An unexpected link between Notch signaling and ROS in restricting the differentiation of hematopoietic progenitors in *Drosophila*

Chiyedza Small*§†‡, Johnny Ramroop*§‡, Maria Otazo*, Lawrence H. Huang*, Shireen Saleque*§, Shubha Govind*§.

* Biology Department, The City College of the City University of New York, New York, NY 10031

§ The Graduate Center of the City University of New York, New York, NY 10016

† Present address: Department of Biology, Medgar Evers College, The City University of New York, Brooklyn, NY, 11225

‡ Equal contribution
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Corresponding author:

Shubha Govind

Biology Department,

The City College of the City University of New York,

138th Street and Convent Avenue,

New York, NY 10031

USA

sgovind@ccny.cuny.edu

Phone: 212-650-8571

Fax: 212-650-8585
ABSTRACT

A fundamental question in hematopoietic development is how multipotent progenitors achieve precise identities, while the progenitors themselves maintain quiescence. In *Drosophila melanogaster* larvae, multipotent hematopoietic progenitors support the production of three lineages, exhibit quiescence in response to cues from a niche, and from their differentiated progeny. Infection by parasitic wasps alters the course of hematopoiesis. Here we address the role of Notch (N) signaling in lamellocyte differentiation in response to wasp infection. We show that Notch activity is moderately high and ubiquitous in all cells of the lymph gland lobes, with crystal cells exhibiting the highest levels. Wasp infection reduces Notch activity, which results in fewer crystal cells and more lamellocytes. Robust lamellocyte differentiation is induced even in *N* mutants. Using RNA interference-knockdown of *N*, *Serrate*, and *Neuralized*, and twin clone analysis of a *N* null allele, we show that all three genes inhibit lamellocyte differentiation. However, unlike its cell-autonomous function in crystal cell development, Notch’s inhibitory influence on lamellocyte differentiation is not cell-autonomous. High levels of reactive oxygen species in the lymph gland lobes, but not in the niche, accompany *N*\(^{RNAi}\)-induced lamellocyte differentiation and lobe dispersal. Our results define a novel dual role for Notch signaling in maintaining competence for basal hematopoiesis: while crystal cell development is encouraged, lamellocytic fate remains repressed. Repression of Notch signaling in fly hematopoiesis is important for host defense against natural parasitic wasp infections. These findings can serve as a model to understand how reactive oxygen species and Notch signals are integrated and interpreted *in vivo*.
INTRODUCTION

In mammalian hematopoiesis, multipotent stem cells follow a tightly-regulated developmental program to specify the correct proportions of at least eight mature lineages. A complex set of hierarchical decisions are executed to supply the body with fully functional cells, while also renewing the original stem/progenitor population. Various lines of experimental evidence suggest that signaling pathways and molecular networks of transcription factors constitute key aspects of stem cell regulation (ORKIN and ZON 2008). Functional homologs of many of these signaling pathways and transcription factors are present in Drosophila and mutations in their respective fly genes give rise to hematopoietic defects (CROZATIER and MEISTER 2007; FOSSETT 2013; HONTI et al. 2013; KRZEMIEN et al. 2010a).

The future larval hematopoietic organ in Drosophila, called the lymph gland, differentiates from a cluster of embryonic mesodermal cells; Notch limits cardioblast (vascular) development in favor of embryonic lymph gland tissue (MANDAL et al. 2004); lymph gland lobe development continues through the first and second larval instars. By the mid third instar, the organ has a pair of anterior lobes, and two sets of smaller posterior lobes, that flank the dorsal vessel. Genetic experiments suggest that progenitors of the three major blood cell types quiesce in the medullary zone or medulla (JUNG et al. 2005; KALAMARZ et al. 2012; KRZEMIEN et al. 2010b; MINAKHINA and STEWARD 2010). The cortex harbors a mixture of maturing and fully-differentiated blood cells. Lineage development in the cortex is tightly regulated. The medulla/cortex boundary is not sharp; cortical cells in an intermediate state of differentiation are closer to the medulla; the terminally-differentiated cells are most peripheral in location (JUNG et al. 2005; KALAMARZ et al. 2012; KRZEMIEN et al. 2010b). Plasmatocytes (90-95%) appear as single cells or in clusters and crystal cells (5-10%) are distributed singly. Crystal cells produce
enzymes for melanization. Like mammalian macrophages, plasmatocytes engulf bacteria and apoptotic cells and make up the majority of circulating cells. Plasmatocytes and crystal cells circulating in the hemolymph have an embryonic origin and represent a developmental compartment that is distinct from the lymph gland [reviewed in (CROZATIER and MEISTER 2007; FOSSETT 2013)]. A non-hematopoietic niche, also referred to as the posterior signaling center, located at the base of the anterior lobes maintains progenitor quiescence in the anterior lobes (KRZEMIEN et al. 2007; LEBESTKY et al. 2003; MANDAL et al. 2007). It also mediates lamellocyte differentiation but mainly in response to wasp infection via the transcription factor Knot/Collier (CROZATIER et al. 2004).

