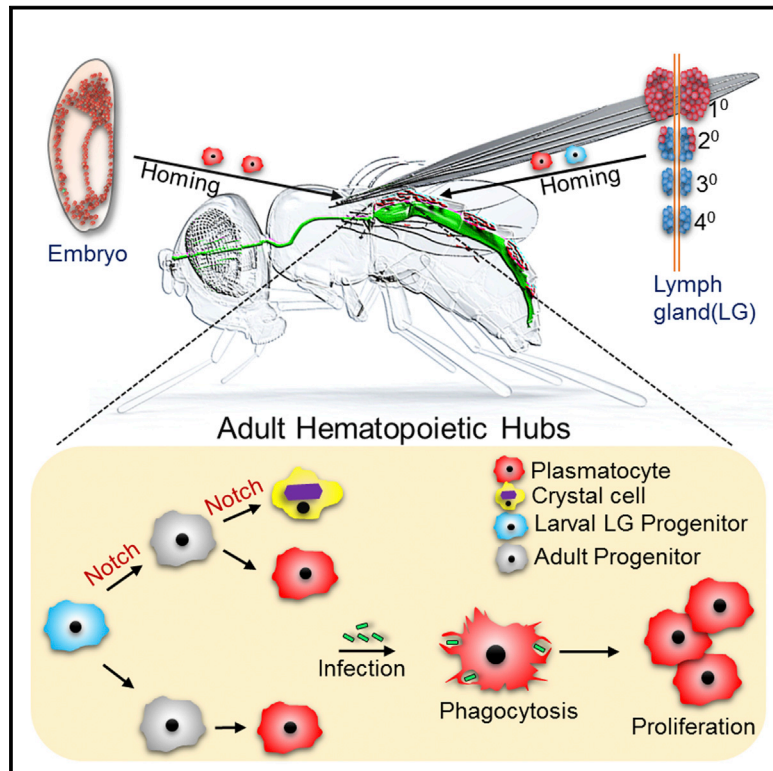


Developmental Cell

Active Hematopoietic Hubs in *Drosophila* Adults Generate Hemocytes and Contribute to Immune Response

Graphical Abstract



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In Brief

Definitive hematopoiesis in *Drosophila* was thought to occur only in the larval hematopoietic organ, which ruptures upon pupation. Ghosh et al. now demonstrate the presence of active hematopoietic sites in the abdomen of adult flies, which can give rise to new blood cells and can respond to immune challenges.

Highlights

- An active hematopoietic hub exists in the abdomen of adult *Drosophila*
- Progenitors within the hub can give rise to plasmatocytes and crystal cells
- Resident plasmatocytes show immune responses and proliferate upon infection
- Progenitors residing in the hub originate from the posterior lobes of lymph gland



Active Hematopoietic Hubs in *Drosophila* Adults Generate Hemocytes and Contribute to Immune Response

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SUMMARY

Blood cell development in *Drosophila* shares significant similarities with vertebrate. The conservation ranges from biphasic mode of hematopoiesis to signaling molecules crucial for progenitor cell formation, maintenance, and differentiation. Primitive hematopoiesis in *Drosophila* ensues in embryonic head mesoderm, whereas definitive hematopoiesis happens in larval hematopoietic organ, the lymph gland. This organ, with the onset of pupation, ruptures to release hemocytes into circulation. It is believed that the adult lacks a hematopoietic organ and survives on the contribution of both embryonic and larval hematopoiesis. However, our studies revealed a surge of blood cell development in the dorsal abdominal hemocyte clusters of adult fly. These active hematopoietic hubs are capable of blood cell specification and can respond to bacterial challenges. The presence of progenitors and differentiated hemocytes embedded in a functional network of Laminin A and Pericardin within this hematopoietic hub projects it as a simple version of the vertebrate bone marrow.

INTRODUCTION

In the recent past, *Drosophila* has been established as one of the best invertebrate model systems to study hematopoiesis. Studies from several groups have elucidated in great details the process of blood cell formation in *Drosophila* both during larval and embryonic stages (Evans et al., 2007; Hartenstein, 2006; Waltzer et al., 2010) and have demonstrated a significant level of conservation of signaling molecules and pathways across metazoans. While the hemocytes during embryonic phase develop from the head mesoderm, the larval lymph gland serves as the hematopoietic organ that ruptures in the pupal phase to release the differentiated hemocytes into circulation (Grigorian et al., 2011). Although the active sites for hematopoiesis have been identified in *Drosophila* embryo and larvae, it is

generally perceived that there are no active centers for hematopoiesis in adult flies. Rather, it has been demonstrated that the circulating hemocytes in the flies are primarily of embryonic and larval origin (Holz et al., 2003; Honti et al., 2014). We argued that compared with larvae, since the adults experience diverse challenges during their lifetime, it is difficult to conceive that they would be able to circumvent these crises merely depending upon the reservoir of long-lived hemocytes of earlier stages. This intriguing yet uncharted aspect motivated us to explore for active center of hematopoiesis in adult flies.

