3, and JNK. This scaffolding protein is evolutionarily conserved including in *Drosophila* (80) and *Caenorhabditis elegans* (81). The *Drosophila* protein is called Sunday Driver (SYD). In future studies it will be interesting to see if this model of cross-activation of JNK by Toll holds up in sterile injury response by manipulating the *syd* gene. It is likely that additional mechanisms also play a role in this process that would link stress and inflammatory responses. For example, the cross-talk could be bidirectional as in the context of a different *Drosophila* tissue (78), the JNK pathway is shown to directly or indirectly regulate the transcription up-regulation of Spz. If such a mechanism were to operate in injury, it will initiate a positive feedback loop for Toll activation.

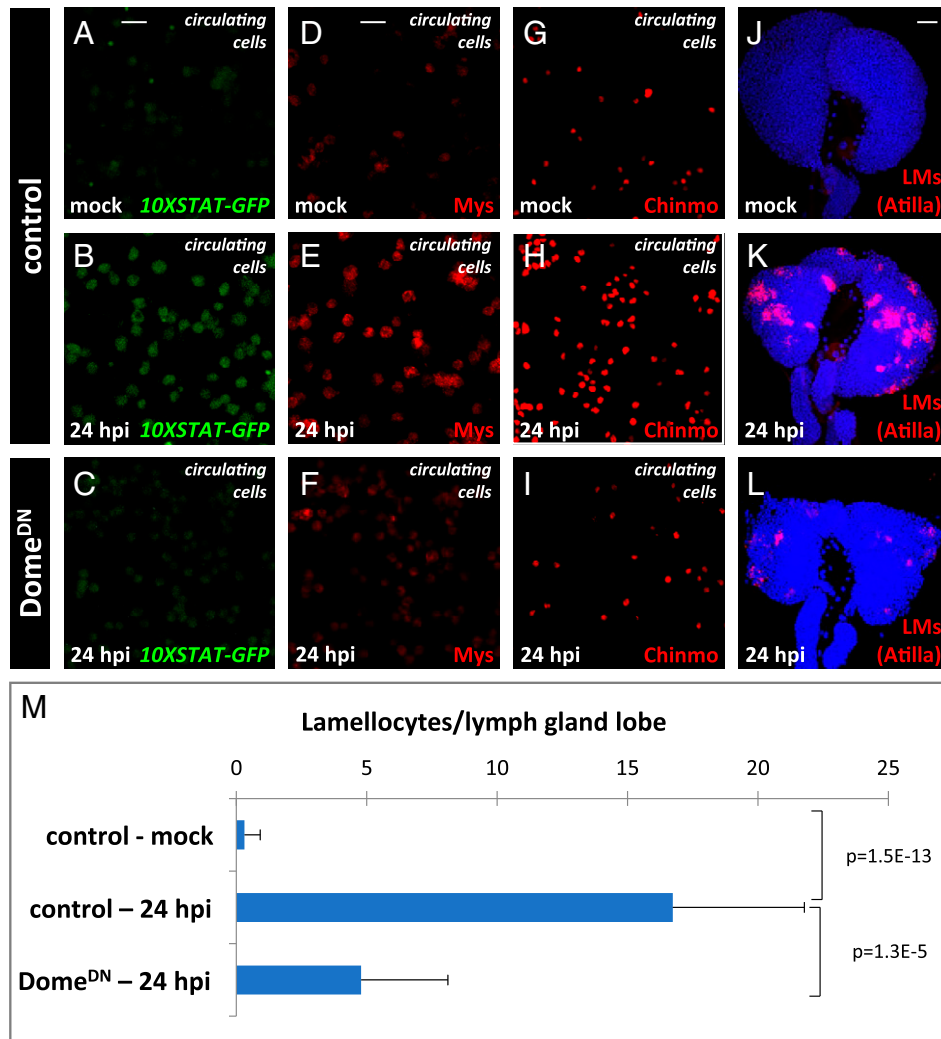
We have also demonstrated that epidermal injury causes the up-regulation of the cytokine-like gene *upd3* in blood cells, and that this occurs along a delayed timeline relative to the other injury-induced markers (6 hpi instead of 3 hpi). The timescale of *upd3* induction in other tissues varies, but is within this range (16, 28). The delay in *Upd3* expression, compared with Toll/JNK activation, fits well with our model that *upd3* is transcriptionally controlled by Toll/Dl, and then functions as a secondary cytokine signal. Importantly, injury-induced and

Toll-dependent *upd3* expression in *Drosophila* hemocytes is very similar to the up-regulation of secondary proinflammatory cytokine genes, such as IL-6 and IL-10, by TLR signaling in mammalian macrophages (82).

