An innate immune response of blood cells to tumors and tissue damage in Drosophila

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SUMMARY

Studies in mice and humans have demonstrated a role for the immune system in preventing the growth of tumors. Deciphering the mechanisms involved in the immune response to tumors is essential to our understanding of immune recognition and cancer progression. Here we report an innate immune response to tumors in Drosophila melanogaster. We found that circulating blood cells, termed hemocytes, adhere to tumors upon detection of basement membrane disruption, and subsequently counter their growth. Basement membrane components are remarkably conserved throughout the animal kingdom, providing a unique structure for the immune system to sense tissue integrity. Further, we show that tissue damage activates JNK signaling in both tumors and aseptic wounds, causing expression of JAK/STAT-activating cytokines. Cytokine secretion from the injured tissue is amplified through the induction of additional cytokine expression in the hemocytes and the fat body, resulting in hemocyte proliferation. Our findings reveal common mechanisms in the response to tumors and wounds in flies. A similar innate reaction may underlie the response to tumors and tissue damage in vertebrates and humans.

INTRODUCTION

Evidence in mice and humans supports the once controversial notion that immune responses, in addition to defending the organism against external threats, have a role in preventing the growth of tumors (Dunn et al., 2002). This function of the immune system as an extrinsic tumor suppressor is known as tumor immune surveillance. The interactions between tumors and the immune system of cancer patients, involving both adaptive and innate mechanisms, are complex and not well understood. It is known that the adaptive immune system can react to altered tumor antigens (Boon et al., 1994); however, studies have shown that cells of the innate immune system are also required for effective tumor surveillance (Girardi et al., 2001; Smyth et al., 2000).

The similarities between wound healing and the formation of the tumor stroma led to the prediction that many aspects of tumor biology would be shared between the two processes, and that insights into tumor-host interactions could be gained by thinking of tumors as chronic wounds (Balkwill and Mantovani, 2001; Chang et al., 2005; Dvorak, 1986). It is believed that innate immune sensing of the distress that tumors cause in a tissue is crucial for the initiation of the response (Dunn et al., 2002). The specific mechanisms by which tumors or tissue damage stimulate innate immune cells are largely unknown. Furthermore, the immune recognition of tumors and tissue damage raises the important question, essential for both immunologists and cancer biologists, of how self tissue is targeted by the immune system.

Innate immunity is the most ancient form of immune defense in animals; the origin of many of its mechanisms dates back to a common ancestor of insects and vertebrates in the metazoan lineage (Hoffmann et al., 1999). Insect immunity, despite some evidence for primed responses (Pham et al., 2007), lacks true memory and therefore relies completely on innate mechanisms for protection against external threats. Drosophila melanogaster has proven to be a powerful model for the study of innate immunity, because mammalian immune signaling pathways, such as the Toll, NF-kB and Janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) pathways, are also crucial for the regulation of immune responses in flies (Lemaitre and Hoffmann, 2007).

Insects have a comparatively simple and robust innate immune system that protects the animal against different kinds of pathogens and parasites by orchestrating a number of defensive responses (Brennan and Anderson, 2004; Lemaitre and Hoffmann, 2007). Circulating blood cells, known as hemocytes, are the cellular arm of the fly immune system (Evans et al., 2003; Meister and Lagueux, 2003). Insect hemocytes share many characteristics of their development and function with mammalian blood cells, and probably share a common origin in evolution (Evans et al., 2003; Hartenstein, 2006). As effectors of the fly immune response, hemocytes phagocytose and kill invading microbes in a similar way to vertebrate macrophages, by encapsulating parasites and other foreign bodies, mediating coagulation in open wounds and controlling melanization reactions that release toxic oxygen species. In addition, secretion of JAK/STAT-activating cytokines by hemocytes has been shown to serve a regulatory role in the humoral response to septic injury (Agaisse et al., 2003).

In this study, we have used a Drosophila tumor model to explore the interactions between tumors and the immune system. We report that malignant tumors in Drosophila that are derived from imaginal discs elicit an innate response from hemocytes. Furthermore, we show that common mechanisms underlie the immune response to tumors and tissue damage in flies.

