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Letter to the Editor

Natural infection of *D. melanogaster* by virulent parasitic wasps induces apoptotic depletion of hematopoietic precursors

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Dear Editor,

Insects, like humans, are endangered by infectious microbial pathogens and parasites, and while their immune systems are much simpler, they successfully defend themselves against natural pathogens via innate humoral (antimicrobial genes) or cellular immune responses.[1] In turn, pathogens have evolved virulence mechanisms and other strategies to resist insect immunity, although the underlying mechanisms behind immune suppression are not well understood.[2] Using the model insect system *Drosophila melanogaster* and its virulent parasitic wasps, *Leptopilina heterotoma* and *L. victoriae*, we provide evidence for a novel mechanism of immune suppression in which infection by wasps specifically induces the removal of hematopoietic precursors within the *Drosophila* host by apoptosis. This report provides the first evidence for such a mechanism for any invertebrate host/parasite system.

When larvae of *Drosophila* are invaded by naturally-occurring parasitic wasps, the humoral immune response genes remain quiescent. Instead, the cellular 'arm' of the innate immune system becomes activated. While most species of naturally-occurring parasitic wasps of *Drosophila* are virulent and carry immune-suppressive factors, at least one avirulent strain *Leptopilina boulardi*, strain G486, has been identified.[3] Infection by this avirulent parasitic wasp leads first to the proliferation and differentiation of blood cell precursors in the hematopoietic organ, the lymph gland[4,5] (Figure 1B), and then to encapsulation of the wasp egg. The three mature blood cells types are plasmatocytes, lamellocytes and crystal cells. Phagocytic plasmatocytes are most abundant (more than 90% of all cells in circulation), both before and after wasp infection. Crystal cells, present in small numbers prior to and after infection (less than 5%), mediate melanization reactions. Lamellocytes are large, flat and adhesive, and appear in large numbers specifically after parasite infection. Plasmatocytes, and the newly-differentiated lamellocytes, rapidly aggregate around parasite eggs to form a cellular capsule, while crystal cells lyse, releasing substances that cause melanization of the capsule.[1,4,5] Successful encapsulation of eggs of *L. boulardi* G486, therefore, involves activation of immune cells within the lymph gland.

Parasitic wasps of *Drosophila* that are highly virulent have adopted active or passive means of avoiding the egg encapsulation process. Previous work on *L. heterotoma* has shown that females of this species produce virus-like particles (VLPs) within the fluid of the long gland, an accessory organ. During oviposition, these wasps deposit VLPs into the larval hemolymph along with their eggs. VLPs induce lamellocytes to become bipolar in shape, lose cytoplasm from their ends, and eventually lyse[6,7] (Figure 1A). Destruction of lamellocytes reduces the host's immune capacity, allowing the wasp egg to develop unhindered.

In earlier experiments with virulent strains of parasitic wasps, including *L. heterotoma*, we reported that parasitic wasp infection also correlates with the absence of the lymph gland lobes.[8] This observation suggested that, in addition to destroying the lamellocyte population, infection of the *Drosophila* host by virulent parasitic wasps might involve the specific removal of hematopoietic precursors by apoptosis. To test this idea, we studied the effect of infection by *L. heterotoma* and its closely-related species *L. victoriae*,[9] on the morphology of host lymph glands.

Lymph glands dissected from control and wasp-infected wild-type *Drosophila* hosts were subjected to TUNEL staining (*in situ* terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling technique; Figure 1B-D). Two to three days after infection by *L. heterotoma*, most of the lobes of the larval lymph glands are absent. When the lobes were present, they were found to be TUNEL-positive (n=16; Figure 1C). The effect of *L. victoriae* infection on lymph gland apoptosis is similar to that of *L. heterotoma* infection (Figure 1D), although, it is slightly delayed. Two days after infection, glands from eight animals infected by *L. victoriae* remain intact and TUNEL-negative (Figure 1D, top). However, 3–4 days after infection, all five glands dissected from *L. victoriae*-infected hosts are TUNEL-positive or have lost lobe cells (Figure 1D, bottom). Lymph glands from uninfected larvae (Figure 1B), and larval brain, fat body, and pericardial cells from infected or uninfected larvae remain negative for TUNEL staining (not shown). Finally, under the same conditions of infection and time points of observation, lymph glands from the avirulent strain of *L. boulardi* strain G486 do not show TUNEL-positive staining or loss of the lymph gland lobes (not shown). Clearly, hematopoietic precursors in the host are targeted specifically for destruction by parasitic wasps *L. heterotoma* and *L. victoriae*.