Collectively, injury-induced Toll, JNK, and JAK/STAT signaling in hemocytes leads to a number of intrinsic as well as systemic inflammatory responses. Importantly, in a longer time-scale response (24 h), when wound healing has progressed significantly, a new blood cell type arises within the hematopoietic system, expanding on early evidence (27) suggesting that the injury stimulus is sufficient to initiate and mediate lamellocyte formation even in the absence of wasp parasitization. Differentiation of lamellocytes in the context of parasitization and mutant conditions is Toll- and JAK/STAT-dependent (6, 7, 83–85). We show this to hold true for sterile injury.

Why lamellocytes form in the absence of any foreign object to encapsulate is an interesting question for future work, but may be related to the idea that in nature, there is no chance of a truly sterile breach of epidermis. Specifically, breach of the larval epidermis is generally a prequel to the injection of wasp embryos. We hypothesize that the response to injury is anticipatory of wasp parasitization, and would dampen when no such foreign invasion is realized. The injury-induced lamellocytes express common lamellocyte markers (L1/Attila, myospheroid, and  $\alpha$ PS4), they form over approximately similar timescales as mature lamellocytes induced by wasp infestation (57), and they exhibit similar average size reported for mature wasp-induced lamellocytes (56). Therefore, by several measures, injury-





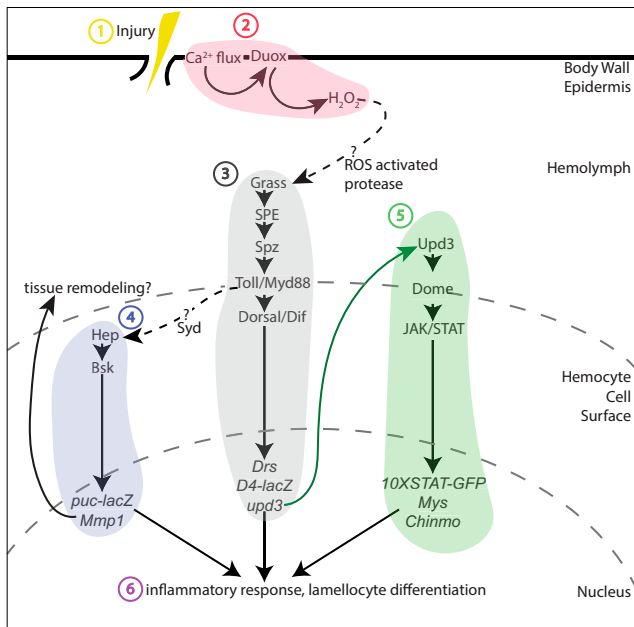
**Fig. 6.** JAK/STAT signaling in blood cells is activated by injury and is required for lamellocyte differentiation. (A–C) The JAK/STAT reporter gene *10xSTAT-GFP* (green) is not expressed in circulating hemocytes of uninjured (A; mock) larvae. It is induced by 24 hpi (B) and suppressed at 24 hpi in larvae expressing a dominant negative form of the receptor *Domeless* (C; *Hml<sup>Δ</sup>-GAL4 UAS-Dome<sup>DN</sup>*). (Scale bar, 20  $\mu$ m.) (D–I) The same results as in A–C are also seen for endogenous JAK/STAT target genes *mys* (D–F, red) and *chinmo* (G–I, red). (Scale bar, 20  $\mu$ m.) (J–M) Blocking the function of the *Dome* receptor (*Hml<sup>Δ</sup>-GAL4 UAS-Dome<sup>DN</sup>*) also suppresses injury-induced lamellocyte differentiation (*Atilla*<sup>+</sup> cells, red) in 24-hpi lymph glands. (Scale bar, 20  $\mu$ m.) Quantitation shown in M.

induced lamellocytes appear to be fully mature. However, functional characterization of these cells to see if they can perform encapsulation is a necessary avenue for future studies.