The lymph gland lobes are immune-responsive (LANOT et al. 2001; SORRENTINO et al. 2002). Parasitic wasps of the Leptopilina genus attack second and early third instar larvae. Wasp infection alters the course of basal hematopoiesis and triggers cell division, lamellocyte differentiation, aggregation, and melanization (LEE et al. 2009b). Lamellocytes are dedicated to encapsulating wasp eggs. Wasp attack reduces the abundance of crystal cells in the anterior lobes (KRZEMIEN et al. 2010b). It also activates NF-κB signaling (GUEGUEN et al. 2013) and oxidative stress (SINENKO et al. 2012) in the niche. Activated NF-κB signaling in the lobes favors lamellocyte differentiation and inhibits crystal cell development (GUEGUEN et al. 2013). The effects of wasp-induced increase in reactive oxygen species is mediated by EGF signaling (SINENKO et al. 2012), although its effects on crystal cell development are not known. While it is clear that the lymph gland disperses after wasp infection and some plasmatocytes and lamellocytes arising in the lymph gland transfer into the hemolymph (LANOT et al. 2001; SORRENTINO et al. 2002; SORRENTINO et al. 2004), how wasp attack alters hematopoiesis to promote lamellocyte differentiation in the first place is not well understood.
In this study we analyze the role of Notch signaling in lamellocyte and crystal cell development. Notch signaling is evolutionarily conserved and regulates the diversification of cell fates in animals ranging from flies to mammals (BRAY 2006; KOCH et al. 2013). It also plays a role in the maintenance of hematopoietic stem cells (BIGAS et al. 2012). In Drosophila, Notch’s role in crystal cell development is well-documented. It regulates embryonic crystal cell development (BATAILLE et al. 2005). In the late embryo, N restricts the developmental potential of multipotent progenitors: at restrictive temperature, late stage-16 embryos, \( N^{ts} \) embryos are unable to specify crystal cells (KRZEMIEN et al. 2010b). In larval stages, Notch signaling regulates commitment to crystal cell lineage (DUVIC et al. 2002; LEBESTKY et al. 2003); in pro-crystal cells, the Notch receptor is activated by Serrate (Ser), expressed in the niche cells. Pro-crystal cells express high levels of AML-like transcription factor, Lozenge, and Suppressor of Hairless (\( Su(H) \)), a transcriptional activator mediating Notch signaling (LEBESTKY et al. 2003; TERRIENTE-FELIX et al. 2013). Notch and Lozenge select specific target genes to mediate crystal cell development (TERRIENTE-FELIX et al. 2013). Ligand-independent non-canonical Notch signaling also promotes crystal cell survival during basal hematopoiesis and hypoxic stress (MUKHERJEE et al. 2011).

Notch was reported to be essential for wasp-induced differentiation of lamellocytes (DUVIC et al. 2002), although the mechanism behind its requirement is not known. We now report an unexpected non cell-autonomous role for Notch signaling in restricting and not promoting differentiation of hematopoietic progenitors into lamellocytes. This inhibition appears to be independent of oxidative stress levels in the niche, although surprisingly, knockdown of \( N \) leads to increased reactive oxygen species (ROS) production in the lobes. Thus, Notch signaling plays an essential role in host defense against parasites in Drosophila. These results highlight an
unexpected pleiotropy for Notch and ROS signaling in regulating the production of alternative lineages during larval hematopoiesis.
MATERIALS AND METHODS

Stocks, crosses, and wasp infections

*HmlΔ-GAL4* (SINENKO et al. 2004) was received from S. Bhattacharya. *Dome-GAL4* (GHIGLIONE et al. 2002) was a gift from M. Crozatier, *Antp-GAL4* received from S. Minakhina (EMERALD and COHEN 2004). *y w; UAS-mCD8-GFP* (SINENKO et al. 2004) was incorporated into appropriate GAL4 backgrounds via standard crosses. UAS-RNAi lines for *Notch* (Valium 1 and 10), *Serrate* (Valium 10) *neutralized* (Valium 10), and *Su(H)* (VDRC line KK) were obtained from the TRiP collection (NI et al. 2009; NI et al. 2008). *msnf9-cherry* (TOKUSUMI et al. 2009) was a gift from R. Schulz. *arm-lacZ 19A/FM7, hs-FLP* (VINCENT et al. 1994) was obtained from N. Baker. A chromosome bearing null allele (*N^{55e11},* HEITZLER and SIMPSON 1991) *N^{55e11} FRT19A/FM7* (OHLSTEIN and SPRADLING 2007) was obtained from B. Ohlstein. *UAS-NICD* encoding the Notch intracellular domain, NICD (STRUHL and ADACHI 1998) was obtained from K. Irvine. *12X Su(H)-lacZ* (GO et al. 1998) was obtained from S. Artavanis-Tsakonas. The *Dome-MESO-GFP* (medulla is GFP-positive) stock was a gift of M. Crozatier. *y^{1} N^{ts1} and w^{a} N^{ts2} rb* flies were obtained from the Bloomington Stock Center.

Females from respective driver stocks containing *y w; UAS-mCD8-GFP; Antp-GAL4/TM6 Tb Hu, y w Dome-GAL4/FM7; UAS-mCD8-GFP* and *y w; HmlΔGFP/HmlΔGFP* were crossed to males from respective RNAi stocks *y v/Y; N^{RNAi}/N^{RNAi} and w^{118}/Y; UAS-NICD/TM3*. Third instar animals, selected from 6-8 hour egg-lays and reared at 27°C, were genotyped by GFP expression. Lymph gland basement membrane dispersal was scored according to (SORRENTINO et al. 2002; SORRENTINO et al. 2004).

For loss-of-function Notch clones (XU and RUBIN 1993), *N^{55e11} FRT19A/FM7* females were crossed to *arm lacZ19A; hs-FLP/TM6 Tb Hu* males to create *N^{55e11}/N^{55e11}* clones marked by
the absence of β-galactosidase. Homozygous wild type \(N^+/N^+\) clones were detected by higher than background β-galactosidase expression. \(N^{55e11}\) mutant clones were generated by \(37^\circ C\) heat shock of second instar larvae for 30-40 minutes. To induce flp-out clones (STRUHL and BASLER 1993), developmentally-synchronized 4-day old larvae with the hybrid flip-out and Gal4 activation system [\(hsp70-flp; Actin>CD2>Gal4\)] and \(UAS-NLS-GFP\) transgenes or those with an additional \(UAS-NICD\) transgene, were heat-shocked at \(37^\circ C\) in a water bath for 15 min. \(N^{RNAi}\) (Valium 10) flp-out clones were similarly induced with a 1 hr heat shock of developmentally-synchronized mid-to-late second instar animals. After recovery at \(25^\circ C\), lymph glands were dissected on day 6 from mid third instar larvae, 20 hrs after heat-shock. \(N^{ts1}\) or \(N^{ts2}\) stocks or progeny from crosses were maintained at \(18^\circ C\). At the early second larval instar stage, animals were shifted to \(29^\circ C\) through the third instar. Animals were matched for biological age to compare phenotypes. Most mutant males did not emerge; escapers showed notched wings.