Our effort has led to the identification of four hematopoietic blood cell clusters in dorsal side of the fly abdomen. The hemocytes within these clusters are embedded in a network of Laminin A and Pericardin. Apart from housing hemocytes originating from both embryonic and larval lineages, these clusters harbor progenitor cells that trace their origin to the tertiary and quaternary lobes of larval lymph gland. Importantly, the precursors are capable of de novo generation of both plasmatocytes and crystal cells. The surge of post-larval hematopoiesis partly relies on Notch signaling, and the resident plasmatocytes are capable of responding to bacterial challenges. All these observations, put together, establish these active hematopoietic hubs as a simpler version of vertebrate bone marrow.

RESULTS AND DISCUSSION

Identification of Hematopoietic Clusters in Adult *Drosophila*

Employing *hemoclectin-Gal4, UAS-GFP*, we have identified four hematopoietic blood cell clusters along the dorsal midline in the abdominal segments A1–A4 of adult flies (Figures 1A–1C and S1A–S1E; Movies S1 and S2). Of the four clusters, the one in the abdominal segment A1 has the maximum aggregation of cells (Figure S1F) that occupies the area that spans the lateral and dorsal sides of the heart (Figures 1D–1H and S1G–S1K). Located just below the dorsal cuticle of the abdominal cavity, the cells are assembled in a groove defined by transverse heart muscles and body wall muscles (Figures 1D–1F, S1G, and S1G'). The longitudinal heart muscle forming the dorsal diaphragm separates the heart and the cluster from abdominal cavity (Figures 1E and 1F). With respect to the pericardial diaphragm formed by pericardial cells present on either side of the cardiac tube,

these hemocytes are located dorsally (Figure 1G). Thus, these clusters remain secluded from rest of the abdominal cavity by the dorsal and the pericardial diaphragm (Wasserthal, 2007) (Figures 1H and S1N).

The hemocytes within the clusters are embedded in an extensive network of extracellular matrix proteins surrounding the heart and the pericardial cells. One of the important components of this network is the type IV collagen-like protein, Pericardin (Figure 1I; Movie S3). In homozygous mutant for *lonely heart* (*loh*), a gene encoding a secreted receptor of Pericardin (Drechsler et al., 2013), the hemocytes fail to form the cluster (Figures 1J–1M, S1M, and S1M'), as this network gets significantly affected. Similar result is observed upon knocking down the expression of Laminin A (Figure 1N), another important component of the network, by driving *UAS-laminin A RNAi* in the cardiac tube by *mef2-Gal4* (Figure 1O). Based on expression and functional analyses, we conclude that both Pericardin and Laminin A function in maintaining adhesive interaction with the hemocytes aiding in formation of the clusters. Interestingly, Laminin A polypeptides (Gu et al., 2003; Siler et al., 2000) and collagen IV (Nilsson et al., 1998) are also prevalent in vertebrate bone marrow. Our finding that the blood cells are fenestrated in a functional network of Laminin A and Pericardin raised the speculation that these sites might function as bone marrow-like tissues in adult flies and thereby demanded an in-depth analysis of the cell types present therein.

Presence of Embryonic and Larval Lineages in the Cluster

For detailed characterization of the cell types, we focused on the largest aggregation present in the segment A1. Primarily based on the expression of *peroxidase-GFP* (*pxn-GFP*) and NimC1/P1, we found the cluster to house a large number of plasmatocytes (Figures 1P and 1R), the most predominant differentiated blood cell type (Jung et al., 2005).

Interestingly, the cells in the cluster express *croquemort* (*crq*), an embryonic marker for plasmatocytes (Figure 1Q). We further validated the embryonic origin of the plasmatocytes by using G-TRACE construct (Evans et al., 2009) (Figure S3D; see Supplemental Information for details) that enables us to detect cells that had once expressed any particular gene prior to the time of investigation (lineage traced) as well as those in which the gene is expressed at the time of observation (live expression). Activation of G-TRACE system by a *Gal4* for *glial cell missing* (*gcm*), a gene known to express exclusively in embryonic plasmatocytes (Lebestky et al., 2000), results in the detection of few P1-positive *gcm* lineage traced (enhanced Green Fluorescent Protein [EGFP]) cells (Figure 1R), thereby confirming that the cluster harbors plasmatocytes of embryonic origin. In addition to these markers, the cells in the cluster express several lymph gland hemocyte-specific markers like *ZCL2897* (Jung et al., 2005) (Figure 1S), and are *Serpent* (*Srp*) and *dorothy-GFP* (Honti et al., 2010) (Figures 1T–1T''') positive, even some of them are lineage traced (Figure 1U) for *collier* (Crozatier et al., 2004). Thus, the hemocyte clusters is a medley of embryonic and larval lineages (Figures 1V and S1L).