The pathways discussed in this paper are broadly conserved (16, 18, 22, 44, 76–78, 86). In the proposed model (Fig. 7), we are able to place the events following injury in a temporal sequence based on the order of observed phenotypes. The propagation of the injury-induced calcium wave within the epidermis is essentially instantaneous (53). The next series of events, from calcium-dependent Duox activation and the production of hydrogen peroxide, to activation of Toll pathway and JNK pathway signaling in blood cells, all occur within the first few minutes to hours. The phenotypic effects of these events are observable within 3 hpi. Transcription of *upd3* and its activation of the JAK/STAT pathway require some more time, but these are fully apparent by 6 hpi. The consequences of these signals in mounting a cellular response is seen with full differentiation of lamellocytes within 24 h of the injury.

In addition to the series of linked activation events (Fig. 7), each individual pathway has its own downstream function in this inflammatory response. *Mmp1*, downstream of JNK,

remodels the tissue surrounding the injury site (64, 87). Antimicrobial peptides downstream of Toll anticipate any bacterial challenge (2), and NF- $\kappa$ B has multiple functions in inflammatory response (31). In addition to lamellocyte formation, the *Upd3* cytokine signal likely primes other tissues, such as the fat body, for a possible innate immune response (67). Differentiation of *Drosophila* lamellocytes from macrophage-like cells (i.e., plasmatocytes) or progenitors is reminiscent of the differentiation of specialized macrophage classes in mammals in response to cytokine signaling. An example of this is the transformation of activated macrophages into nonphagocytic epithelioid cells during granuloma formation (88). In the future, it will be important to determine if proinflammatory signaling in *Drosophila* involves prostaglandins and other eicosanoids, which have important roles in mammals (30, 89). The role of inflammatory prostaglandin signaling has not yet been investigated in *Drosophila*, although a COX-like enzyme, called *Pxt*, has been identified (90). Evolutionarily, a response to any breach of the body cavity precedes innate and acquired immune responses. Understanding the molecular events and their sequential function, we hope, will further mammalian studies on injury and healing.



**Fig. 7.** Model of injury-induced inflammatory signaling in the larval blood system. Injury to the larval epidermis (1, in yellow) leads to a wave of calcium signaling (53) that activates epidermal Duox and produces ROS (2, in red) that activates the serine proteases Grass and SPE, which in turn, activate Spz and initiate Toll signaling in blood cells (3, in black). The Toll/Myd88 complex activates Dorsal, translocating it to the nucleus. Dorsal/Dif transcriptionally up-regulates several Toll targets, including the cytokine-like *upd3* gene. Cross-talk between Toll and JNK signaling components JNK (Bsk) and JNKK (Hep) (4, in blue) at the level of the Toll receptor complex is proposed here based on published data on the scaffolding protein JIP3 (SYD) (76). As a result, the expression of JNK targets in the cell, including *puc* and *Mmp1* is Toll/Myd88-dependent. *Mmp1* is a secreted protein that accumulates in circulating and lymph gland blood cells and upon secretion, functions in tissue remodeling, as cells are recruited to the injury site. Spz has been proposed to be a downstream target of JNK (78), allowing the possibility of a positive feedback loop in Toll activation following injury. After a short delay that allows synthesis and secretion of Upd3, this cytokine signals via its receptor, Dome and activates JAK/STAT target genes, including *mys* and *chinmo* (5, in green). Collectively, rapid Toll and JNK signaling, along with secondary JAK/STAT signaling, mediate the proinflammatory response to injury within the blood system and drive the differentiation of lamellocytes (6, in purple).