Wasp infections were performed as follows: from a six-hour egg-lay, second instar wild type or mutant larvae were subjected to infection by \(L. boulardi\) \(G486\) (SORRENTINO et al. 2002) or \(L. boulardi\) \(Lb17\) (SCHLENKE et al. 2007) for six to twelve hours, after which wasps were removed and host larvae were allowed to develop to third instar stage. Uninfected controls followed the same growth regimen.

**Immunohistochemistry and ROS detection**

Unless otherwise specified, mid third instar larvae from 6 h or 12 h egg-lays were used in antibody staining experiments. Staining was performed according to (PADDIBHATLA et al. 2010) Primary antibodies: mouse anti-Notch (C17.9C6, detects the intracellular product; Developmental Studies Hybridoma Bank, (FEHON et al. 1990) 1:10, mouse anti-Nimrod C/P1
and mouse anti-L1/Atilla (1:10) (KURUCZ et al. 2007), mouse anti-prophenol oxidase (1:10, Dr T. Trenczek, University of Giessen), 1:10, anti-integrin βPS (CF.6G11; Developmental Studies Hybridoma Bank; (BROWER et al. 1984)) 1:10, anti-proPO (CHRISTOPHIDES et al. 2002), anti-β-galactosidase from Capell (1:2,000 clone analysis) or from Immunology Consultants, Inc (1:200 for Notch activity). Secondary antibodies: Cy3 AffiniPure Donkey Anti-Chicken (1:500 Jackson Immuno Research) or anti-mouse (1:200 Jackson Immuno Research or 1:500 Invitrogen). Rhodamine-, Alexa Fluor 488-, or Alexa Fluor 647-tagged Phalloidin and nuclear dye Hoechst 33258 (all Invitrogen) were used to examine cell morphology. ROS detection (Invitrogen D11347) was carried out according to (OWUSU-ANSAH and BANERJEE 2009).

Lamellocytes were visualized by (a) their cell shape (filamentous F-actin); or (b) by expression of integrin βPS (STOFANKO et al. 2008); or (c) Atilla expression (L1; (KURUCZ et al. 2007); or (d) by the misshapen transcriptional enhancer msnf9 linked to cherry expression (TOKUSUMI et al. 2009). msnf9-cherry-positive lamellocytes are also strongly positive for F-actin with Alexa Fluor 488-labeled Phalloidin (data not shown).

**Data collection and statistical analysis**

Samples were imaged with Zeiss LSM 510 confocal microscope; 0.8 μm -1.5 μm thick optical images were analyzed according to (GUEGUEN et al. 2013). Select images were imported into Adobe Photoshop CS5. The eyedropper tool was used to define and extract a signal intensity value from a 5 X 5 square of 25 pixels in random regions of the lobes. Intensity data were collected from 20 cells per lobe in at least 12 animals. Images showing signal saturation were not used for quantification. Niche areas (in μm²) were measured on confocal images using Path Tool of AxioVision 4.8 software. The average number of niche cells per lobe was scored by manually
counting the mCD8-GFP- and Hoechst-positive cells in merged z-stacked images of the niche. For niche size and cell number, data were tested for normality before performing statistics using the R software (R-DEVELOPMENT-CORE-TEAM 2010). All other data were analyzed using Microsoft Excel 2010.
RESULTS

Notch expression in third instar lymph glands

To examine the expression of the intracellular domain of Notch, we stained lymph glands with anti-Notch antibody. Notch signal is highest in some GFP-negative cells (arrow) while the staining signal is low in cortical $Hml>GFP$ cells (Fig. 1A-A”). Notch staining is high in $Dome> mCD8-GFP$-positive cells of the medulla of anterior (Fig. 1B-B”) and posterior lobes (data not shown), as well as $Antp>mCD8-GFP$-positive niche cells (Fig. 1C-C”). In none of these populations, is the Notch signal nuclear. We were surprised to find that Notch levels are low or undetectable in lamellocytes induced by wasp attack (Fig. 1D-D”) or those in the gain-of-function $hopscotch^{Tumorous-lethal} (hop^{Tum-})$ (LUO et al. 1995) background, where ectopic JAK-STAT signaling promotes lamellocyte differentiation in the absence of parasite attack (data not shown). Specificity of Notch staining and successful knockdown of Notch protein was confirmed by staining $Antp>N^RNAi$ glands, where the Notch signal was either reduced or not detected in GFP positive niche cells (Fig. S1). Differences in Notch levels and its subcellular localization in the lymph gland suggest different states of Notch activity in different cell populations, and likely distinct functions of Notch in hematopoietic development.

$N$ activities in larval lymph gland lobes

To test if $N$’s influence on progenitor differentiation is more or less ubiquitous, or if Notch activity is localized to crystal cells, we examined Notch activity using specific reporters. We found that a reporter of Notch and Su(H) transcription, $Su(H)-lacZ$ (GÖ et al. 1998), is expressed at moderate levels in wild type anterior lobe cells in all three larval stages, although the reporter activity levels are distinctly higher in the third instar lobes (Fig. 2A-D).
Additionally, only at the third instar stage, high N target gene activity is also detected in scattered cells (Fig. 2D arrows). A significant fraction of these cells (37.3%; Fig. 6C, C’ stars) are also pro-PO-positive, suggesting ontogenetic relationship among the Su(H)-lacZ- and pro-PO-positive cell populations. Developmentally, the appearance of mature crystal cells in the lymph gland, positive for Lozenge and Su(H) proteins, coincides with the second-to-third instar molt (Lebestky et al. 2000).