RESULTS

Hemocytes adhere to RasV12/scr+ and scr tumors

Clones of cells with a mutant polarity determinant scribble (scrib), that simultaneously express an oncogenic form of the Ras protein (RasV12/scr–/– clones), generate tumors when induced in the eye-
antennal imaginal discs of Drosophila larvae (Pagliarini and Xu, 2003). This tumor model, previously established in our laboratory, reproduces crucial steps in the progression of human cancers. The tumors dramatically overproliferate, outcompete wild-type cells, and give rise to masses of cells, which degrade the basement membrane (BM) (Pagliarini and Xu, 2003; Srivastava et al., 2007). Our work on the immune response to tumors in Drosophila started following the observation that RasV12/scrib–/– tumors (GFP-positive) display many cells on their surface that do not belong to the tumor (Fig. 1A). These GFP-negative cells are hemocytes, as revealed by their expression of the GATA transcription factor Serpent (Srp), a marker for hemocytes (Lebestky et al., 2000) (Fig. 1A).

Hemocytes are the cellular branch of the fly immune system, and are known to mediate several responses to pathogens. The attachment of hemocytes to the surface of RasV12/scrib–/– and scrib tumors led us to speculate that an immune reaction might be taking place in these larvae. Thus, we decided to look for additional signs of an immune response to the tumors. Most hemocytes in wild-type larvae are found circulating in the hemolymph, a blood-like liquid filling the body cavity, which is set in circulation by the pumping of the heart. We found that the number of circulating hemocytes in RasV12/scrib–/– and scrib larvae were dramatically increased when compared with wild-type larvae (Fig. 2A). Hemocyte counts were also increased in RasV12/scrib–/– and scrib larvae when compared with the morphology-defective edysioseless1 (ecd1) mutant (Garen et al., 1977) (Fig. 2A), which was used as a control for the extended larval development that animals hosting tumors undergo. The proportions of the three hemocyte subtypes remained similar to those of wild-type animals (see Methods section). In addition to scrib tumors, we also analyzed the effect in hemocytes of tumors caused by mutations in two other polarity determinants, lethal giant larvae (lgl) and discs large (dlg). Similar results were obtained in animals with dlg, lgl, RasV12/dlg–/–, and RasV12/lgl–/– tumors, with elevated numbers of circulating hemocytes but no observable changes to subtype proportions (data not shown).

Next, we addressed the question of how circulating hemocyte counts are elevated in RasV12/scrib–/– and scrib larvae. In addition to the circulating hemocytes, a population of undifferentiated hemocytes resides in the lymph glands that, normally, is only released to the hemolymph during metamorphosis (Holz et al., 2003). Early lymph gland emptying in larvae is part of a well-characterized immune response to eggs injected by parasitic wasps (Lemaître and Hoffmann, 2007). In RasV12/scrib–/– or scrib larvae the BM surrounding the lymph gland lobes remains intact, and we did not observe any sign of lymph gland emptying (supplementary material Fig. S2). This rules out an early release of hemocytes as a cause for their greater number in circulation, and suggests that the increase is the result of increased proliferation induced by the tumors. Indeed, anti-phosphohistone 3 (PH3) staining revealed that a significantly higher proportion of circulating hemocytes were undergoing mitosis in larvae hosting scrib and RasV12/scrib–/– tumors (Fig. 2B-E). These data indicate that the presence of tumors stimulates proliferation of hemocytes and that, in addition to the local presence of hemocytes on the surface of the tumor, a systemic reaction is taking place in these larvae.

**Fig. 1. Adhesion of hemocytes to tumors.** (A) GFP-expressing RasV12/scrib–/– tumors (green) in the eye-antennal discs display GFP-negative cells on their surface (yellow square magnified) that express the transcription factor Serpent (Srp), a hemocyte marker (anti-Srp staining, red nuclei). (B) A confocal section of a RasV12/scrib–/– tumor, perpendicular to the surface, shows hemocytes (anti-Srp staining, red) adhered to an area of the tumor where the BM, visualized with a collagen IV GFP protein trap (Vkg-GFP, green), is disrupted. (C) scrib tumors (all cell nuclei stained with DAPI, blue), resulting from fusion of the wing, third leg and haltere imaginal discs, showing hemocytes on their surface (anti-Srp staining, red). These hemocytes are plasmatocytes, as revealed by their expression of the plasmatocyte-specific protein NimC1 (P1 antibody staining, purple).
Hemocytes restrict tumor growth

In humans, the immune system is thought to play a role in preventing the development of cancer (Dunn et al., 2002); at the same time, inflammatory responses triggered by tumors can promote their own development (de Visser et al., 2006). We therefore decided to test whether the immune response we observed had an effect on the tumors. To do this, we compared the sizes of tumors from control scrib larvae with scrib larvae in which hemocyte numbers were reduced by overexpression of the proapoptotic protein Hid (Zhou et al., 1997) (supplementary material Fig. S3). Tumors within the Hid-expressing larvae were significantly larger than in scrib controls (Fig. 3A,B,D). Therefore, the immune response of blood cells does counter the growth of tumors.