Despite one report that suggests the presence of C4 expressing crystal cells in adult circulation (Kurucz et al., 2007b), it is considered that crystal cells are not present in adults (Binggeli et al.,

2014). Primarily, this is due to the absence of any Prophenoloxidase (proPO) expressing crystal cell in circulation. We, however, observed that 5-days post-eclosion (dpe), there are some Hindsight (*Hnt*)-positive crystal cells (Terriente-Felix et al., 2013) present within the cluster (Figure 1W). Co-localization of *lozenge-GFP* (*lz*) with proPO (Rizki and Rizki, 1984) further supports our findings (Figures 1X–1X'). To have a functional correlate, we induced heat activation of proPO in crystal cells (Rizki and Rizki, 1984). This results in formation of melanized crystal cells on dorsal side of the abdomen, corresponding to the position of first cluster (Figures 1Y and 1Y'). These results clearly establish the presence of resident functional crystal cells in the clusters.

The Cluster Houses Hemocyte Precursors

GATA factor *Serpent* (*Srp*) is expressed in low levels in all hemocytes, including plasmatocytes and crystal cells (Figure 2A). However, the hemocyte precursor cell can be identified by the presence of high levels of *Srp* expression (Rehorn et al., 1996). Analysis of developing cluster at 2 dpe reveals the presence of cells positive for both *Srp* and *hml* (plasmatocytes) (Figures 2B–2B''), and a small subset of cells exclusively expressing *Srp*. No crystal cells (*Hnt*) are present in the cluster. In contrast, at 5 dpe, along with the two cell types mentioned above, some *Srp*- and *Hnt*-positive crystal cells are seen (Figures 2C–2C''). Quantitative analysis of the above observations clearly demonstrate an increase in the number of differentiated cells (plasmatocytes and crystal cells) with a concomitant decline in the number of cells exclusively expressing *Srp* at 5 versus 2 dpe (Figure S2A). These results also indicate that the *Srp*-positive cells within the cluster that do not express either *hml* or *Hnt* might be the precursor cells, yet to turn on differentiation.

We then followed the crystal cell development in the cluster. Since activation of Notch (*N*) pathway precedes *Lz* expression in crystal cells (Duvic et al., 2002), we generated a recombinant fly line with *12XSu(H)lacZ* in the background of *lz-GFP*. *Su(H)lacZ*-positive cells are first seen in the cluster 2 dpe (Figure 2D), whereas the expression of *lz-GFP* is observed only on 3 dpe (double arrowhead; Figure 2E). Interestingly, some of these *lz-GFP*-positive cells still have low levels of *Su(H)lacZ* expression (arrowhead; Figure 2E). By 5 dpe, we observe an increase in the number of cells that are either expressing *lz-GFP* (double arrowhead; Figure 2F) or have low levels of *Su(H)lacZ* expression along with *lz-GFP* expression (single arrowhead in Figure 2F). However, at 7 dpe, while an increase in number of *lz-GFP* cells can be seen, there is a decrease in the number of double-positive cells (Figure 2G). The number of cells expressing only *Su(H)lacZ* that remain more or less unaltered till 5 dpe demonstrates a sharp decline on day 7. Quantitative analysis of the cell types present in the cluster based on the expressions of *Su(H)lacZ* and *lz-GFP* further ascertains the above observations (Figure S2B). These results, therefore, clearly demonstrate de novo origin of *lz-GFP*-positive crystal cells from *Su(H)lacZ*-positive cells within the cluster.