Ubiquitous moderate activity from the Su(H)-lacZ reporter is detected in the N^{ts1} or N^{ts2} lobes reared at permissive 18°C temperature (one Su(H)-lacZ copy in N^{ts}/Y males; Fig. 3A, B, E), although high signal in crystal cells is rarely observed in the N^{ts} backgrounds presumably due to the inhibitory effect of the mutation. At non-permissive temperature however, both the moderate ubiquitous and high local Su(H)-lacZ signals are reduced in the N^{ts1} and N^{ts2} backgrounds (Fig. 3C-E). Unlike crystal cell differentiation, in N^{ts}/Y lobes, neither the medulla/cortex sizes (assessed by Dome-MESO-GFP expression (Fig. 7A, B) nor plasmatocyte development (anti-Nimrod C staining; data not shown) appear to be affected at 29°C. Thus, it appears that, consistent with Notch expression, Notch signaling is widespread in third instar anterior lobes. We hypothesized that the moderate ubiquitous Notch activity is needed for selection and patterning, i.e., the distributed appearance of crystal cells in the cortex, in a step prior to their maturation, which requires robust cell autonomous Notch activity.

NICD expression promotes development of crystal cell clusters

We first examined Notch’s role in crystal cell development. We were surprised to find that the expression of the Notch intracellular domain (NICD) in the niche (Antp>NICD) results in a significant increase in crystal cell number (average 67 crystal cells/lobe in Antp>NICD...
glands, compared to 18 crystal cells/lobe in control glands, n = 6 lobes for each genotype; p < 0.05) (Fig. 4A, B). Many of these crystal cells develop in clusters, a pattern rarely observed in wild type glands. Conversely, RNAi knockdown of N via Antp led to a significant reduction in crystal cell number (Fig. 4C).

“Flp-out” clones of constitutively-active NICD strongly induced crystal cell fates in third instar lymph glands: All (99%, n = 89 cells) GFP-positive clonal cells expressing NICD are also pro-PO-positive (Fig. 5C-D’), compared to 0.5%, (n = 236 cells) of pro-PO-positive cells in control clones (Fig. 5A-B’). We also observed rare pro-PO-expressing cell clusters in NICD-expressing posterior lobes (Fig. S2C-D’). Crystal cells normally do not develop in posterior lobes (Fig. S2A-B’). Thus, ectopic NICD expression makes all daughter cells unresponsive to their normal internal and external developmental cues and, instead, programs them into crystal cells.

Consistent with the Antp>NICD result above (Fig. 4A-C), numerous crystal cells were also found outside the actin>NICD “flp-out” clones (Fig. 5D, D’). This observation further supports an indirect positive influence of Notch activity on crystal cell differentiation. In addition, actin>NICD “flp-out” lobes contain mature anti-Nimrod C/P1-positive plasmatocytes at the lobe periphery. These plasmatocytes do not express NLS-GFP and are therefore NICD-negative (Fig. 5C, C’’). Together, these results not only confirm that high Notch activity is sufficient for crystal cell development as described in previous studies (DUVIC et al. 2002; LEBESTKY et al. 2003) but also that Notch-mediated non cell-autonomous mechanism patterns the cortex with dispersed crystal cells.
**Notch activity is quenched after wasp infection**

To examine if wasp infection might decouple the ubiquitous and localized N activities, we infected second instar Su(H)-lacZ transgenic animals (wild type for N) and stained lymph glands from mid-third instar animals with anti-β-galactosidase antibody. We found a significant, two-fold reduction in the ubiquitous signal in lobes of wasp-infected Su(H)-lacZ animals compared to lobes from uninfected animals (Fig. 6G). In addition, the number of cells with high Notch activity (some of which also express the pro-PO enzyme (green, arrow)), is significantly reduced (Fig. 6A-F), supporting the idea that moderate ubiquitous Notch activity may be needed to specify crystal cell development, while acquisition of their final fates requires higher Notch activity in the maturing crystal cells, consistent with Notch’s cell autonomous role (LEBESTKY et al. 2003). The anterior lobes of wasp-infected Su(H)-lacZ glands revealed strong lamellocyte differentiation (Fig. 6H, I), raising the possibility that reduction in Notch activity may not compromise lamellocyte differentiation as previously reported (DUVIC et al. 2002).

**Notch RNAi promotes lamellocyte differentiation**

To examine if Nts/Y; Dome-MESO-GFP/+ mutant lymph glands support differentiation of lamellocytes, we exposed second instar animals to L. boulardi. Unexpectedly, wasp infection of temperature-restricted Nts1 or Nts2 animals results in robust lamellocyte differentiation and lobe dispersal in mid third-instar animals (Fig. 7A-F).

The expression of Serrate, the Notch ligand, is restricted to few cells of the niche (LEBESTKY et al. 2003). To examine if reduction in Notch signaling in this cell population induce lamellocyte differentiation, we conducted cell-specific knockdown using UAS-NRNAi in either Valium 1 or Valium 10 vector (NI et al. 2009; NI et al. 2008) in the niche or cortex, utilizing the
Antp and Hml GAL4 drivers, respectively. The driver stock contains the UAS-GFP reporter transgene, marking the location of GAL4-positive cells. In each case, lamellocytes were detected in N\textsuperscript{RNAi} lobes, but not in control lobes. Lamellocytes themselves were GFP-negative, and were located in the vicinity of the GFP-positive cells (Fig. 8A-D”), suggesting an inductive relationship. The strongest effects were observed upon knockdown with either Valium 1 or Valium 10 in the niche (Table S1; Fig. 8). As is the case with wasp-infected animals, mature lamellocytes were most often in the cortical zone, rarely at the medulla/cortex junction, and never in the niche or posterior lobes.

Since basement membrane disruption correlates with the presence of lamellocytes (SORRENTINO et al. 2002; SORRENTINO et al. 2004), we inspected the integrity of the basement membrane in both control and N\textsuperscript{RNAi} lobes (Table S1). Basement membrane in the control lobes were largely intact (Fig. 8A, C), but N\textsuperscript{RNAi} lobes were frequently dispersed (Fig. 8B, D, D’, D”, Table S1). Lamellocytes were also observed in smear preparations of circulating hemolymph of N\textsuperscript{RNAi} animals (Fig. 8F) but not in control smears (Fig. 8E). With both drivers, knockdown with Valium 10 UAS-N\textsuperscript{RNAi} resulted in stronger dispersal relative to Valium 1 UAS-N\textsuperscript{RNAi} knockdown. These results imply that lamellocyte precursors are sensitive to Notch activity in neighboring cells.