Hemocytes react to tissue damage

We tried to dissect the mechanisms by which the innate immune system in flies recognizes the presence of tumors. As noted above, hemocytes were preferentially found in areas of the RasV12/scrib-/- and scrib tumors where the BM of the disc was disrupted (Zhou et al., 1997) (supplementary material Fig. S3). Tumors within the Hid-expressing larvae were significantly larger than in scrib controls (Fig. 3A,B,D). Therefore, the immune response of blood cells does counter the growth of tumors.

In wing discs that were dissected at different times after wounding, hemocytes were shown to adhere to the damaged tissue (Fig. 4A-D). The number of adherent hemocytes peaked 24 hours after wounding (Fig. 4C,E). At 48 hours, BM healing was evident and concurrent with fewer adherent hemocytes (Fig. 4D,E), further suggesting a link between disruption to BMs and hemocyte attachment. Moreover, we observed that, similar to larvae hosting tumors, the number of circulating hemocytes in wounded larvae was significantly increased 24 hours after the operation (Fig. 4F). These experiments show that tissue damage causes a reaction similar to the response observed in tumors. We conclude from the wounding experiments that hemocytes are capable of reacting to tissue damage.
We decided to specifically test the possibility that BM disruption was sufficient to trigger the response from hemocytes. In order to do this, we examined imaginal discs where the BM had been degraded by targeted expression of matrix metalloproteases (MMPs); these enzymes are capable of degrading most extracellular matrix components (Mott and Werb, 2004). Both our group and others have shown that MMPs are involved in BM degradation in RasV12/scrib–/– tumors (Srivastava et al., 2007; Uhlirova and Bohmann, 2006). Expression of Mmp2, one of two MMP proteins present in Drosophila (Llano et al., 2002), which is driven by patched-Gal4 (ptc-Gal4) in a stripe of cells across the wing disc, degrades the underlying BM (Fig. 5A,B). We found that hemocytes adhered to the disc in the region where Mmp2 expression had degraded the BM (Fig. 5A,B). In these same animals, adhesion of hemocytes was also observed in the salivary glands (Fig. 5C,D), where the BM was similarly disrupted, thus showing that hemocyte adhesion to regions of BM disruption was not a phenomenon restricted to imaginal discs. We infer from the above experiments that BM disruption alone is sufficient to cause recruitment of hemocytes.

To ascertain whether BM disruption was also sufficient to trigger the production of a proliferative response in hemocytes, we bled these larvae and measured their hemocyte content. No significant difference was observed when compared with control larvae (Fig. 5E), indicating that although disruption of the BM was enough to produce adhesion of hemocytes, it is not sufficient to induce their proliferation. This suggests that in addition to BM disruption, one or more signals, which are probably common to tumors and wounds, are responsible for the proliferation of hemocytes in response to tissue damage.

**JAK/STAT signaling increases hemocyte numbers in response to tissue damage**

Among the signaling pathways already known to promote hemocyte proliferation (Harrison et al., 1995), we found that scrib larvae exhibit high levels of JAK/STAT activity throughout the third instar (Fig. 6A,B and supplementary material Fig. S4). This was revealed by using a STAT-GFP reporter (Ekas et al., 2006), which expresses GFP under the control of binding sites for the STAT transcription factor. The reporter was highly expressed in the tumors (supplementary material Fig. S5), but was also expressed systemically. Importantly, STAT-GFP reporter activity was detected in the circulating hemocytes of scrib larvae, but not in the
hemocytes of wild-type larvae (Fig. 6C,D). The specificity of the STAT-GFP reporter to detect JAK/STAT signaling in hemocytes was confirmed by overexpression of a dominant negative mutant form of Domeless (DomeΔCYT, deletion of the cytoplasmic domain), a receptor from the JAK/STAT pathway (Brown et al., 2001), driven by He-Gal4. This was effective at inhibiting reporter expression in the hemocytes of scrib larvae (Fig. 6E). Conversely, expression of a constitutively active form of the JAK kinase Hopscotch (HopTum) (Harrison et al., 1995) induced STAT-GFP expression in the hemocytes of wild-type larvae (Fig. 6F). In addition, we examined STAT-GFP activation in the circulating hemocytes of wild-type larvae, 24 hours after performing in situ wounding of their wing discs. In situ wounding results in STAT-GFP reporter expression in circulating hemocytes (Fig. 6G) and DomeΔCYT expression, as seen in scrib larvae, was able to abolish STAT-GFP expression (Fig. 6H).