To determine whether *Su(H)lacZ*-positive cells originate from the *Srp* positive precursors, we monitored the expression of both *Srp* and *Su(H)lacZ* within the cluster. Initially, while some cells that turn on *Su(H)lacZ* have high levels of *Srp* expression (Figures 2H and 2H'), in subsequent days, as the *Su(H)lacZ* expression gets stabilized, a reduction in *Srp* expression is

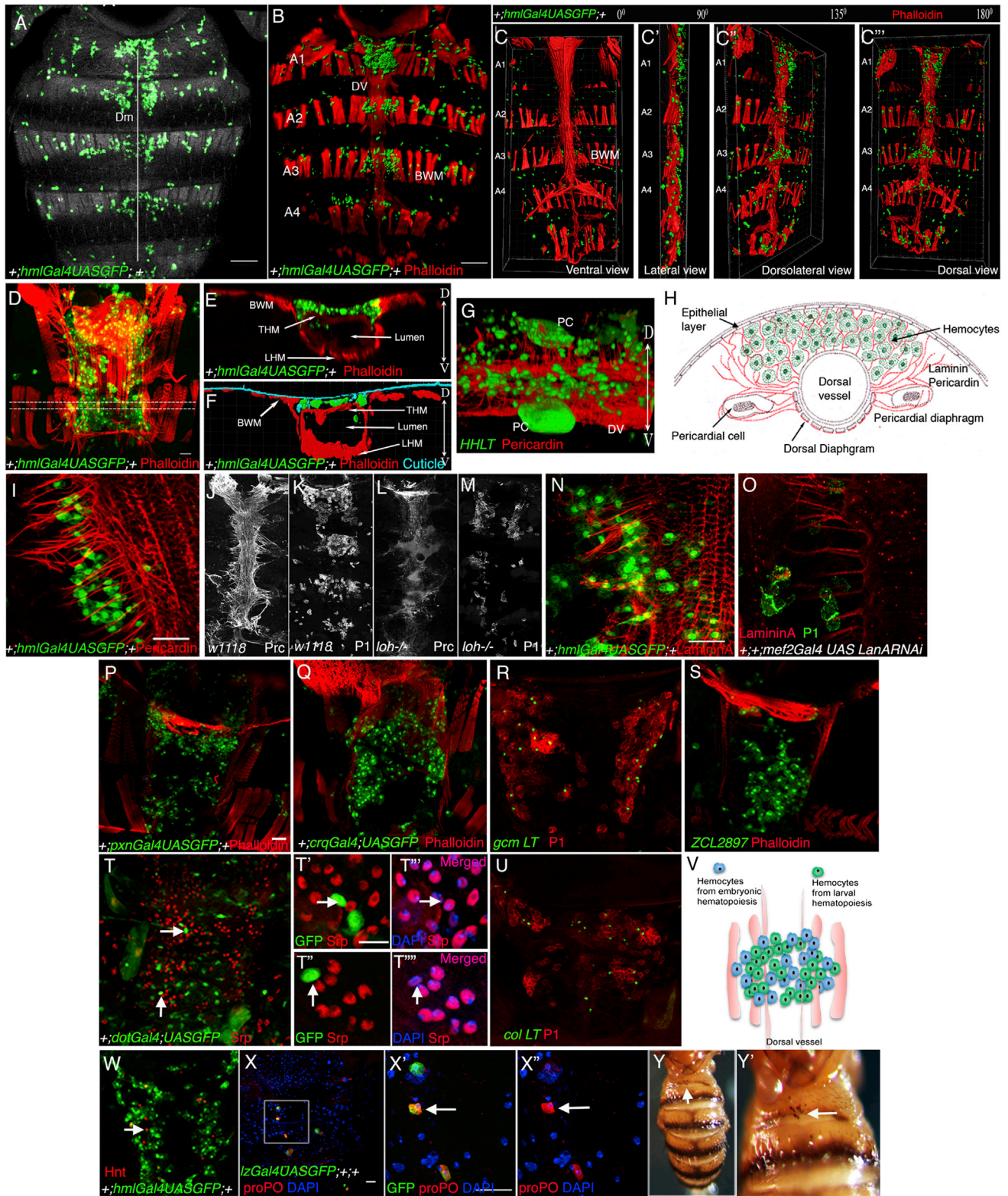


Figure 1. Adult Hematopoietic Hubs *Drosophila*

(A and B) Hemocyte clusters (A) in the abdominal segments on either side of dorsal midline (Dm) are closely associated with dorsal vessel (DV) (B). (C–C''') 3D reconstruction of a fillet showing the position of four abdominal hemocyte clusters (green) above DV (red). Horizontal rotation angles are as mentioned. (C') Lateral view of (C), whereas (C'') and (C''') are rotation of (C') toward dorsal side.

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observed (Figures 2I, 2I', and S2C–S2G'). This result in conjunction with our earlier observation establishes that crystal cells develop in adult cluster from high Srp-positive precursor cells, and this process requires N signaling. As a functional correlate to establish the presence of precursor cells in the cluster, we twitched N signaling to determine its effect on differentiation of crystal cells. Since the onset of *Su(H)lacZ* and *Iz-GFP* expression in the cluster is observed at 2 and 3 dpe, respectively, we impaired N signaling in the precursors by driving *UAS-N RNAi* using *hemese-Gal4* from 2 dpe. As shown in Figure 2K, this result in complete loss of crystal cells compared with that observed in WT clusters (Figure 2J). Interestingly, the marginal increase in the number of plasmatocytes observed by knocking down N correlates with the number of crystal cells missing in this genetic background when compared to control (Figure 2M). Likewise, overexpressing N in these cells results in almost 7-fold increase in the number of crystal cells with a significant drop in the number of plasmatocytes (Figures 2L and 2M).