The above results identify a novel strategy that virulent parasitic wasps use to counteract host immunity: By specifically deleting a significant proportion of hematopoietic precursors that would otherwise be directly engaged in the immune response of encapsulation, the wasps ensure the immediate protection of their progeny without compro-

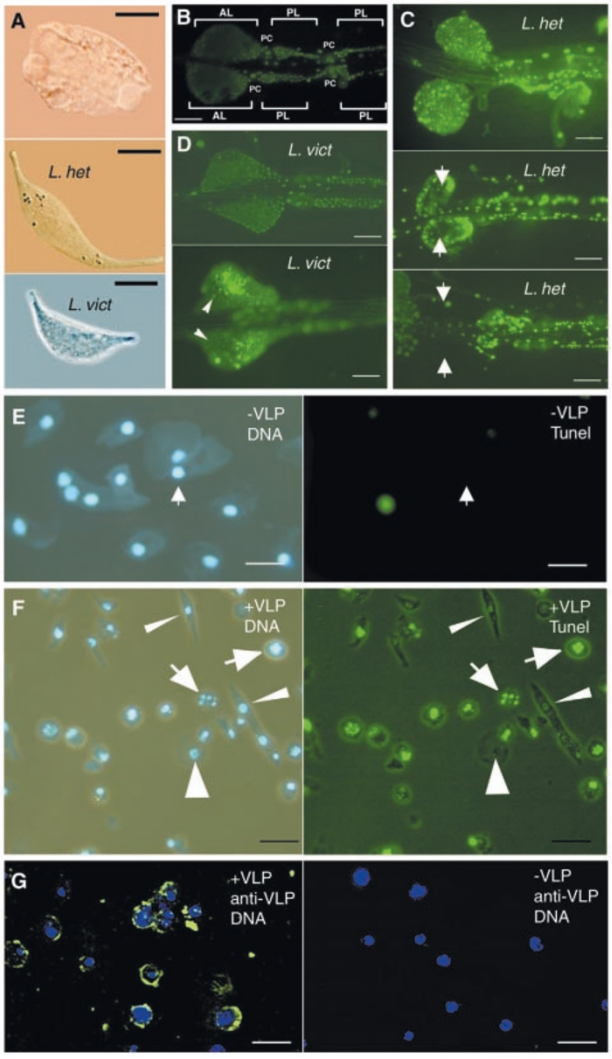

Figure 1 (**A**) VLP treatment promotes bipolar morphology of lamellocytes followed by their lysis. Top, lamellocyte from *hop*$^{Tum\text{-}l}$/Y larva, 7 h after incubation in 10% fetal bovine serum. Middle, bipolar lamellocyte in the same medium, 5 h after addition of *L. heterotoma* VLPs and (bottom), 7 h after addition of *L. victoriae* VLPs. Scale bar, 25 μm. (**B–D**) Lymph glands from wild-type control and infected hosts, prepared for visualizing TUNEL assay (Molecular Probes kit, catalog # 1684795). (**B**) Uninfected control; anterior-most lobe pair (anterior lobe, AL) is the largest. Remaining posterior lobes (PLs) are smaller; pericardial cells (PC) intercalate these posterior lobes. The lymph gland continues posteriorly with the pulsating dorsal vessel, which directs the flow of the hemolymph and blood cells into the body cavity. The uninfected control is largely negative for TUNEL. When hosts are infected with *L. heterotoma* (**C**), 2 days after infection, overall TUNEL staining is strong (top). Three days after infection, either some AL cells are missing (**C**, middle panel, arrows), or entire ALs are missing (**C**, bottom panel, arrows). Lymph glands of hosts infected with *L. victoriae*, 2 days after infection, are TUNEL-negative (**D**, top panel). However, 3 days after infection, loss of cells within strongly TUNEL-positive ALs, is observed (**D**, bottom panel, arrowheads). Scale bars for panels **B–D**, 100 μm. (**E–G**) TUNEL (**E, F**) or VLP staining (**G**) of *hop*$^{Tum\text{-}l}$ *blood cells treated with L. heterotoma* fluid. (**E, F**). TUNEL staining of blood cells incubated with *L. heterotoma* fluid containing VLPs show strong TUNEL positivity and have fragmented nuclear material (arrows, panel **F**). In contrast, very few blood cells when treated with buffer alone are TUNEL-positive (E). Cells in panels E and F are counterstained with fluorescent DNA dye Hoechst 33258. Some lamellocytes with bipolar morphology show very weak TUNEL-positive staining (narrow arrowheads). Plasmatocytes negative for TUNEL reaction are indicated by a triangle. Scale bars, 25 μm. (**G**) Confocal images depicting the association of VLP antigen with plasmatocytes from *hop*$^{Tum\text{-}l}$ cells incubated with VLP protein-specific antibody (left, green) and negative control (right). Cells are counterstained with TOTO-3 (blue, Molecular Probes). All samples were mounted in 50% glycerol containing 2.5% DABCO. Scale bars, 25 μm

