

Salmonella pathogenesis reveals that BMP signaling regulates blood cell homeostasis and immune responses in *Drosophila*

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Inter cellular signaling by bone morphogenetic proteins (BMPs) regulates developmental decisions in virtually all animals. Here, we report that Decapentaplegic (Dpp; a *Drosophila* BMP family member) plays a role in blood cell homeostasis and immune responses by regulating a transcription factor cascade. The cascade begins with Dpp repression of Zfh1, continues with Zfh1 activation of Serpent (Srp; a GATA factor), and terminates with Srp activation of U-shaped (Ush) in hematopoietic cells. Hyperactivation of Zfh1, Srp, and Ush in *dpp* mutants leads to hyperplasia of plasmatocytes. *Salmonella* challenge revealed that in *dpp* mutants the misregulation of this cascade also prevents the generation of lamellocytes. These findings support the hypothesis that Ush participates in a switch between plasmatocyte and lamellocyte fate in a common precursor and further suggests a mechanism for how all blood cell types can arise from a single progenitor. These results also demonstrate that combining *Drosophila* and *Salmonella* genetics can provide novel opportunities for advancing our knowledge of hematopoiesis and innate immunity.

Serpent | GATA | plasmatocyte | lamellocyte | Decapentaplegic

Signaling molecules of the Decapentaplegic (Dpp)–bone morphogenetic protein (BMP) family are important for embryonic development and adult homeostasis in many species. In this family *Drosophila* Dpp shows greatest amino acid similarity to mammalian BMP2/4, and cross-species functional conservation has been observed for these proteins (1). Conservation between flies and mammals also exists for mechanisms that define the cardiogenic mesoderm during early embryonic development. In both organisms, Dpp/BMP proteins induce the expression of Nkx transcription factors via conserved cardiac enhancers (2). Recent studies in flies and mammals suggest that Dpp/BMPs signal to the cardiac field a second time late in development to restrict heart cell proliferation. For example, loss of late-stage Dpp signaling in *dpp*^{Δ6} mutant embryos causes pericardial cell hyperplasia that can be rescued by overexpression of Dpp (3).

The *Drosophila* lymph gland (LG) surrounds the anterior-most region of the heart and is composed largely of pericardial cells derived from the cardiogenic mesoderm. Definitive hematopoiesis takes place in the LG, producing three types of cells. Plasmatocytes (normally 95% of blood cells) are phagocytic cells, whereas crystal cells (normally 5% of blood cells) are nonphagocytic cells that participate in wound healing. Lamellocytes are large cells generated during organismal defense that encapsulate objects too bulky for plasmatocyte phagocytosis (4). Conserved genes that participate in hematopoiesis in flies and mammals include the GATA family transcription factor Serpent (Srp) and U-shaped (Ush). Several GATA proteins regulate key steps in mammalian hematopoiesis, often via interactions with FOG proteins (the Friend of GATA family includes Ush from *Drosophila*; refs. 5 and 6).

In the LG, Srp is expressed in all hematopoietic cell types (precursor cells, prohemocytes, and mature blood cells). In all prohemocytes Srp activates the transcription factor Ush. In a

subset of prohemocytes, signals from the Notch pathway synergize with Srp to activate the transcription factor Lozenge. The activation of Lozenge leads to suppression of Ush and facilitates crystal cell formation. In the absence of Notch signaling, Srp forms a complex with Ush that represses crystal cell formation and facilitates plasmatocyte formation. Ush expression is seen throughout the remainder of plasmatocyte development. Thus, under normal conditions prohemocytes give rise to crystal cells and plasmatocytes (4). Lamellocytes are thought to arise from a plasmatocyte or a common precursor of plasmatocytes and lamellocytes. Lamellocytes form when a balance between Ush and the Janus kinase Hopscotch, normally favoring Ush and plasmatocyte formation, is shifted toward Hopscotch (7).

Here, we describe studies of hematopoietic cells in *dpp* mutants. These studies were conducted by using a noninvasive method for infecting *Drosophila* larvae with *Salmonella*. The data suggest that Dpp dorsal ectoderm to mesoderm signaling late in embryogenesis controls blood cell homeostasis by regulating a transcription factor cascade. The cascade includes Zfh1, Srp, and Ush, and the misregulation of this cascade leads to hyperplasia of plasmatocytes. Further, *Salmonella typhimurium* challenge revealed that this aspect of Dpp signaling also regulates immune responses, as *dpp* mutants cannot generate lamellocytes in response to infection.

Results

Recently, we reported that late in embryonic development, Dpp dorsal ectoderm to mesoderm signaling restricts the number of pericardial cells expressing the transcription factors Zfh1, Odd-skipped (Odd), and Tinman in the heart (3). Reexamining data from this study revealed a significant excess of Zfh1- and Odd-expressing cells in the LG of *dpp*^{Δ6} embryos. The reexamination also showed that in the heart both Odd and Tinman are downstream of Dpp via Zfh1 but in the LG only Odd is downstream. To determine whether overgrowth of Odd-expressing LG cells effected hematopoiesis we examined *dpp*^{Δ6} mutant embryos and larvae in more detail.