Once we knew that JAK/STAT signaling was active in hemocytes in response to wounds and tumors, we wanted to investigate the effect of inhibiting the JAK/STAT signaling in these hemocytes. In scrib larvae, DomeΔCYT expression lessened hemocyte expansion caused by the tumors (Fig. 6I). Furthermore, this reduction in the number of circulating hemocytes resulted in significantly larger tumors (Fig. 3C,D). DomeΔCYT expression also prevented the increase in hemocyte numbers observed in wounded larvae (Fig. 6I); whereas, consistent with previous reports (Zettervall et al., 2004), it had no effect on hemocyte numbers in unwounded controls. Together, these data indicate that JAK/STAT signaling is activated in hemocytes in response to wounds and tumors, and that this activity is required for their increased proliferation following these insults.

An amplification loop in JAK/STAT signaling mediates the systemic response to tissue damage

Having established the importance of JAK/STAT signaling in the response to tumors and wounds, we wanted to study how its activity is regulated to achieve a systemic reaction in response to local damage. The JAK/STAT pathway in Drosophila is activated by three closely related cytokines, with homology to human interleukins, encoded by the genes unpaired, unpaired 2 and unpaired 3 (upd, upd2 and upd3) (Agaisse et al., 2003). Therefore, we decided to investigate the expression pattern of these genes in tumors and wounds. We found that expression of upd3, monitored with an upd3-Gal4 reporter (Agaisse et al., 2003), was clearly upregulated in both scrib tumors (Fig. 7A,B) and mechanically wounded discs (Fig. 7C,D). Similarly, upd and upd2 were upregulated (supplementary material Fig. S6), indicating that tissue damage, caused by either tumors or wounds, induces local expression of all three Drosophila JAK/STAT-activating cytokines.

As a possible pathway involved in expression of the Unpaired cytokines in response to tissue damage, we tested the JNK mitogen-activated protein kinase (MAPK) signaling cascade. JNK signaling has been shown to increase locally, in external wounds in the larval epidermis (Ramet et al., 2002) and in cultured imaginal discs wounded ex vivo (Bosch et al., 2005). In our in vivo system of aseptic wounding, we confirmed that JNK activity is upregulated (Fig. 7S and supplementary material Fig. S7), as shown by expression of reporters for the puckered (puc) gene, a JNK-phosphatase that lies downstream of JNK and is a negative regulator of the pathway (Martin-Blanco et al., 1998; Morin et al., 2001; Pastor-Pareja et al., 2004). Further, JNK activity was dramatically increased in scrib tumors (Fig. 7T). We checked expression of upd3 after wounding by using hep75 larvae, which contain a strong hypomorphic mutant in the gene encoding the Drosophila JNK-kinase hemipterous (hep) (Glise et al., 1995). Twenty-four hours after wounding, expression of upd3 is either absent or reduced at the wound site in hep75 mutant discs (Fig. 7E). Also, upd upregulation, monitored with an upd-lacZ reporter (Chao et al., 2004), was prevented when the level of JNK activity was reduced by expression of Puckered (supplementary material Fig. S8). Conversely, expression of a constitutively active form of the JNK-kinase (Hep.CA) induced ectopic expression of the upd-lacZ reporter (supplementary material Fig. S9). These results show that local activation of JNK signaling is required, and sufficient, for expression of Unpaired cytokines in wounds and tumors.