mising the viability of the host. We do not yet know if this effect of infection on *Drosophila* hematopoiesis is reversible, but for the brief period of host development between the second and the early third larval instars when the host is immune-competent, the selective deletion of hematopoietic precursors appears to ensure the developmental success of the parasite.

To examine if the VLP-containing wasp fluid synthesized in the long gland can also induce apoptosis of circulating blood cells *in vitro*, we examined blood cells from *hop*$^{Tum\text{-}l}$ larvae, 17 h after exposure to *L. heterotoma* fluid. (Due to a single point mutation, *hopscotch*$^{Tumorous\text{-}lethal}$, *hopTum-l* http://flybase.bio.indiana.edu/ larvae have an overabundance of plasmatocytes and lamellocytes and are a convenient source of blood cells). Whereas only 5.2% (10/193) of all cells treated with buffer alone are TUNEL-positive (Figure 1E), more than 30% (66/195) of the cells treated with buffer-diluted wasp fluid (fluid contains VLPs) are TUNEL-positive ($P<0.01$; Z-test; Figure 1F). The overwhelming majority of these TUNEL-positive cells (60/66; 91%) are plasmatocytes. Cell death in TUNEL-positive plasmatocytes was confirmed by the visualization of condensed and fragmented nuclear material with nuclear dye Hoechst 33258 (Figure 1F, arrows). Few, if any, lamellocytes undergoing bipolar transformation are TUNEL-positive and cells that do show staining are only weakly positive (Figure 1F, narrow arrowheads). This observation suggests that lamellocyte lysis is mediated by molecular mechanisms distinct from those that govern the apoptosis of plasmatocytes.

To determine if *L. heterotoma* VLPs can be found within the fluid-treated plasmatocytes, we stained isolated cells with a VLP-specific antibody (Chiu and Govind, unpublished). Confocal micrographs in Figure 1G clearly reveal the presence of VLP proteins in plasmatocytes treated with *L. heterotoma* fluid under experimental conditions similar to those in the TUNEL experiment. As expected, this antibody also revealed the incorporation of VLPs in lamellocytes (Chiu and Govind, unpublished). The observation that VLP proteins are present in plasmatocytes undergoing apoptosis supports the idea that reduction in the number of mature plasmatocytes *in vitro* might be due, in part, to their uptake of *L. heterotoma* VLPs. There is precedence for virus-induced apoptosis in both vertebrate and invertebrate cells. Adenovirus, papillomavirus and human immunodeficiency virus type 1 actively promote apoptosis of mammalian cells.[10] Apoptosis in insect host blood cells has also been reported: polydnaviruses from the braconid wasp *Microplitis demolitor* (MdPDV) induce the apoptosis of granular cells of its larval host *Pseudoplusia inculdens*.[11]

Hosts and their parasites have co-evolved for millions of years. Studying naturally-existing hosts and their pathogens is likely to reveal well-conserved molecular mechanisms and strategies that provide the specificity in host/parasite

interactions. Because of the wealth of genetic tools and genomic information available for *Drosophila*,[12,13] *Drosophila* and its parasitic wasps provide a powerful experimental system in which molecular mechanisms regulating host/parasite interactions can be defined. Identification of the mechanisms that trigger and mediate the depletion of immature and mature immune cells in *Drosophila* will yield fundamental insights into our understanding of the function, regulation and evolution of host immunity and parasite resistance.

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