***dpp* Mutants Have Excess Srp and Ush but Not Lozenge and Antennapedia.** We began by examining the embryonic expression of Srp, a marker for all hematopoietic cells of the LG. Consistent with previous reports, we found that Srp expression in the LG

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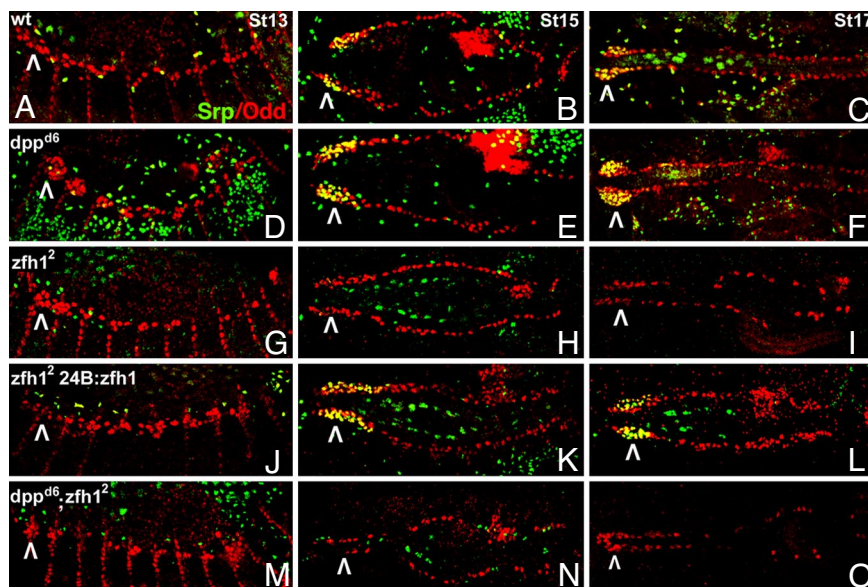


Fig. 1. *zfh1* mutants do not express Srp in the embryonic LG, and *dpp* mutants have an excess of Srp-expressing LG cells. Embryos are labeled for Srp (green) and Odd (red). Anterior is to the left, and the LG is indicated by an arrowhead. Stage-13 embryos are shown in lateral view. Stage-15 and -17 embryos are shown in dorsal view. The large red object in a subset of embryos is part of the digestive system. (A–C) WT. (A) Odd is expressed in pericardial cells including in the LG and in stripes in the ectoderm. No Srp is visible in pericardial cells but fat body expression is visible. (B) Srp is seen in a subset of Odd LG cells (yellow) and in fat body. (C) Srp persists in Odd-expressing cells (yellow) and fat body. (D–F) *dpp*^{d6}. (D) The number of Odd pericardial cells in the LG is slightly increased but no Srp is visible. (E) The number of Odd- and Srp-expressing LG cells (yellow) is clearly greater than in WT. (F) The overgrowth of Srp- and Odd-coexpressing LG cells (yellow) persists. (G–I) *zfh1*². (G) The number of Odd LG cells is roughly the same as for WT. No Srp is visible. (H) The number of Odd LG cells is slightly lower than in WT and no Srp is visible. (I) The number of Odd cells is significantly lower than in WT and no Srp is visible. (J–L) 24B:Gal4:UAS:Zfh1; *zfh1*². Pan-mesodermal Zfh1 expression in *zfh1*² mutants rescues Srp expression in LG cells. (K and L) The number of Srp cells is roughly the same as in *dpp*^{d6}. (M–O) *dpp*^{d6}; *zfh1*². (M) The number of Odd LG cells is roughly the same as for WT. No Srp is visible. (N) The number of Odd LG cells is slightly lower than in WT, and no Srp is visible, like *zfh1*². (O) The number of Odd cells is significantly lower than in WT, and no Srp is visible, like *zfh1*². (Magnifications: $\times 400$.)

does not initiate before stage 15 (Fig. 1). Subsequently, at the earliest stage of Srp expression there is already a greater number of Srp-expressing cells in the LG of *dpp* mutants than in WT (Fig. 1 *B* and *E*). *dpp* mutant embryos continue to have an excess of Srp-expressing cells in the LG through stage 17 (Fig. 1 *C* and *F*). This overgrowth phenotype is similar to the response of Zfh1- and Odd-expressing LG pericardial cells (3). Also in both genotypes all Srp pericardial cells coexpress Odd (Fig. 1 *B*, *C*, *E*, and *F*, yellow double-labeled cells). We conclude that Dpp regulates Srp as it does Zfh1 and Odd in the LG; late-stage Dpp signals limit the number of Srp-expressing cells.