It is therefore quite evident from our results that the clusters of blood cells on dorsal side of adult fly are not a mere aggregation of hemocytes of embryonic and larval origin but also houses true progenitors. The very fact that they house blood cell precursors and exhibit dynamicity as de novo crystal cells get differentiated within them qualifies them to be considered as active hubs of hematopoiesis in adult.

Origin of the Hematopoietic Precursors of the Hub

Upon identifying the hemocyte precursors, we sought to define their origin. In WT larvae, differentiation of plasmatocytes and crystal cells takes place in the primary lobe of the lymph gland, while secondary, tertiary, and quaternary lobes harbor the reserve blood cell precursors (Jung et al., 2005) (Figure 2N) that undergo differentiation only upon infection (Sorrentino et al., 2002). As reported earlier (Mondal et al., 2011), we found that driving *PvrRNAi* by *hml-Gal4*, *UAS-GFP* leads to differentiation of all cells present in the primary lobe of the lymph gland (Figure 2O). However, cells in the tertiary and quaternary lobes remain undifferentiated. Analyses of hematopoietic hub of

UAS-PvrRNAi; *hml-Gal4*, *UAS-GFP* flies reveal a drastic reduction in the number of plasmatocytes when compared with control (Figures 2P and 2Q). We attribute this reduction in plasmatocyte number to the block in migration of differentiated hemocytes in circulation that results from knocking down *Pvr* (Brückner et al., 2004). Interestingly in these clusters we do find a large number of Srp-positive precursor cells (Figures 2Q and S2I–S2L). We presume that this relative increase in number of Srp positive cells when compared with control might be an outcome of preventing circulating plasmatocytes from migrating into the cluster. More importantly, the presence of Srp-positive precursors in the clusters together with the observation that the hemocyte precursors only reside in the tertiary and quaternary lobes of the lymph glands of larvae of this genotype strongly indicates that these precursors actually originate from tertiary and quaternary lobes of larval lymph gland.

Our analysis of *collier* expression in late third instar larval lymph gland reveals that apart from being expressed in the posterior signaling center (PSC) of the primary lobe (Crozatier et al., 2004), it is also expressed at a very high level in the hemocyte precursors of tertiary and quaternary lobes of the lymph gland (Figure 2R). On activation of G-TRACE construct by *col Gal4* from late third instar larvae several *col* lineage traced cells (EGFP) are detected in the adult cluster (Figures 2S–2U and S2H–S2H''), thereby establishing that the hemocyte precursors present in the tertiary and quaternary lobes of larval lymph gland do migrate into these clusters. Some of these GFP positive *collier* lineage traced cells have high levels of Srp expression demonstrating their precursor state, while some have low levels of Srp expression (Figures 2S–2S''). Immunostaining of adult clusters having lineage traced *collier* EGFP cells with P1 and Hnt antibodies reveals that they are positive for either of this differentiation marker (Figures 2T–2U''). In addition, there are also *collier* lineage traced cells that express neither of these markers (arrows in Figures 2T, 2T', and S2H–S2H'') and therefore represent undifferentiated precursors. These results demonstrate that *collier* lineage traced progenitors in the hub originates from the hemocyte precursors present in the tertiary and quaternary lobes

(D) Position of hemocyte cluster 1 with relation to DV.

(E) Transverse section (dotted line through D) showing the cluster is nested in a groove created by body wall muscle (BWM) and transverse heart muscle (THM). LHM, longitudinal heart muscle.

(F) Location of hemocyte cluster with relation to dorsal cuticle (cyan).

(G) The hemocytes are dorsal to pericardial cells (PC). Genotype used: *w*; *hand-Gal4*, *hml-Gal4*, *UAS-FLP.JD1*, *UAS-2xEGFP/+*; *P{Gal4-Act5C(FRT.CD2).P}S/+*.

(H) Scheme depicting anatomical position of the cluster based on our observation.

(I) The hemocytes are fenestrated in a network of Pericardin.

(J and L) Compared with control (J), there is a drastic reduction in expression of Pericardin in *loh^{MB05750}* (L).

(K and M) The normal clusters of hemocytes (K) get disrupted in *loh^{MB05750}* (M).