When examining upd3 expression in wounds and scrib tumors, we noticed that upd3 was expressed in the hemocytes and fat body of these larvae (Fig. 7H,J,N,P). Strong upd3 upregulation is detected in wounded wing discs within 6 hours of wounding (Fig. 7C); however, upd3 upregulation was not detected this early in the hemocytes and fat body (Fig. 7I,O), showing that upd3 expression in the damaged tissue precedes its upregulation in the hemocytes and fat body. Since JAK/STAT activity is systemically elevated as a result of wounds or tumors, we decided to examine the possibility that JAK/STAT signaling itself could be activating upd3 expression in the hemocytes and fat body in a positive feedback loop. To test this, we examined upd3 expression in hopTum larvae; this gain-of-function mutant causes abnormally high levels of JAK/STAT activity.
signaling (Harrison et al., 1995). We found that the fat body and hemocytes of hopTum mutant larvae constitutively expressed upd3 (Fig. 7L,R), indicating that activation of JAK/STAT signaling in these two cell types can induce upd3 expression. upd3 ectopic expression in hopTum larvae was not observed in imaginal discs (Fig. 7F) or any other tissues (not shown), suggesting that hemocytes and fat body cells are specifically involved in a positive feedback loop to amplify JAK/STAT activity.

Altogether, the above data support a model in which JNK signaling in damaged tissues activates the expression of JAK/STAT-activating Unpaired cytokines; this local response is subsequently amplified by additional expression of the Unpaired cytokines from the hemocytes and the fat body. Consistent with this model, reduced expression of JAK/STAT-activating cytokines at the local site of damage in hopTS mutants resulted in reduced, or non-existent, upd3 expression in the fat body and circulating hemocytes (Fig. 7K,O). In addition, expression of DomeΔCYT in hemocytes reduces systemic expression of the STAT reporter in larvae hosting scrib tumors (Fig. 7U,V), providing further support for the existence of this positive feedback loop in JAK/STAT signaling. Conversely, expression of HopTum in hemocytes, driven by He-GAL4, causes high systemic activation of the STAT-GFP reporter (Fig. 7W,X).

DISCUSSION
An innate immune response to tumors in Drosophila
We investigated interactions between tumors and the immune system in Drosophila, and found that malignant tumors derived from imaginal discs elicit an innate immune response in their hosts. The fly hemocytes have a central role in this response. In the presence of tumors, circulating hemocytes attach to the tumor surface (Fig. 1), increase in number (Fig. 2) and restrict tumor growth (Fig. 3). Our experiments, which were designed to further dissect the phenomenon, revealed that hemocytes react in a similar way to aseptic wounds in imaginal discs (Fig. 4) and that they attach to tumors and wounds as a consequence of BM disruption (Fig. 5).

Previous studies have suggested that Drosophila hemocytes are able to discriminate intact, self BM from disrupted or non-self BM, and showed that lamellocytes in tu(1)Sz1 mutant larvae encapsulate transplanted damaged tissues, as well as undamaged tissues from sufficiently distant drosophilid species (Rizki and Rizki, 1980). However, tu(1)Sz1 is a mutation that causes a constitutive autoimmune encapsulation response; similar reactions were not observed when transplants were made into wild-type hosts (Rizki and Rizki, 1980), leaving the relevance of these findings to the normal function of the immune system unclear. Migration of hemocytes towards septic wounds in the body wall of Drosophila embryos has previously been reported (Wood et al., 2006). Our experiments show, unambiguously, that the lack of an intact BM causes recruitment of hemocytes to a tissue. Given the vigorous circulation of the hemolymph in larvae, this recruitment of hemocytes probably results from the capture of randomly impacting hemocytes by the damaged tissue, rather than from directed hemocyte migration. In addition, hemocytes are capable of adhesion to undamaged imaginal discs; a population of hemocytes is consistently found under the posterior region of the eye imaginal disc throughout the third larval instar. In very late third-instar larvae/white prepupae, we also noticed occasional adhesion of hemocytes to wing, leg and haltere imaginal discs. The reason for normal hemocyte adhesion is not known.

The structure and components of the BM are remarkably conserved in species ranging from flies to vertebrates (Fessler and
Our work suggests that the BM, which surrounds every organ, has a general role as an indicator of tissue integrity, thus allowing the immune system to sense damage by assessing BM status. However, the proliferation of circulating hemocytes is not stimulated by BM disruption, but is actually a consequence of JAK/STAT activation within the hemocyte (Fig. 6). Consistent with this, MMP-mediated BM degradation failed to induce $upd^3$ expression in the wing disc (supplementary material Fig. S9).