We tested whether Dpp restricts the number of Srp-expressing cells in the LG expression via the same mechanism for restricting Odd, by restricting Zfh1. When we assayed for Srp in *zfh1* null mutant embryos we saw no Srp LG expression at any stage (Fig. 1 *G–I*). These data indicate that *zfh1* is required for Srp expression and corresponds with reduced Odd expression in *zfh1* mutants. This result is specific because the loss of Srp-expressing cells in *zfh1* mutants can be rescued by overexpressing Zfh1 by using the pan-mesodermal driver 24B:Gal4 (Fig. 1 *J–L*). To determine whether *zfh1* expression is epistatic to Dpp in the specification of Srp-expressing cells we examined Srp expression in *dpp*^{d6}; *zfh1*² embryos. We observed no Srp-expressing LG cells at any stage, a phenocopy of *zfh1* single mutants (Fig. 1 *M–O*). This result indicates that *zfh1* is downstream of *dpp* in regulating Srp expression and Dpp limits Srp expression in the LG by restricting Zfh1.

We examined Srp and Odd LG expression in WT and *dpp* mutant third-instar larvae. We were able to compare gene expression in WT and *dpp* mutant larvae because *dpp*^{d6} homozygous individuals survive to adulthood. As seen in the embryo, Srp and Odd were expressed in nearly all LG cells in WT. The hypertrophic LG of *dpp* mutants also expressed Srp and Odd in

all cells, suggesting that the overabundance of Srp- and Odd-expressing cells in *dpp* mutant embryos persist into the third instar (Fig. 2 *A* and *B*).

Srp directly activates Ush to repress crystal cell fate and facilitate plasmatocyte development (8). Ush is expressed in nearly all Srp-expressing LG cells in WT, in crystal cell precursors before being repressed, and in plasmatocyte precursors that will become 95% of circulating cells. The overgrown LG of *dpp* mutant larvae also express Ush in nearly all cells. This result suggests that in *dpp* mutants Srp functions as in WT and activates Ush, leading to an overabundance of Ush-expressing cells (Fig. 2 *C* and *D*).

Srp directly activates Lozenge to stimulate crystal cell formation (9). Examination of a Lozenge reporter marking crystal cell precursors revealed no significant difference in the number of Lozenge-expressing cells between WT and *dpp* mutants ($n = 6$, $P > 0.29$; Fig. 2 *E* and *F*). Srp overexpression leads to excess Ush but not Lozenge in *dpp* mutants because Srp only activates Lozenge in the presence of Notch signaling. As Notch signaling should be unaffected in *dpp* mutants the number of Srp-expressing cells receiving Notch signals where Lozenge would be activated is normal.

The effect of embryonic Dpp signaling on Srp and Odd expression in third-instar larvae suggested that *dpp* mutants might contain an overabundance of hematopoietic niche cells. Niche cell precursors in the cardiogenic mesoderm are specified independently of Dpp by Antennapedia but they coalesce with Srp- and Odd-expressing cells at roughly the same time that Dpp signaling effects Srp and Odd (10). Thus, Dpp could impact the coalescence process or subsequent niche cell development. However, examination of Antennapedia in a large number of third-instar LG did not identify any meaningful difference in the