To further assess the role of Notch signaling in lamellocyte differentiation, we created RNAi knockdown lymph glands where levels of other N pathway components, Ser, Neuralized (neur), or Su(H) were similarly reduced in the niche. In all cases, supernumerary lamellocytes were induced (Fig. 9 and data not shown). In some Antp>neur\textsuperscript{RNAi} glands, ectopic lamellocytes were observed close to the GFP-positive cells (Fig. 9A-B”), but away from the niche. Knockdown of Ser (Fig. 9C-D”) or Su(H) (data not shown) had similar effects.
Thus it appears that \( N \) pathway components alter cellular properties of the niche, which in turn affects progenitor differentiation. Knockdown of these proteins however affects neither the number of \( \text{Antp}^{mCD8-GFP} \)-positive niche cells, nor the size of the niche (Fig. 9E). In none of these six backgrounds, did we detect actin-rich processes arising from the niche into the progenitor population as reported by others (CROZATIER et al. 2007; MANDAL et al. 2007; TOKUSUMI et al. 2012). Thus, Notch signaling does not affect specification or growth of the niche itself, but instead Notch signaling contributes to the production of a signal needed to maintain lamellocyte progenitor quiescence. RNAi results above (Fig. 8, 9 and Table S1) suggest that this inhibitory influence on lamellocyte differentiation, while stronger in the niche, is not limited to this compartment, and can also be exerted in other parts of the lobe.

**Notch’s inhibitory influence on lamellocyte differentiation is non cell-autonomous**

Non cell-autonomy of \( N \) was examined in two independent experiments. Flp-out clones, marked with GFP and expressing \( N^{\text{RNAi}} \) produce descendants that fail to differentiate into lamellocytes. However cells in the vicinity of GFP-positive \( N^{\text{RNAi}} \) cells (but not neighboring GFP-expressing cells without the \( N^{\text{RNAi}} \) transgene) show lamellocyte morphology (Fig. 10A, B arrow) and strong lobe dispersal (Fig. 10C, D). We also induced twin clones (Xu and RUBIN 1993) in heterozygous \( \text{N}^{55e11/N^{+}} \) glands. \( \text{N}^{55e11} \) is a strong loss-of-function allele (DIAZ-BENJUMEA and GARCIA-BELLIDO 1990). In this technique, somatic recombination in heterozygous cells produces daughter cells that are either homozygous mutant (\( \text{N}^{55e11/N^{55e11}} \)) or wild type (\( \text{N}^{+/N^{+}} \)). Wild type clones are identified by two copies of the marker protein, \( \beta \)-galactosidase, encoded by the \( \text{lacZ} \) transgene present in \( \text{cis} \) to \( \text{N}^{+} \). Fig. 11 shows that integrin \( \beta \)-positive-lamellocytes also express high levels of \( \beta \)-galactosidase. These double-positive cells are
not found within the mutant $N$ clone that is negative for the $\beta$-galactosidase signal (Fig. 11A-C’’), and a majority of newly-differentiated cells are observed in the cortex (Fig. 11). Thus, consistent with the $N^{RNAi}$ experiments, lamellocyte differentiation is non cell-autonomous and a majority of newly-differentiated cells are observed in the cortex (Fig. 11).

$\text{Antp}>N^{RNAi}$ leads to changes in ROS levels in lymph gland lobes

Recent work has linked increased ROS levels in the niche to lamellocyte differentiation after wasp infection ((SINENKO et al. 2012) and Fig. S3). To examine if reduction in Notch activity has a similar effect on ROS levels, we stained control and $\text{Antp}>N^{RNAi}$ lymph glands with dihydroethidium (DHE). This cell-permeable probe reacts with cellular superoxide ions to generate oxyethidium (ZHAO et al. 2003). The interaction of oxyethidium with nucleic acids is detected as red fluorescence (TARPEY et al. 2004). Fig. 12 shows strong red fluorescence in the medulla in control glands, indicating high ROS levels. However, ROS are not detected in the niche and they are low in the cortex, where cells retain the unreacted probe (Fig. 12A, A’’, blue).

In $\text{Antp}>N^{RNAi}$ lymph glands, the “high ROS” population (presumed medulla) is depleted (Fig. 12B). However, $\text{Antp}>mCD8-GFP$-positive cells with $N^{RNAi}$ show no change in ROS levels and exhibit significantly low or no ROS (Fig. 12B’’), much like the niche of control lobes (Fig. 12A’’). Even NICD expression in the niche did not alter ROS levels in the niche (Fig. 12C’’). However, ROS levels are significantly higher in the $N^{RNAi}$ cortex (Fig. 12B) but not in NICD cortex (Fig. 12C). These observations suggest that a ubiquitous effect of Notch signaling is to maintain low ROS in the anterior lobes, which may in turn maintain lamellocyte progenitors in an undifferentiated state.
DISCUSSION

In this study we report a dual role for Notch signaling in larval hematopoiesis: First, Notch appears to play a reciprocal role in the development of crystal cells and in lamellocyte development. Moderate levels of wild type Notch signaling promote crystal cell selection, but, contrary to expectation, support quiescence and not differentiation of lamellocyte progenitors. The inhibitory influence on lamellocyte differentiation is exerted non cell-autonomously and correlates with high oxidative stress in lobes undergoing lamellocyte differentiation and dispersal.