(N and O) The nested hemocytes are intercalated in Laminin A (N), downregulation of which by *mef2-Gal4* affects cluster formation (O).

(P and Q) Plasmatocytes in hematopoietic cluster express *pxn* and *crq*.

(R) In a cluster of *w*; *UAS-GTRACE/gcmGla4*; *+/+fly*, some plasmatocytes (P1) are *gcm* lineage traced (EGFP).

(S) Some hemocytes in the cluster are positive for *ZCL2897*.

(T) Expression of *dorothy* (green) in the cluster (T).

(T'–T''') Zoomed in regions of T showing that *dorothy*-positive cells (arrow, green) express low levels of Srp (red).

(U) Presence of *collier* lineage traced cells (EGFP) in the *w*; *UAS-GTRACE/+*; *kn-Gal4/+* cluster.

(V) Schematic representation of the cluster and its components.

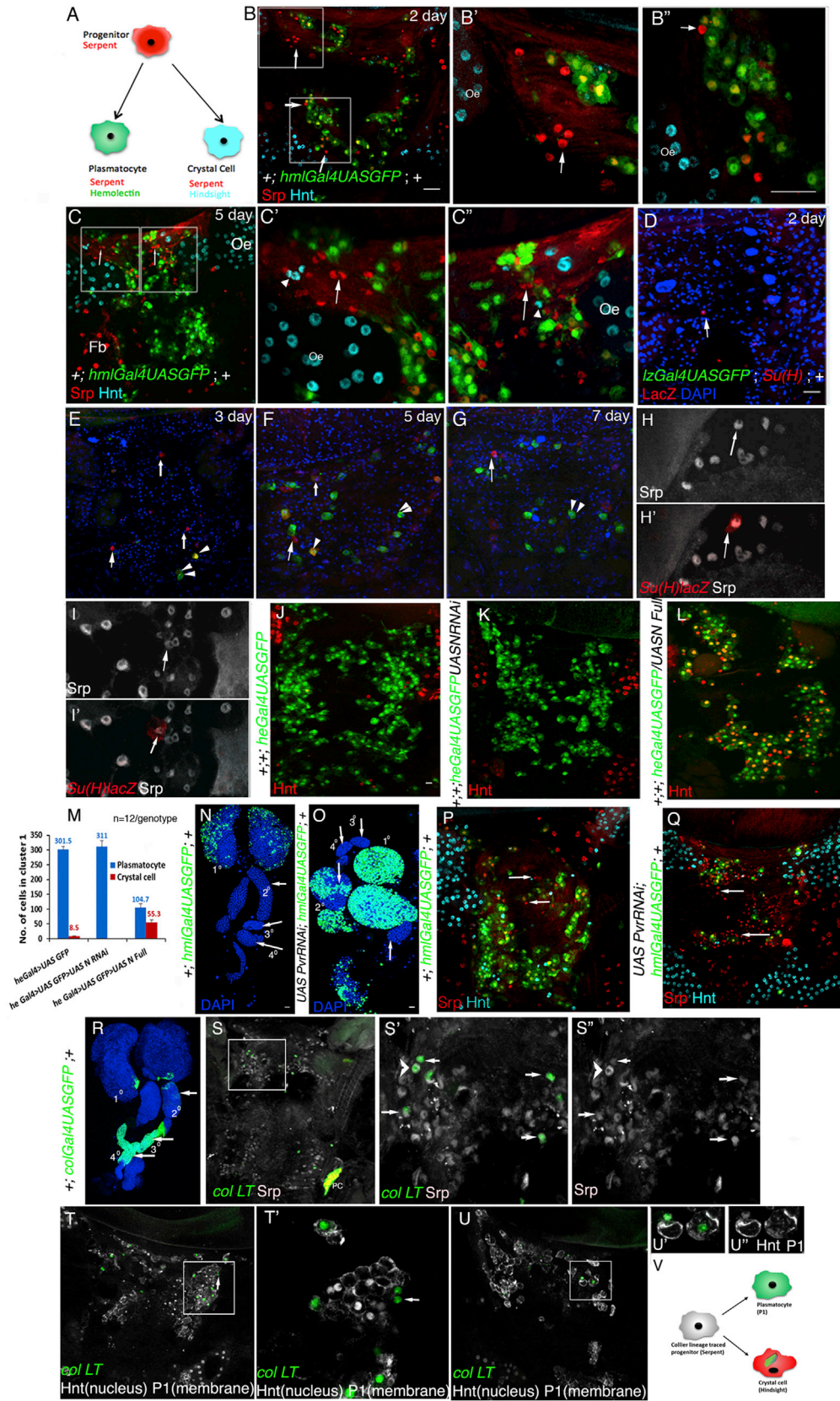
(W) Presence of crystal cells in the cluster.