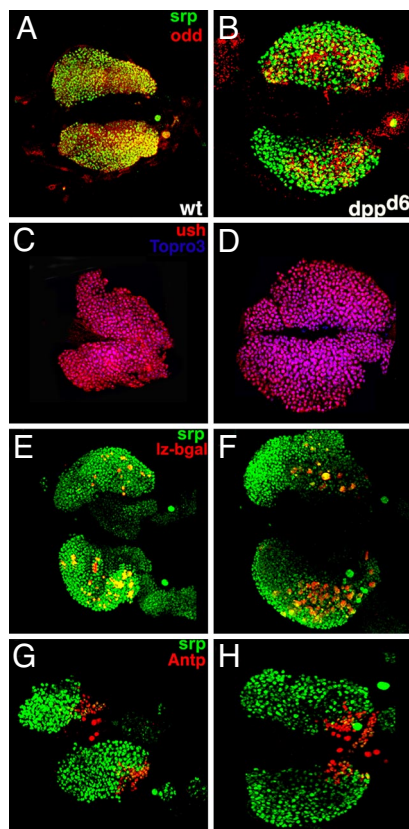


Fig. 2. *dpp* mutant larvae have enlarged LG with an excess of Srp- and Ush-expressing cells. (A and B) Dissected primary lobes of LG from WT and *dpp* mutant third-instar larvae labeled for Srp (green) and Odd (red). Srp is a marker for all hematopoietic cells. The enlarged LG of *dpp*^{d6} larvae displays an overabundance of Srp- and Odd-coexpressing cells. (C and D) LG labeled for Topro3 (blue) and Ush (red). Ush is a marker for prohemocytes and plasmatocytes. The enlarged LG of *dpp*^{d6} larvae displays an overabundance of Ush-expressing cells. (E and F) LG labeled for Srp (green) and a Lozenge reporter gene (red). Lozenge is a marker for crystal cells. Cell counts revealed that there is no meaningful difference in the number of Lozenge-expressing cells between WT and *dpp* mutants. (G and H) LG labeled for Srp (green) and Antennapedia (red). Antennapedia is a marker for cells of the hematopoietic stem cell niche. In examining many LGs we could discern no meaningful difference in the distribution or number of Antennapedia cells between WT and *dpp* mutants. (Magnifications: $\times 400$.)

number or distribution of Antennapedia-expressing cells between WT and *dpp* mutants (Fig. 2 G and H).

Salmonella Colonizes the Gut and Infects Circulating Blood Cells of Third-Instar Larvae. Would the overabundance of cells expressing the plasmatocyte markers Srp and Ush lead *dpp* mutants to have abnormal blood cell profiles? Further, as blood cells in flies are primarily for organismal defense would the immune response of *dpp* mutant larvae be impaired? To answer these questions we developed a noninvasive protocol for infecting *Drosophila* third-instar larvae with *Salmonella*.

Salmonella enterica serovar *typhimurium* is naturally infectious to a broad range of vertebrates, including humans. It enters orally then crosses the gut epithelium by invading and eventually killing M cells of the Peyer's patch. Once across the epithelial barrier, *S. typhimurium* infects macrophages and spreads to the mesenteric lymph nodes and subsequently to other organs (11). In response, the host activates an array of immune programs and occasionally host and microbe battle to a draw, resulting in host-adapted Salmonellosis (a form of gastroenteritis).

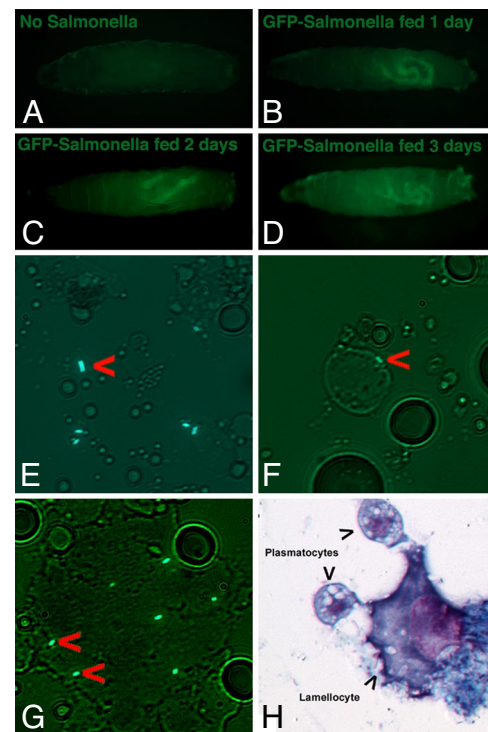


Fig. 3. Ingested GFP expressing *S. typhimurium* colonize the gut and infect circulating blood cells of WT larvae. Live 6-day-old third-instar larvae or their live-mounted blood cells are shown. (A) WT. (B–D) Larvae were fed GFP expressing *S. typhimurium* for 1–3 days then were fed normally for 3–5 days. Note GFP expression in the gut tube and increasing GFP expression outside the gut with increased *S. typhimurium* feeding. (E–G) Blood cells from larvae fed GFP expressing *S. typhimurium* for 2 days. (E) *S. typhimurium* rods freely circulate in the blood. (F) An *S. typhimurium* rod appears integrated into the membrane of a plasmatocyte. (G) Numerous *S. typhimurium* rods are visible within a lamellocyte. Note that *S. typhimurium* is not associated with vacuoles in either cell, suggesting they did not enter via phagocytosis. (H) Fixed and Wright-Giemsa-stained blood cells (plasmatocytes and a lamellocyte) from a larva fed *S. typhimurium* for 3 days (image at slightly reduced magnification versus E–G). (Magnifications: A–D, $\times 25$; E–H, $\times 1,000$.)

S. typhimurium does not typically infect *Drosophila*, but we identified a genetically modified strain that is capable of infecting larvae via feeding. The GFP-expressing strain of *S. typhimurium* we use is almost as virulent in mammals as the WT strain $\chi 3761$. The LD₅₀ of the modified strain is $\approx 5 \times 10^4$ for adult mice and as few as 50 cells are sufficient to kill newborn mice when inoculated orally (Roy Curtiss, personal communication). When *Drosophila* first- or second-instar larvae are fed the modified strain for 1–3 days and then fed normally for 3–5 days *S. typhimurium* persist in the gut into the third instar (Fig. 3 A–D).