Dual and reciprocal non-autonomous effects of N on lineage decision

Division and development of a heterogeneous hematopoietic progenitor population in the third instar medulla is arrested; cortical cells in proximity to the medulla are less fully developed than peripheral cortical cells. Signals from the niche, cortex, and the medulla maintain medullary quiescence, and some of these signals must be modified to release progenitors from their quiescent state (reviewed in (Fossett 2013). Our experiments reveal that (1) Notch protein, while ubiquitously expressed, shows considerably variable subcellular localization suggesting different states of N activities in the organ (Fig. 1); (2) Notch activity is low and ubiquitous in first and second instar lobes, but increases in the third instar stage as the host becomes competent for defense against parasite attack ((Sorrentino et al. 2002) and Fig. 2); (3) Although reduction in Notch activity does not affect Antp>GFP expression, niche size, or the development of the plasmatocyte lineage, it has divergent effects on the other two lineages. While moderate Notch activity encourages the selection and development of individual crystal cell in the lobe cortex, the same activity maintains lamellocyte progenitors in an undifferentiated state (Fig. 4-10).
Lamellocytes themselves do not express high levels of Notch (Fig. 1) and it is quite possible that Notch protein levels are downregulated during the differentiation process.

Notch’s autonomous effects on crystal cell development are understood in some detail (Lebustky et al. 2003; Mukherjee et al. 2011; Terriente-Felix et al. 2013). Our results suggest that ubiquitous Notch activity in third instar lobes (Fig. 2) in basal hematopoiesis is required for the selection of the correct proportion of crystal cells in the third instar lobe resulting in their dispersed distribution pattern. Other cell signaling mechanisms including Notch signaling itself, build on this Notch activity “pre-pattern” for continued crystal cell development and maturation. Temperature-restricted $N^{ts}$ lobes exhibit normal medulla/cortex zonation (Fig. 7) and produce Nimrod-C-positive plasmatocytes in their correct location (our unpublished results) but are unable to produce crystal cells ((Lebustky et al. 2003) and Fig. 3). However, forced expression of NICD (or $N^{RNAi}$) in the niche alone is sufficient to encourage (or discourage) crystal cell development (Fig. 4). More significantly, ectopic NICD expression in mitotic clones alters and overcomes the effects of the normal “pre-pattern” in basal hematopoiesis and programs all clonal cells into crystal cells (Fig. 5).

We were surprised to discover that temperature-restricted $N^{ts}$ lobes are immune competent and can produce lamellocytes (Fig. 7). Thus, reduced Notch does not compromise lamellocyte differentiation (i.e., Notch may not be needed for lamellocyte differentiation). Instead, under low Notch, lamellocyte progenitors are specified and are developmentally competent. The inhibitory effect of Notch activity on lamellocyte progenitors in the absence of infection is observed upon RNAi knockdown in the niche or cortex (Fig. 8, 9) or removal of $N$ function in somatic clones (Fig. 10, 11). We propose that a second role for moderate ubiquitous Notch activity is to generate a signal that shelters lamellocyte progenitors in the absence of
infection. Infection reduces this ubiquitous Notch activity releasing lamellocyte progenitors to
mature (Fig. 6).

Are the reciprocal effects of N on crystal cells and lamellocytes linked? Recent studies
have uncovered unexpected plasticity in hematopoietic development (AVET-ROCHEX et al. 2010;
HONTI et al. 2010; KROEGER et al. 2012; STOFANKO et al. 2010). Wasp infection induces
proliferation and lamellocyte differentiation (KRZEMIEN et al. 2010b; SORRENTINO et al. 2002),
but infection reduces the abundance of crystal cells (KRZEMIEN et al. 2010b). Our results provide
a mechanism whereby reduction in Notch activity would coordinate alternative fate choice in
basal versus activated hematopoiesis; such a switch would allow progenitor reprogramming to
tailor host defense to promote wasp egg encapsulation. As such, lymph gland dispersal and
release of plasmatocytes and lamellocytes into the hemolymph alters the circulating blood cell
population, enhancing the host’s immune competence. Our data reveal that Notch plays a critical
role in this process. Similar scenarios for Notch mediated restriction of alternative cell fates has
been observed at multiple stages of vertebrate hematopoiesis. Notch signaling in zebra fish
promotes the production of hematopoietic stem cells at the expense of endothelial cells from a
common bi-potent hemangioblast (LEE et al. 2009a). Subsequently Notch1 signaling is observed
in the mouse thymus, where Notch manages the checkpoint for T-cell commitment at the
expense of the B-lineage program; and Notch1 collaborates with GATA-3 to commit progenitors
to the T cell lineage (GARCIA-OJEDA et al. 2013). Further downstream Notch favors adoption of
an αβ over γδ T cell fate (RADTKE et al. 2005). Our study expands the recurrent and burgeoning
theme of selecting alternative cell fates by activating Notch signaling at crucial lineage branch
points in multiple development contexts and in diverse organisms.
Notch signaling blocks the production of high ROS in anterior lobes

Reduction of Notch signaling appears to influence progenitor quiescence via increased levels of superoxide ions in most lobe cells, but not in the niche (Fig. 12). Surprisingly, cells in the lobe where levels of N (or N pathway components were reduced), show high levels of oxyethidium in response to high superoxide. Thus, it appears that Notch signaling can have systemic effects on the oxidative stress level of the organ. Aberrant increase in cellular ROS is associated with loss of quiescence and precocious differentiation of medullary progenitors (OWUSU-ANSAH and BANERJEE 2009). In addition, the hypoxia protein HIF-α interacts with NICD to promote transcription of target genes that support crystal cell survival (MUKHERJEE et al. 2011). Wasp infection induces high ROS in the niche; infection results in the secretion of the epidermal growth factor-like cytokine, Spitz, whose activation of rhomboid and the EGF receptor induces lamellocyte differentiation in the cortex (SINENKO et al. 2012). Thus, oxidative stress via ROS has multiple and complex effects on hematopoietic cell quiescence, differentiation or survival. Our results point to the possibility that Notch signaling maintains low oxidative stress in cells of the cortex (but not the niche) to preserve lamellocyte progenitor quiescence and promote crystal cell development and survival. Reduced N, or an increase in ROS signals wasp-induced lamellocyte differentiation. The mechanism underlying ROS changes in the cortex remain to be explored.