(X–X'') Co-localization of *Iz-GFP* with proPO (red). (X') and (X'') are zoomed in region of (X).

(Y) Melanization of crystal cells (arrow).

(Y') Melanized crystal cells (arrow) are detected in a position that corresponds to the first cluster of hemocytes in A1.

Scale bar represent 100 μ m (A and B); 10 μ m (T'–T'''); the rest represent 20 μ m. See also Figure S1 and Movies S1, S2, and S3.



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of larval lymph gland and that they can give rise to both plasmacytes and crystal cells (Figure 2V).

Existence of a Post-larval Surge of Hematopoiesis in *Drosophila*

Next we wanted to investigate whether like crystal cells there is de novo differentiation of plasmacytes within the hub. For this purpose, we activated the G-TRACE cassette using *hml-Gal4* with the aim to lineage trace (by EGFP expression) all plasmacytes that are formed during embryonic and larval stages, as well as to mark by RFP expression any plasmacyte that is formed de novo within the adult cluster (Figures 3B' and S3D). Analysis of the cluster in adults at 5 dpe reveals the existence of plasmacytes that are either only lineage traced for *hml* expression (EGFP) or positive for both lineage traced and live expression (EGFP and RFP), or the ones that exhibit only RFP expression (arrow in Figures 3B–3D''). While RFP expression in the plasmacytes confirms current expression of *hml* in these cells, the very existence of RFP-positive hemocytes that are not lineage traced definitely implies de novo origin of these plasmacytes in the hub. Analyses of the hub at 4 and 8 dpe give identical results (Figures S3A–S3G'').

Next, we wondered about the signal that triggers this surge of hematopoiesis in adult. For this purpose, we generated flies where the lineage-tracing cassette can be activated by *notch-Gal4* in a genetic background that has a *Gal80^{ts}* allele. Presence of *Gal80^{ts}* allele would allow us to have a temporal control over the induction of the cassette by *Gal4*. Flies reared at 18°C for entire developmental period till 5 dpe (Figure 3E') to keep the *Gal80* active and in turn the *Gal4* inactive served as control. In these flies none of the plasmacytes (P1) are found to be N lineage traced, validating the tight regulation of *Gal80* on the G-TRACE construct (Figures 3E–3F'). Interestingly, in the same genetic background upon activating G-TRACE system by *notch-Gal4* 10-hours post-pupation, we found that all the differ-

entiating crystal cells are positive for both lineage traced and active N expression (Figures 3G–3H''). This observation is in tune with our finding that *Su(H)*, a downstream target of N is active in differentiating crystal cells in the cluster.