We noted that larvae feeding on *S. typhimurium* experienced a developmental delay of 1 full day, a 20% increase in the normal time from hatching to pupation. To determine the bacterial load responsible for his delay we measured the internal titer of *S. typhimurium* in third-instar larvae that had been fed for 3 days. In two assays of ≈ 20 larvae we found on average that each larva contained 560 *S. typhimurium* cells (range 408–712). We repeated the assay twice with added gentamycin to kill any *S. typhimurium* that had not entered larval cells and found that on average 65% (315–345 *S. typhimurium* cells) were intracellular.

Examination of blood from infected third-instar larvae revealed that *S. typhimurium* were freely circulating and had infected both types of defensive blood cells (plasmatocytes and lamellocytes; Fig. 3 E–G). The presence of vacuoles was used to distinguish between *S. typhimurium* internalized by phagocytosis

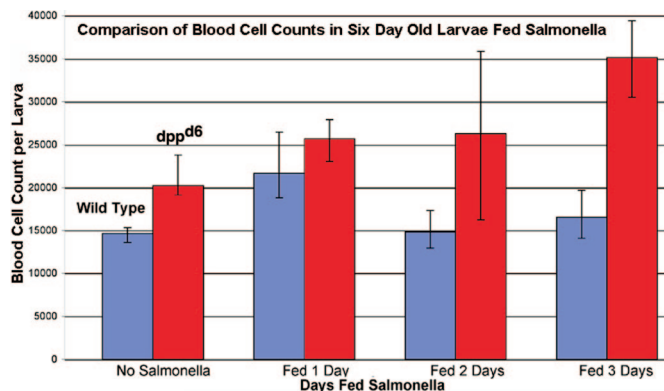


Fig. 4. *dpp* mutant larvae have significantly more circulating blood cells than WT, and the disparity is exacerbated with *S. typhimurium* exposure. The mean and range of blood cell counts for 6-day-old larva are shown ($n = 30$) in WT and *dpp* mutants. Note that there are $\approx 38\%$ more blood cells in *dpp* mutants than in WT under normal conditions (blue columns). This difference is exacerbated to 112% upon 3 days of exposure to *S. typhimurium* (red columns).

(vacuoles present) versus infection (no vacuoles). We chose this criteria because *S. typhimurium* within epithelial cells of the gut were unaccompanied by vacuoles (data not shown).

dpp Mutants Have Excess Plasmatocytes and Defective Immune Responses to *S. typhimurium*. *S. typhimurium* infection leads to an equal developmental delay in WT and *dpp* mutant larvae. However, the infection is eventually lethal only for *dpp* mutants. When exposed to *S. typhimurium* both WT and *dpp* mutant larvae will pupate but *dpp* mutants never eclose as adults. To better understand the underlying cause of lethality in *dpp* mutants we conducted three hematology studies of third-instar larvae under normal and *S. typhimurium* environments.

First, we counted the number of blood cells in 6-day-old WT and *dpp* mutant larvae (Fig. 4) by using Trypan blue staining. We found that under normal conditions *dpp* mutants generated significantly more (38%) blood cells per larva than WT ($P < 0.001$). We then extended this study to immune stress conditions of increasing severity (larvae were fed *S. typhimurium* for 1–3 days followed by normal feeding for 3–5 days; Fig. 4). When exposed to *S. typhimurium* for 1 day the overall number of blood cells per larva significantly increases in both WT ($P < 0.004$) and *dpp* mutants ($P < 0.001$), but the disparity between the genotypes is reduced to 18%. However, this difference remains statistically significant ($P < 0.028$). Thereafter in WT increasing exposure to *S. typhimurium* leads to a reduction in the number of blood cells, back to a level not significantly different from a normal environment ($P > 0.096$). Alternatively, in *dpp* mutants the number of blood cells continually increases with increasing exposure to *S. typhimurium* to the point where there are significantly more (74%) cells in the 3-day-fed *dpp* mutant larvae than in *dpp* mutant larvae in a normal environment ($P < 0.001$). The blood cell increases in *dpp* mutants yield significantly more blood cells in 2-day-fed (78% more; $P < 0.022$) and 3-day-fed (112% more; $P < 0.001$) *dpp* mutants than in similarly treated WT larvae.