Our data indicate that the initial expression of Unpaired cytokines in the damaged tissue is amplified through a positive feedback loop (Fig. 7) in which JAK/STAT activation in hemocytes and the fat body causes expression of Upd 3, which then increases JAK/STAT signaling. This amplification loop causes systemic activation of JAK/STAT signaling following local damage. Since hemocytes are capable of expressing Upd 3, the resulting increase in the number of hemocytes caused by JAK/STAT activity may act as another positive input in the amplification loop. Although not addressed in this study, there are probably additional effects of systemic JAK/STAT signaling in response to tissue damage. The fat body, given its role as the main source of antimicrobial peptides in the humoral response to infection, could secrete the same products in response to both tissue damage and infection or, it could secrete tissue damage-specific products. In larvae with tumors, we observed an increase in the size of the lymph glands’ secondary lobes (not shown), suggesting that although the lymph glands were not releasing hemocytes, the hemocytes there were reacting to tissue damage by increasing their proliferation rate.

Our results show that hemocyte adhesion and hemocyte proliferation are dependent on different stimuli, namely BM disruption and secretion of JAK/STAT-activating cytokines, respectively. BM degradation by MMPs caused local recruitment of hemocytes, but did not cause an increase in the number of circulating hemocytes (Fig. 5E). Conversely, hemocytes expressing DomeΔCYT were less abundant in circulation, but still able to adhere to tumors (Fig. 3C) and wounds (not shown), suggesting that JAK/STAT activation does not affect their adhesive properties.
Overall, the results support a model in which convergence of the two stimuli determines a fully effective tissue damage response (TDR), increasing the number of available hemocytes and localizing them to the damaged tissue (Fig. 8). In the event of a wound, the activation of an immune response to tissue damage could clearly benefit the animal by priming the immune response in preparation for the entrance of pathogens that would normally follow. Our experiments indicate that the TDR counters tumor growth (Fig. 3), although further experiments are needed to address this. Our previous work, and studies by other research groups, found that BM degradation in tumors, mediated by MMP expression (Srivastava et al., 2007; Uhlirova and Bohmann, 2006), enhances tumor growth. Insect hemocytes, unlike imaginal disc cells, are able to secrete BM components (Fessler and Fessler, 1989), providing a possible mechanism through which the immune reaction to tumors could restrict tumor growth by healing the BM. Hemocytes may also influence the growth of the tumor through phagocytic activity; hemocytes are known to phagocytose apoptotic cells during normal embryonic development (Tepass et al., 1994).

We found that the incidence of apoptosis in the imaginal discs of *scrib* and RasV12/*scrib*−/− flies was not significantly different to that found in wild-type flies (not shown). However, it is known that apoptotic stimuli in immortalized Drosophila imaginal disc cells dramatically promote autonomous and non-autonomous proliferation causing aberrant overgrowths (Ryoo et al., 2004). Therefore, the removal of only a few of these half-dying or ‘undead’ cells could have a big effect in preventing further tumor growth. Additional or alternative mechanisms, involving for instance the production of reactive oxygen species or other more specific signals sent from the hemocytes to the tumor to prevent its growth, remain an interesting possibility for further studies.

**Common mechanisms in the response to tumors and tissue damage**

The data presented here uncovers the existence of an innate mechanism for the detection of, and response to, tissue damage in Drosophila, at work in both tumors and wounds. It has been proposed that the immune system, beyond recognition of the self versus non-self paradigm (Janeway, 1989), may not only respond to foreign or abnormal antigens, but also to so-called ‘danger signals’ (Matzinger, 1994; Matzinger, 2002). According to this model, known as the ‘danger hypothesis’, the immune system would be alerted by endogenous stress signals released from injured tissues. Recent findings seem to confirm the activation of the immune system by this proposed kind of signals (Ogura et al., 2006; Shi et al., 2003).

In species ranging from yeast to vertebrates, JNK signaling has been implicated in the response to many forms of environmental stress, including radiation, osmotic stress, redox stress and nutrient imbalance (Weston and Davis, 2007). In particular, in Drosophila imaginal discs, it has been shown that JNK activation can be induced by loss of cell-cell adhesion (Igaki et al., 2006), and by abrupt discontinuities in positional values (Adachi-Yamada et al., 1999) or proliferation rates (Moreno et al., 2002) across the tissue, resulting in apoptosis. High levels of JNK signaling are observed in the damaged tissue of both aseptic wounds and tumors. JNK signaling subsequently induces the expression of the Unpaired cytokines, thus promoting hemocyte proliferation. Physical damage from a wound naturally indicates a breakage of the BM; however, in tumors, BM degradation requires MMP expression, which is induced by JNK signaling (Srivastava et al., 2007; Uhlirova and Bohmann, 2006), suggesting that JNK activation is sufficient to induce the whole TDR program. Defining the precise molecular mechanisms by which JNK is activated in all these different situations could lead to the identification of common, or different, danger signals and should yield insights into how these seemingly diverse phenomena take place. It could also help to ascertain whether the homology between wounds and tumors extends upstream of JNK activation.