## Materials and Methods

**Fly Stocks.** The following fly stocks were used in this work: *D4-lacZ* (A. Courey, The University of California, Los Angeles [UCLA], Los Angeles, CA); *Drs-GFP*, *modSP<sup>1</sup>*, *Grass<sup>Herrade</sup>*, *SPE<sup>Pasteur</sup>*, *GNBP1<sup>osinis</sup>*, *GNBP3<sup>Δ40</sup>*, *psh<sup>1</sup>*, *spz<sup>m7</sup>*, *da-gal4*, *Dif<sup>2</sup>* (B. Lemaitre, Ecole Polytechnique Fédérale de Lausanne [EPFL], Lausanne, Switzerland); *Myd88<sup>kra1</sup>* (S. Wasserman, University of California at San Diego [UCSD], San Diego, CA); *Df(2L)J4*, *Df(2L)TW119* (Y. T. Ip, UMass Medical School, Worcester, MA); *ea<sup>4</sup>*, *snk<sup>2</sup>*, *gd<sup>1</sup>*, *ndl<sup>10</sup>* (D. Stein, University of Texas at Austin, Austin, TX); E. Lemosy, Medical College of Georgia, Augusta, GA); *UAS-Duox-IR*, *UAS-Catalase*, *UAS-dome<sup>DN</sup>*, *10xSTAT-GFP* (E. Bach, NYU School of Medicine, New York, NY); *upd3 > GFP* (N. Perrimon, Harvard Medical School, Boston, MA); *Mmp1-lacZ* (D. Bohmann, University of Rochester Medical Center, Rochester, New York); *A58-Gal4* (M. Galko, The University of Texas MD Anderson Cancer Center, Houston, TX); *Hml<sup>A</sup>-GAL4* (S. Sinenko, Institute of Cytology, Russian Academy of Sciences, Moscow, Russia); *UAS-Pxn*, *Toll<sup>10b</sup>*, *puc-lacZ* (U.B.);  $\alpha$ PS4-GFP (VDRC v318086); *Df(3L)ED4743* (*GNBP1*; DrosDel); *Df(3L)ED4413* (*GNBP3*; DrosDel); and each of the following from BDSC: *w<sup>1118</sup>* (stock 5905), *UAS-bsk<sup>DN</sup>*, *hep<sup>75</sup>*, *Df(3R)JI-P* (*spz*, *grass*), *Df(3R)Exel6205* (*spz*), *Df(3R)Exel6195* (*SPE*), *Df(3R)Exel6208* (*grass*), *Df(3R)Exel6270* (*modSP*), *Df(3R)BSC741* (*ea*), *Df(3R)Exel8157* (*snk*), *Df(1)BSC542* (*gd*) (91), and *Df(3L)BSC411* (*ndl*) (71).

**Axenic Cultures and Injuries.** The axenic culture protocol was adapted from Brummel et al. (92). Briefly, a sterile working environment was created by first washing a work hood with 70% ethanol followed by UV irradiation. Standard fly

food was autoclaved and then poured into sterile vials or Petri dishes inside the sterilized hood and allowed to cool before use. *Drosophila* embryos were collected from plates and washed with purified water using standard procedures, and then transferred to a 1.5-mL microfuge tube. Embryos were sterilized using twofold diluted bleach followed by two washes in 70% ethanol and two washes in sterile water. Sterilized embryos were then transferred via sterile pipet to the previously prepared sterile food cultures. Fly cultures were grown at room temperature inside the sterilized work hood until the point of injury, and injured, sterile larvae were returned to the hood for recovery. For injury of axenic larvae, dissection plates were washed in 70% ethanol and UV-irradiated, while forceps, pins, and pin holders were sterilized by autoclave. Water and 1× PBS were sterilized by vacuum filtration into sterile bottles. Cultures were verified as sterile using standard colony forming unit (CFU) assays sampling both cultures and larvae. Briefly, for cultures, 2 mL of sterile water was washed over the surface of the food for approximately 1 min, then 1 mL was retrieved via sterile pipet to a sterile 1.5-mL microfuge tube. The microfuge tube was centrifuged briefly to pellet food debris, and 100  $\mu$ L of supernatant was spread onto LB plates. For larvae, five larvae were collected in sterile water in a 1.5-mL microfuge tube and pulverized using a sterile, disposable micropestle. Larval carcass debris was pelleted by quick centrifugation and 100  $\mu$ L of supernatant was spread onto LB plates. Standard fly cultures of similar developmental age were used as a positive control, while the sterile water vehicle alone served as a negative control. Seeded LB plates were sealed with Parafilm and left at room temperature for 4 to 5 d, by which point microbial colonies could be readily observed on positive control plates.