In a pair of studies we determined which of the three blood cell types were in excess in *dpp* mutants. We first visualized crystal cells in WT and *dpp* mutant larvae under normal conditions. After conducting cell counts this study showed no significant difference in crystal cell numbers between the genotypes [$P > 0.43$; [supporting information \(SI\) Fig. S1](#)]. This result is consistent with our demonstration that there is no difference in Lozenge LG expression between WT and *dpp* mutant larvae (Fig. 2 *E* and *F*). Next, we visualized plasmatocytes and lamel-

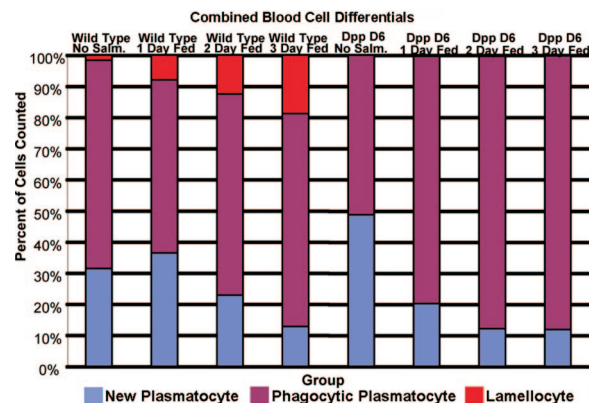


Fig. 5. *dpp* mutant larvae rarely differentiate lamellocytes in response to *S. typhimurium*. The mean percentage of blood cells per larva for lamellocytes (red) and new (blue) and phagocytic (purple) plasmatocytes are shown in WT and *dpp* mutants under normal and *S. typhimurium* conditions. New and phagocytic plasmatocytes were distinguished by the presence of vacuoles in phagocytic plasmatocytes (Fig. S2). Six-day-old larvae ($n = 12$) were assayed 3–5 days after *S. typhimurium* feeding. In WT the percentage of circulating lamellocytes increases >10-fold in response to increases in *S. typhimurium* exposure. In contrast, lamellocytes are extremely rare in *dpp* mutants.

lyocytes in WT and *dpp* mutant larvae under normal and increasing *S. typhimurium* environments and determined blood cell differentials by using Wright-Giemsa staining (Diff-Quik). In this study we ascertained the percentage of new plasmatocytes (without vacuoles), phagocytic plasmatocytes (vacuoles present), and lamellocytes (Fig. 3H) in WT and *dpp* mutants. In WT, in the absence of infection, 31% of the blood cells were new plasmatocytes (Fig. 5). In *dpp* mutants, in the absence of infection, 48% of the blood cells were new plasmatocytes. In WT with *S. typhimurium* exposure the fraction of new plasmatocytes first increased with 1 day of feeding to 38% but then decreased to below normal in 2-day-fed (26%) and 3-day-fed larvae (16%). In *dpp* mutants with *S. typhimurium* exposure the level of new plasmatocytes decreased to 23% and continued to decrease to below the level of comparable WT larvae (20% in 1 day, 12% in 2 days, and 11% in 3 days of feeding).

When comparing WT and *dpp* mutants in normal environments, *dpp* mutants displayed a greater proportion of new plasmatocytes than WT, which was consistent with our data that *dpp* mutants have an excess of Ush-expressing plasmatocyte precursors. Thus, in normal environments excess plasmatocytes generated in *dpp* mutants do not have an immediate opportunity to engage in phagocytic activity. This discrepancy disappears after 3 days of *S. typhimurium* exposure. At this point in both genotypes, the fraction of new plasmatocytes is roughly equal and below the level for normal conditions. The erasure of the distinction can be explained by an increase in *S. typhimurium* encounters with extended feeding.

In addition, *S. typhimurium* exposure uncovered a cryptic phenotype of *dpp* mutants. In response to infection, *dpp* mutants display a significantly impaired ability to differentiate lamellocytes, the most potent class of defensive cells (Fig. 5). In WT, the percentage of circulating lamellocytes increases >10-fold in response to *S. typhimurium* (from 1–2% in nonfed to ≈18% in 3-day-fed larvae). In *dpp* mutants we rarely saw a lamellocyte even under the most extreme *S. typhimurium* challenge, which suggests that in addition to an excess of Ush-expressing plasmatocyte precursors *dpp* mutants have an excess of Ush expression in individual precursor cells that blocks them from entering the lamellocyte pathway.

Given our data that crystal cell numbers are normal and lamellocytes are absent in *dpp* mutants we provide a hypothesis

for the ability of *S. typhimurium* to exacerbate the disparity in total blood cell numbers between *dpp* mutants and WT (Fig. 4). In WT larvae under an *S. typhimurium* challenge, the number of plasmatocytes falls to below normal levels because these larvae generate significant numbers of lamellocytes to fight the infection. Alternatively, in *dpp* mutants under the same conditions an increasing number of plasmatocytes is generated because plasmatocytes are their only defense. The ability of *dpp* mutants to compensate for the lack of lamellocytes by ramping up plasmatocyte production suggests the existence of a previously undetected feedback circuit connecting pathogen-detecting cells of the immune system and hematopoietic cells that provide the defensive response.