Insects and vertebrates share many innate immune mechanisms (Hoffmann et al., 1999); the response to damaged tissues, or ragged cells such as tumors, is crucial to all multicellular organisms. Spontaneous tumors in wild specimens of all the major invertebrate phyla, including insects, have been widely documented by field naturalists (Harshbarger and Taylor, 1968; Scharrer and Lochhead, 1950), a fact often overlooked in
contemporary discussions of the powers and limitations of lower organisms as cancer models. Given that the main factors involved in the Drosophila TDR, which we have characterized, such as MMPs (Page-McCaw et al., 2003), BM molecules (Fessler and Fessler, 1989), stress sensing through JNK (Stronach and Perrimon, 1999) and cytokine-activated JAK/STAT signaling (Hombria and Brown, 2002), are all conserved in humans, similar TDR mechanisms may exist in humans and other vertebrates. Based on our findings, we believe that Drosophila will provide a powerful system to further the understanding of innate immune reactions to cancers and wounds in humans.

METHODS
Fly strains and culture
The following strains were used: y w;FLP1;AyGAL4,UAS-GFP.S65T;FRT82B,Tub-Gal80; w;UAS-RasV12,FRT82B,scrib–/–;TM6B; y w;FLP1;AyGAL4,UAS-MyRFbvg4854/Cyo;FRT82B,Tub-Gal80; w;FRT82B,scrib–/–;TM6B; w118, ec1° st1° ca1°; w;ub-GAL4.A.K; w;UAS-GFP.S65T; w;vg4854/Cyo; w;He-GAL4.85; w;UAS-myr-GRP;TM6B; w;UAS-myr-GRP (II); wpct-GAL4.4; w;10XSTAT92E-GFP1; w;UAS-dome.Acyt2.1; y w;UAS-hopTum/Cyo; w;udp-lacZ; w;UAS-hid; y w hep25°/FM7a,act-GFP; w;UAS-puc (III); w;UAS-Mmp2 (III); w;pupd3-GAL4;UAS-GFP.S65T/Cyo; y w hep25°/FM7a,act-GFP; y v hopTum–/– BasC; w;puc4862/TM6B; w;UAS-GFP.S65T.puc4862-1–Gal4/TM6B; w;UAS-hep.CA (III).

He-Gal4 was chosen as a driver for hemocyte marking and overexpression. Using He-Gal4 to drive GFP expression under the set conditions of our experiments (heterozygous, 25°C), we did not observe GFP signals in other tissues that could be involved in the immune response, such as the imaginal discs, fat body or larval epidermis. We confirmed this by crossing w;He-Gal4,UAS-GFP flies to a strain bearing a flip-out cassette (actin-FRT+y°-FRT-Gal4) and a UAS-Flipase construct, which amplifies and permanently marks all of the lineage of cells that express Gal4 during development. Additionally, these tissues, unlike hemocytes, were unaffected when expression of Hid was driven with He-Gal4, further supporting the lack of a significant amount of Gal4 expression.

The genotypes of the animals employed in each experiment are detailed in the supplementary material. Whenever staging of the larvae was required, parental flies were transferred to a fresh vial and left there to lay eggs for 1 day; we considered the time of removing the flies from the vial to be 12 hours (±12) AEL (after egg laying). Careful attention was given to avoiding overcrowding in these vials, since this can cause delayed and asynchronous development of the larvae. Cultures were maintained at 25°C, except for ecd1 mutant cultures, which were placed at 29°C (restrictive temperature) for 5 days AEL.