The developing lymph gland has emerged as a powerful system to study mechanisms governing hematopoietic stem/progenitor cell quiescence and differentiation. We show that Notch activity controls quiescence versus development for divergent lineages. Moderate Notch activity correlates with low ROS levels and lamellocyte quiescence. Experimental evidence suggests that a low-oxygen milieu of the bone marrow protects mammalian hematopoietic stem
cells and the supporting niche cells from oxidative stress whereas high ROS correlates with their 
ability to self renew (ITO et al. 2006; PARMAR et al. 2007; PICOLI et al. 2005; TOTHova et al. 
2007; WANG and WAGERS 2011). Primitive hematopoietic stem cells with low ROS exhibit 
properties of quiescent stem cells which include slow cycling and expression of Notch1 (Jang 
and SHARKIS 2007). Thus, it is possible that intracellular ROS levels coordinate with signaling 
pathways to specify quiescence versus proliferation and differentiation. ROS have traditionally 
been considered to be agents of cell damage and are associated with many human pathologies 
(BALABAN et al. 2005). Recent data link ROS more directly to cellular function and homeostasis 
via signaling pathways (HAMANAKA et al. 2013). Given the extensive conservation of N 
functions in fly and mammalian hematopoiesis (Fossett 2013; Krzemien et al. 2010a), our 
studies open the possibility of more thoroughly understanding how signal transduction and ROS 
are integrated and how this integration coordinates basal and activated hematopoietic 
development.
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LITERATURE CITED


to lock haemocytes into a differentiation programme. Development 137: 27-31.


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Fig. 1. Notch intracellular domain expression in third instar lymph glands

(A) Variable Notch expression (red) in the cortical cells of a mid third instar lymph gland. (A’) Magnification shows weak Notch signal in Hml>GFP-positive cells, whereas some Hml>GFP-negative cells have high punctate cytoplasmic staining (arrow). (B-B’’) In the medulla, high Anti-Notch staining colocalizes with the Dome>mCD8-GFP membrane signal (arrow). (C-C’’) Notch also colocalizes with Antp>mCD8-GFP signal in an early third instar lymph gland niche (arrow). (D-D’’) Notch staining is lower or is not detected in large lamellocytes (Alexa 488-Phalloidin positive, green) in wild type mid-third instar lymph glands from wasp-infected animals. Lamellocytes are rarely found in uninfected wild type lymph glands (see Fig. 6H). Hoechst (blue) stains DNA.

Fig. 2. Ubiquitous and localized N activity in the larval lymph glands

(A-D) Anti-β-galactosidase staining detects moderate ubiquitous expression of Su(H)-lacZ reporter transgene in lobes of first, second, or third instar larvae. Higher expression is detected in a few scattered cells, presumed crystal cells, in lobes from the third instar animals. (Also see Fig. 6).

Fig. 3. Ubiquitous Notch reporter activity depends on functional N product

Lymph glands from N\text{ts}2/Y; Su(H)-lacZ larvae at permissive (A, B), or non-permissive (C, D) temperature, stained with anti-β-galactosidase antibody (red). Moderate Notch activity at permissive temperature (A, B) is significantly reduced at non-permissive temperature (C-E). Standard deviation is shown.
Fig. 4. A non cell-autonomous role for N in crystal cell development

(A) Anterior lobes of Antp>mCD8-GFP lymph gland stained with anti-pro-PO antibody (red) marks crystal cells in control lobes. (B) Antp>NICD lobes stained with anti-pro-PO antibody (red) have more crystal cells compared to control lobes. (C) A significant reduction in crystal cell number is observed in Antp>N^RNAi lobes. Samples were taken from mid-third instar animals.

LG refers to lymph gland; standard error and p-value are shown.

Fig. 5. A cell-autonomous role for the Notch intracellular domain in crystal cell development

(A-B") hsFLP; actin>NLS-GFP control clones marked with GFP are negative for pro-PO (red). High magnification of the defined areas A’ and A” from panel A and defined areas B’ and B” in panel B are shown. (C-D") Most cells in hsFLP; actin>NLS-GFP, NICD clones express pro-PO (red), but not Nimrod C (P1). High magnification of the defined areas C’ and C” in panel C, and D’ and D” in panel D are shown. Hoechst channel is omitted in panels B and D for clarity.

Fig. 6. Notch activity is reduced after wasp infection

Anti-β-galactosidase antibody (red) -stained lobes from Su(H)-lacZ animals co-stained with anti-pro-PO (green) to detect coincidence of high Notch activity in crystal cells (stars). Cells expressing Su(H)-lacZ only make up 25.3% of all labeled cells; cells positive for pro-PO only constitute 37.3% of all labeled cells. The remainder labeled cell population (37.3%) are double-positive (n = 406 cells from 20 lobes). Control animals did not experience wasp attack (A-C’), or animals were exposed to wasps (D-F) before dissection. Upon infection, Notch activity decreases
(D-G). Standard deviation is shown panel G. (H, I) Lamellocyte differentiation in Su(H)-lacZ lobes is normal. Anterior lobes from uninfected Su(H)-lacZ (H), or infected (I) animal. Lamellocytes are labeled with Phalloidin tagged with Alexa Fluor-647 (white), which also labels the dorsal vessel.

**Fig. 7. Lymph glands from Nts animals are competent for lamellocyte differentiation**

Lymph gland lobes (A-F), or circulating cells in the hemolymph (C-F, bottom inserts), stained with Rhodamine-Phalloidin to detect the presence of lamellocytes in Nts1 (A-D) or Nts2 (E, F) mutants reared at permissive (A, C) or non-permissive (B, D-F) temperatures.

**Fig. 8. NotchRNAi correlates with lamellocyte differentiation and lobe dispersal**

Supernumerary lamellocytes are detected with msnf9-cherry reporter (B) or with Rhodamine Phalloidin (D), in Antp>N RNAi lobes (B, D-D”), and not in control lobes (A, C-C”). Lamellocyte differentiation accompanies lobe dispersal (D-D”). Hemolymph smears from control (E) and Antp>N RNAi (F) animals stained with Rhodamine Phalloidin show circulating lamellocytes in experimental but not control samples.