Discussion

***dpp* Mutants Display Disrupted Blood Cell Homeostasis.** Understanding the disruption of blood cell homeostasis (excess plasmatocytes) in *dpp* mutant larvae is simple; an embryonic error in precursor cells is maintained and amplified over time in their descendants all of the way through terminal differentiation. Here, the error is the loss of Dpp dorsal ectoderm to mesoderm signaling late in embryogenesis. This loss of Dpp results in an excess of Zfh1-expressing pericardial cells in embryonic LGs and hearts at the expense of the adjacent dorsal muscle cells (3). Not previously connected to hematopoiesis, Zfh1 sits near the top of a transcription factor cascade that begins in embryos, continues in larvae, and ultimately dictates plasmatocyte homeostasis. We previously showed that Zfh1 activated Odd, and here we show that Zfh1 activates Srp in embryonic pericardial cells. Given that Odd transcription precedes Srp in pericardial cells and that Srp is present only in cells that express Odd, it is tempting to speculate that Odd functions between Zfh1 and Srp. The excess Zfh1 pericardial cells in *dpp* mutants follow their normal developmental path and generate excess Odd and excess Srp pericardial cells. The excess embryonic Srp pericardial cells follow their normal developmental path and by the third instar they have generated excess Ush-expressing prohemocytes. The excess Ush-expressing prohemocytes follow their normal developmental path and become excess plasmatocytes.

Potential Conservation of Dpp/BMP Signaling in Hematopoiesis. This study implicates Dpp signaling in limiting the number of hematopoietic precursor cells in *Drosophila*. It was previously shown that BMP-R1A signaling in osteoblast cells of the inner bone surface limits hematopoietic cell proliferation in the adjacent marrow (12). To date the BMPR-1A ligands BMP2/4 have not been linked to hematopoietic cell growth in adult mammals or during embryonic development. Our data suggest that this hypothesis is worth testing.

Other testable hypotheses relevant to mammalian hematopoiesis involve transcription factors between Dpp/BMP signals and Srp/GATA activation such as Zfh1 and Odd. Zfh1 is related to the duplicated mammalian proteins ZEB1/2. ZEB1 is also known as Δ EF1, and interactions between Δ EF1 and BMP2 have been reported to influence osteoblast differentiation in cell culture (13). Odd is related to the duplicated mammalian proteins OSR1/2, and OSR2 has been reported to regulate osteoblast proliferation in knockout mice (14).

***dpp* Mutants Display Impaired Immune Responses.** Upon *S. typhimurium* challenge *dpp* mutant larvae respond differently from WT in two distinct, but we believe, related ways. First, *dpp* mutants rarely display any lamellocytes, whereas the frequency of lamellocytes in WT increases >10-fold in the most challenging *S. typhimurium* environments. We present evidence that lamellocytes play a critical role in combating bacterial infection. The molecular underpinnings of lamellocyte origins have not been as thoroughly documented as the origin of plasmatocytes or crystal

cells. On this topic, our data strongly support and extend a recent report that the Ush loss of function phenotype includes the generation of lamellocytes under normal conditions and that ectopic Ush suppresses ectopic lamellocytes generated by *hop^{TumL}* (7).

These observations suggest that lamellocytes arise from plasmatocytes or a common precursor in which a balance between Ush and Hopscotch activity, one that normally favors Ush and the plasmatocyte differentiation program, is shifted toward Hopscotch and the lamellocyte program. Our data are wholly consistent with this hypothesis because *dpp* mutants have an overabundance of Ush expression and they do not generate lamellocytes even when challenged by *S. typhimurium*. In *dpp* mutants, it appears that the balance is tilted so far in favor of Ush that even a severe infection cannot overcome it. Our data provide strong evidence in support of this hypothesis because it derives from a *dpp* loss-of-function genotype that has no lamellocytes rather than a gain-of-function *hopscotch* allele with ectopic lamellocytes or suppression of this phenotype with ectopic expression of Ush.

The most parsimonious manner in which to incorporate the existence of a common precursor of plasmatocytes and lamellocytes into our understanding of fly hematopoiesis is to expand the role of Ush-expressing prohemocytes into that of a single progenitor for all three blood cell types. From this perspective, these cells become crystal cells upon Notch signaling and become lamellocytes upon disruption of the balance between Ush and Hopscotch (in respect to a signal that is currently unknown). With plasmatocytes as the default differentiation program it is easy to see why these cells are 95% of circulating blood cells.

The second difference between WT and *dpp* mutant larvae in their immune response is that after an initial increase in circulating plasmatocytes in both genotypes *dpp* mutants continue to increase the number of plasmatocytes with increasing *S. typhimurium* exposure whereas WT larvae reduce circulating plasmatocytes to below normal levels. We have suggested that the increase in plasmatocytes in *dpp* mutants is their way of compensating for a lack of the more potent lamellocytes in battling infection. However, this raises a question: how do plasmatocyte precursors know that no lamellocytes are being generated and they need to accelerate plasmatocyte production?