Wandering third-instar larvae (for 3-hpi experiments) or early third-instar larvae (for 24-hpi experiments) were removed from vials and washed thoroughly with purified water. Larvae were then transferred to a drop of 1× PBS pH 7.4 on a silicone dissection plate (Silgard). Individual larvae were gently stabilized dorsal-side up using forceps while a single puncture injury was carefully made to the lateral body wall at ~75% body length from the anterior. Puncture injuries were made using a sharp minuten pin (Fine Science Tools 26002-15) held in a pin holder (Fine Science Tools 26018-17). Immediately after injury, larvae were transferred to a standard food plate at room temperature for recovery.

**Dissections, Bleeds, Immunofluorescence, and Quantification.** The dissection of larval lymph glands, the collection of circulating blood cells, and their analysis by fluorescence or immunofluorescence was performed using standard procedures, as previously described (93). For lamellocyte counting in lymph glands, dissected samples were either from the  $\alpha$ PS4-GFP reporter line (Fig. 3) or were immunostained for Atilla/L1 expression (Fig. 6), then imaged via fluorescent confocal microscopy. Subsequent z-stack image files were analyzed using ImageJ and the "3D" plug-in in order to more clearly visualize individual lamellocytes. Circulating cells were immunostained for Mys expression (Fig. 3) on glass slides with circular "wells" created by a hydrophobic coating, then imaged using fluorescent confocal microscopy. The 20× objective was focused on the center of each circular well, and the field-of-view was captured as an image. The number of lamellocytes was counted for each image and used for statistical analysis. For immunostaining, the following antibodies were used: mouse anti- $\beta$ -galactosidase (Promega; 1:100), mouse anti-Mmp1 (1:1:1 mixture of DSHB 3A6B4, 3B8D12, and 5H7B11; 1:100), rabbit anti-Chinmo (E. Bach; 1:250), mouse anti-Mys (DSHB CF.6G11; 1:10), mouse anti-L1 (I. Andó, Biological Research Centre, Institute of Genetics, Szeged, Hungary; 1:100), mouse anti-Dorsal (DSHB 7A4; 1:10), mouse anti-Dif (Y. Engstrom, Stockholm University, Stockholm, Sweden; 1:250). Microscope images were quantified using ImageJ and Imaris software.

**Quantitative Real-Time PCR Analysis.** Circulating cells from 10 larvae were isolated in 20  $\mu$ L of 1× PBS for each biological replicate and total RNA was extracted using the PureLink RNA mini kit (Ambion) and quantitated using a spectrophotometer (Implen). The SuperScript III First-Strand synthesis SuperMix kit (Invitrogen) and 150 ng of RNA was used for cDNA synthesis and relative quantitative PCR was performed by comparative  $C_t$  method using Power SYBR Green PCR master mix kit (Applied Biosystems) with a StepOne Real-Time PCR detection thermal cycler (Applied Biosystems). Primers used in this study were either from published literature or designed using Primer3, and the expression level of *RpL32* was used to normalize total cDNA input in each experiment.

Primer sequences (5'-3') are as follows: *Drs*: (forward) CGTGAGAACCTTTCCAA-TATGA, (reverse) TCCAGGACCACAGCAT; *Mmp1*: (forward) GGCAGAGCGGGTGA-GATAG, (reverse) TTCAGTGTTCATAGTCGTAGGC; *upd*: (forward) AACTGGATCGAC-TATCGCAAC, (reverse) CTATGGCCGAGTCTGGCTAC; *upd2*: (forward) CCAGCC AAGGACGAGTTAC, (reverse) GCTGCAGATGCCGTACTC; *upd3*: (forward) ACAAAT GCGCATTCTATAAGG, (reverse) ATGTTCGCGATGCTAGTGAAG; *mys*: (forward) GAT-CACGGTACATGCGAGTG, (reverse) GTACCATGACCGAGCAGAT; *chinmo*: (forward) CAGTGCCAAATGAGGCTAATG, (reverse) TCAAGTCTCCAGTTCACG; *Rpl32*: (forward) GACGCTCAAGGGACAGTATCT, (reverse) AAACCGGTTCTGCATGAG.

**Data Availability.** All study data are included in the main text and *SI Appendix*.

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