**Fig. 9. Lamellocyte differentiation is induced by neurRNAi and SerRNAi**

(A) Control Antp>GFP and (B, B’) Antp>neurRNAi lymph glands stained with anti-L1 antibody (red). L1-positive lamellocytes are found in the cortex adjacent to satellite niche cells (green). (C) Control Antp>GFP and Antp>SerRNAi (D, D’) lymph glands stained with Rhodamine Phalloidin. F actin-rich lamellocytes (red) are adjacent to the GFP positive niche cells. Hoechst stains DNA (blue). (E) Niche size is unaffected by knockdown of N pathway components. Left:
Average number of GFP-positive cells per niche \((n \geq 20)\) are not significantly different in control \((\text{Antp} > mCD8-GFP \text{ and } \text{Antp} > y \text{ w})\) lobes and experimental \((\text{RNAi of } N, \text{ Ser, neur or Su(H)})\) lobes. Right: The average area of GFP positive cells per niche \((n \geq 20)\) is not significantly different between control and experimental lymph glands. Standard deviations for both parameters are shown.

**Fig. 10.** \(N^{\text{RNAi}}\)-flp-out clones encourage lamellocyte differentiation and lobe dispersal

(A, C) Control lobes after induction of somatic recombination with NLS-GFP-positive cells but without \(N^{\text{RNAi}}\), do not induce lamellocyte differentiation (A), or lobe dispersal (C). The edge of the lobe in panel C is continuous (arrows) suggesting the presence of an intact basement membrane. (B, D) Clones with NLS-GFP and \(N^{\text{RNAi}}\) induce lamellocytes (B, arrow) and lobe shows a discontinuous edge indicating disrupted basement membrane (D).

**Fig. 11.** Notch clones reveal non cell-autonomous function in lamellocyte differentiation

(A) Schematic of twin clones (genotypes labeled) and lamellocytes in the cortex of an \(N^{55e11}/N^+\) anterior lobe. The schematic corresponds to an analysis of 14 z-stacked confocal images, each of 0.8 \(\mu\)m thickness; merged z-stacks are shown in panels B-C”. (B) A mutant \((N^{55e11}/N^{55e11})\) clone, marked by the absence of \(\beta\)-galactosidase and a wild type \((N^+/N^+)\) clone detected by high levels of \(\beta\)-galactosidase (red). Lamellocytes positive for integrin \(\beta\) are also \(\beta\)-galactosidase-positive (yellow), and these cells are found outside the clone boundary (B-C”). The region of interest in panel B is shown at a higher magnification in panels C-C”, where double-positive \(N^+/N^+\) lamellocytes with large, thin morphologies are more clearly evident. Hoechst stains DNA (blue).
Fig. 12. \(N^{\text{RNAi}}\) induces high levels of ROS in blood cells, but not in the niche

ROS staining of freshly-dissected lymph glands of control (\(\text{Antp}\rangle mCD8-GFP, \ A-A''\)), experimental (\(\text{Antp}\rangle mCD8-GFP, \ N^{\text{RNAi}}\) (B-B''), and (\(\text{Antp}\rangle mCD8-GFP, \ NICD\) (C-C'')) animals. High ROS is detected in the medulla and cortex of \(\text{Antp}\rangle mCD8-GFP, \ N^{\text{RNAi}}\) lymph glands. ROS levels are low or undetectable in the niche of all three genotypes (A'', B'', C'').
Fig. S1. Specificity of Notch staining

(A-A") Notch staining in Antp > mCD8-GFP background shows Notch signal (red) in all GFP-positive and GFP-negative cells. (B-B") Reduction of the Notch staining signal is observed in GFP-positive, but not in GFP-negative, Antp > mCD8-GFP; N RNAi lobe. (C) Antp > mCD8-GFP; N RNAi lobe stained with Alexa Fluor 488–linked Phalloidin (pseudocolor) to detect F-actin. Cells of lamellocytic morphology and rich in F-actin appear in the vicinity of the niche.

Fig. S2. Cell-autonomous role for N in crystal cell differentiation in posterior lobes

(A, B') hsFLP; actin>NLS-GFP control clones in a posterior lobe do not express pro-PO (red). (B, B') Magnification of the region outlined in A and A'. (C, D') Unlike the control lobes, the hsFLP; actin>NLS-GFP, NICD posterior lobe produced clones that also express pro-PO. (D, D') Magnification of the region outlined in panels C and C'. Hoechst stains DNA. For clarity, this channel is omitted in panels A’, B’, C’, D’.

Fig. S3. Wasp infection increases ROS in the niche

(A-A") ROS levels (red) in the niche of uninfected WT (wild type; Antp > mCD8-GFP) lymph gland are low or undetectable (A") compared to levels in the corresponding lymph gland regions from infected animals (B-B”). ROS levels are noticeably higher in the rest of the lymph gland lobe. Confocal settings of infected glands were lowered to image the intensity of ROS.
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Fig. 2. Ubiquitous and localized $N$ activity in the larval lymph glands
Fig. 3. Ubiquitous Notch reporter activity depends on functional $N$ product

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Fig. 7. Lymph glands from $N^t$s animals are competent for lamellocyte differentiation
Fig. 8. *Notch*<sup>RNAi</sup> correlates with lamellocyte differentiation and lobe dispersal

Fig. 9. Lamellocyte differentiation is induced by *neur*<sup>RNAi</sup> and *Ser*<sup>RNAi</sup>
Fig. 10. $N^{RNAi}$-flp-out clones encourage lamellocyte differentiation and lobe dispersal

Fig. 11. Notch clones reveal non cell-autonomous function in lamellocyte differentiation
Fig. 12. \(N^{RNAi}\) induces high levels of ROS in blood cells, but not in the niche

Table S1. The effects of \(N^{RNAi}\) in the wild type lymph glands

Dissected lymph glands were analyzed for the presence of three or more lamellocytes or signs of dispersal, both of which are criteria for immune activation. The Valium 10 construct elicited a stronger response than the Valium 1 construct.

<table>
<thead>
<tr>
<th>Valium</th>
<th>Genotype</th>
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<th>Lymph Glands with Lamellocytes</th>
<th>Dispersed Lymph Glands</th>
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