One possibility is a previously undetected feedback circuit providing hematopoietic cells with continuous input from pathogen-detecting cells in the innate immune system. Perhaps immune cells in circulation or elsewhere continuously monitor the number of *S. typhimurium* they encounter and relay this information back to the LGs via cytokine-mediated signals. These "infectious titer" signals are received by hematopoietic cells and translated into appropriate actions (e.g., turn on lamellocytes). Thus, in *dpp* mutants the lack of lamellocytes is noted by monitoring cells because the number of *S. typhimurium* they encounter continues to increase and they signal back to hematopoietic stem cells that respond by increasing plasmatocyte production.

One potential hematopoietic protein involved in the interpretation of feedback signals is Hopscotch. Hopscotch mutant phenotypes suggest it plays this role: *hop^{TumL}* yields lamellocytes under normal conditions (constitutively active for feedback) and *hop^{M4}* never generate lamellocytes or increase the number of circulating plasmatocytes when infected (loss of function for feedback). A second potential feedback-interpreting protein in hematopoietic cells is Zfrp8 because loss-of-function mutations in Zfrp8 mimic the *hop^{TumL}* phenotype (15).

***S. typhimurium* Facilitates Studies of Hematopoiesis and Innate Immunity.** The ability to elicit an immune response in flies via feeding has several advantages over current methods. Adding *S.*

typhimurium to the food eliminates the need for labor-intensive injection of bacteria. However, infection by feeding does not sacrifice the quantitation possible with injection; the titer of internal *S. typhimurium*, both freely circulating and within host cells, can be readily calculated. In addition, the fact that all larvae need to eat results in near-universal infection under our experimental conditions.

The ability to generate large numbers of infected individuals allows one to design genetic screens for fly mutants that enhance or suppress the immune response to *S. typhimurium*. Further, modifier screens of infected flies with mutations in genes known to effect larval innate immunity (e.g., GATAd and GATAe; ref. 5) can be conducted to place these genes into pathways. In addition, as *S. typhimurium* is a human pathogen it has been extensively studied and many mutants are available. Incorporating specific mutant strains of *S. typhimurium* into genetic screens may allow increased precision with regard to identifying mutations in a pathway of interest. For example, *S. typhimurium* with a variety of mutations in the secreted MAPK inhibitor AvrA that suppresses JNK-mediated apoptosis in flies (16) could be used in screens for genes associated this interaction. Finally, the parallel between the infectious trajectory of *S. typhimurium* in flies and mammals suggests that studies in *Drosophila* may uncover new information about *S. typhimurium* and its mechanisms of pathogenesis in mammals.

In summary, our protocol for infecting *Drosophila* larvae with *S. typhimurium* revealed that Dpp signaling regulates a transcription factor cascade that controls blood cell homeostasis and immune responses. Our results suggest testable hypotheses regarding conserved BMP-dependent signaling mechanisms governing aspects of hematopoiesis in mammals. We are now in a position to combine the analytical power of *Drosophila* and *Salmonella* genetics to tackle complex questions regarding in-

teractions between the hematopoietic and innate immune systems and to investigate the infectious mechanisms of additional bacterial pathogens.

Materials and Methods

Drosophila Genetics. Strains were as described (3) except Lozenge(-1509/-59)-lacZ (9). The antibodies anti-Odd (17), anti-lacZ (Developmental Studies Hybridoma Bank (University of Iowa, Iowa City), anti-Srp (18), anti-Antennapedia (Developmental Studies Hybridoma Bank), and anti-Ush (19) were as described. The nucleic acid dye ToPro3, secondary antibodies, and signal amplification techniques (Molecular Probes) were as described (3).

Salmonella Genetics. Methods for feeding χ 8276 *S. typhimurium* U.K.-1 Δ asdA16 carrying pYA3552 Asd⁺ and GFP-3 to larvae, determination of larval infectious titer (CFU per larva) in the presence or absence of gentamycin (20), and a detailed strain description can be found in [SI Text](#). *Salmonella* feeding experiments were conducted at room temperature (23°C). Fluorescence microscopy of live third-instar larvae was as described (3).

Hematology. Two dye-based methods in mammalian hematology were adapted for fly blood cells by extrapolating from ref. 21. One method, Trypan blue (Fisher Scientific) staining of all blood cells was used to estimate total blood cell counts by using a hemocytometer. The second method, Wright-Giemsa staining of fixed blood cells (Diff-Quik; Dade Behring) generates distinct color patterns in different cells. This method was used to determine the frequency of each blood cell type in a fixed number of counted cells (a blood cell differential). Detailed procedures can be found in [SI Text](#). Visualization of crystal cells in larvae followed ref. 22. *T* tests for significance in fluorescence and hematology assays were implemented as described (3).

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