Hemocyte counts
Larvae were washed in water, dried and bled by tearing the larval epidermis with two pairs of forceps into an 11 μl drop of PBS placed over a Sylgard (Dow Corning) plate. To maximize the recovery of hemolymph and circulating cells, the larvae were torn inside out, into the drop. The liquid was mixed and loaded into a hemocytometer for immediate counting. For each sample, the hemocytes in five squares (0.1 mm³ each) were counted and added up. Lamellocyte counts in RasV12/scrib–/– and scrib larvae represented 1-2% of the total number of circulating hemocytes

shown), similar to wild-type and ecd1 animals. Crystal cells in circulation in scrib and RasV12/scrib–/– larvae were very rare, as for wild-type flies, which was consistent with the fact that signs of melanization were not observed in these animals. At least 14 larvae were bled and analyzed for each genotype, time point or wounding experiment. In all cases, the distributions of the measurements were found to fit a normal distribution according to K-S tests. We found it very hard to reliably estimate the amount of hemolymph in a larva, owing to its small volume and the interstitial liquid retained by the larval tissues after bleeding. To measure hemocyte density, we used the number of hemocytes per mg of fresh larval weight. To measure larval weight, we weighed 12 larvae together in a Mettler AE100 microbalance and averaged the figure to obtain the following typical larval weights (mg): wild-type 6 days AEL, 1.51; ecd1 6 days AEL, 1.53, 10 days AEL. 2.13, 14 days AEL, 3.19; scrib 10 days AEL, 2.37, 14 days AEL, 3.46; RasV12/scrib–/– 10 days AEL, 2.28, 14 days AEL, 3.28. We used these weights to normalized the absolute hemocyte counts shown in Fig. 2A.

In situ wounding
Mid and late third-instar larvae were operated on whilst immersed in water in a petri dish that also contained ice to decrease larval movement. In situ wounding was performed by gently holding the larva in position with one pair of forceps while closing a second pair of forceps (Dumont #5 straight tips) over the larval epidermis and the underlying disc. Operated larvae were transferred to a fresh vial and left to develop at 25°C. Most larvae reach pupation around 24 hours after performing the operation. Wounded larvae were able to develop into normal adults that present defects only in the operated wings.

Staining and imaging
Staining tissues with antibody was performed according to the standard procedures used for imaginal discs. The following primary antibodies and dyes were used: rabbit polyclonal anti-Srp (1:200 dilution, Deborah Keiko-Hoshizaki), mouse monoclonal L1 antibody (1:1000, Istvan Ando), mouse monoclonal P1 antibodies and dyes were used: rabbit polyclonal anti-PH3 (Ser10) antibody (1:1000, Upstate). Four different primary antibody (1:1000, Istvan Ando), mouse monoclonal anti-βgal (1:500, Sigma), goat Alexa 635-conjugated anti-mouse IgG (1:200, Molecular Probes), goat Alexa 545-conjugated anti-mouse IgG, goat Alexa 488-conjugated anti-mouse IgG, phalloidin-TxR (1:50, stock solution dissolved in DMSO instead of methanol, Molecular Probes). The samples were mounted in Vectashield or DAPI-Vectashield (Vector Labs). Confocal images were taken in a Zeiss LSM510 Meta confocal microscope.

For anti-PH3 staining of circulating hemocytes, four larvae of every genotype were each bled into 5 μl of PBS on a separate glass slide (12 slides). Hemocytes were allowed to settle and attach to the glass for 10 minutes and then fixed with 4% formaldehyde for an additional 10 minutes. Hemocytes were then stained with rabbit polyclonal anti-PH3 (Ser10) antibody (1:1000, Upstate). Four different 10X micrographs per animal were taken and hemocytes counted (>5000 per genotype).

STAT-GFP expression in living hemocytes was imaged by bleeding larvae into 10 μl of PBS on a slide and placing a coverslip on top of the liquid, after adding and mixing 1 μl of DAPI-Vectashield for immediate imaging. The same results were obtained using fixed hemocytes.
Living larvae were immobilized by the tension created by water between a slide and a coverslip, and imaged in a Leica MZ FLIII fluorescence stereomicroscope with a Leica DFC300FX camera.

Tumor size measurement

Tumors were mounted in DAPI-Vectashield and confocal micrographs were taken of the DAPI signal at sample mid-depth. The area occupied by the tumor was measured with the program ImageJ (NIH). Measurement distributions passed K-S tests for normality.

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COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

J.C.P.-P. designed research. J.C.P.-P. and M.W. performed research. J.C.P.-P. and T.X. wrote the manuscript.

SUPPLEMENTAL MATERIAL

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REFERENCES


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