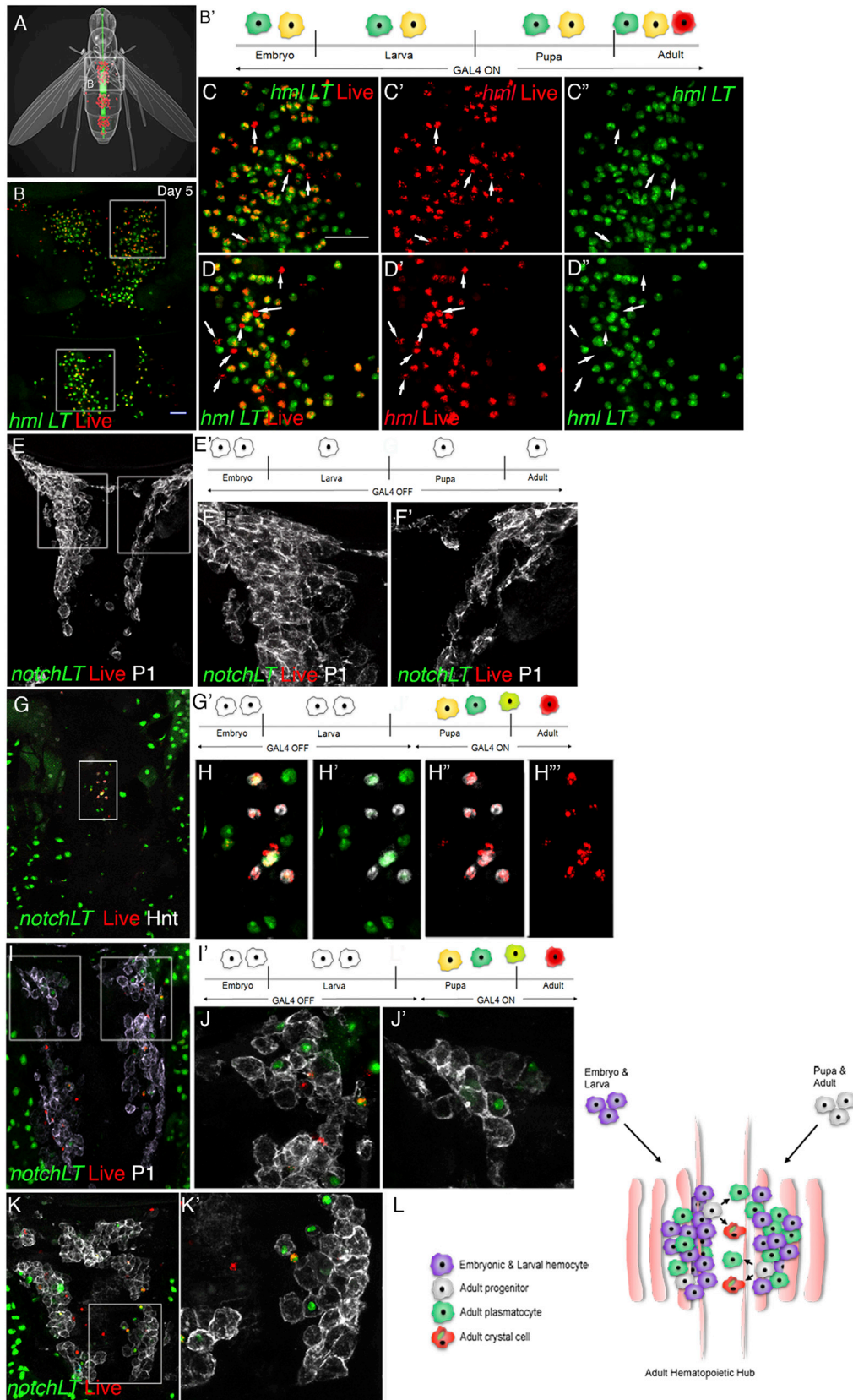
In contrast to this, only a small subset of plasmacytes (P1) is N lineage traced (Figures 3I–3K' and S3H–S3M'). Since the lineage tracing system was activated 10-hr post-pupation, a time point by which disruption of the larval lymph gland takes place, none of the lineage traced GFP-positive cells represents the population of differentiated larval plasmacytes that migrate to the hub. Rather, they represent the plasmacytes that are born in the post-larval period. However, the number of N lineage traced plasmacytes being very less compared with the total number of plasmacytes that actually differentiate in the hub (compared with RFP expressing cells in Figures 3C–3D''), we conclude that while a majority of the plasmacytes generate de novo in the hub by a N independent mechanism, some of them depend on N for their genesis. In sum, our results demonstrate that N signal is involved in the genesis of crystal cells as well as of some plasmacytes in the hematopoietic hubs of adult flies (Figure 3L).

Dynamic Hematopoietic Hub Generate Immune Response

Temporal studies using *hml-GFP* reveal that the number of plasmacytes in the hub do not remain the same during the adult life. There is a gradual increase in the number till 5 dpe; thereafter, it remains more or less constant up to 8 dpe. However, with aging, a decline in the plasmacyte number in the hub is observed (Figures 4A–4F). Since we were able to detect several progenitors till 5 dpe, we speculate that the initial increment in number of plasmacytes is partly due to the formation of new plasmacytes from these precursors. However, upon analysis of the number of plasmacytes present in adult circulation vis-a-vis to those present in the cluster at 2 and 5 dpe, we found that while there

Figure 2. Hemocyte Progenitors Are Present within the Hematopoietic Hub in Adult

- (A) Plasmacyte and crystal cell originate from precursors that express only *Srp*.
 (B) Presence of plasmacytes and few hemocytes that express only *Srp* (arrow) at 2 dpe in the hub.
 (B' and B'') Zoomed in regions of (B). Arrows denote cells that express only *Srp*.
 (C) At 5 dpe, crystal cells, plasmacytes, and several *Srp* positive (arrow) cells are seen in the cluster.
 (C' and C'') Zoomed in of (C) showing crystal cells (arrowhead) and *Srp*-positive hemocyte (arrow).
 (D–G) Temporal kinetics of expressions of *Su(H)LacZ* (red) and *Iz-GFP* (green) in the cluster. At 2 dpe, only *Su(H)LacZ*-positive cells are seen (arrow, D). Expression of *Iz-GFP* is turned on in some *Su(H)LacZ*-positive cells by 3 dpe (arrowhead, E). At 5 (F) and 7 (G) dpe, while the number of *Iz-GFP* (double arrowhead) expressing cells increase, a concomitant decrease in *Su(H)LacZ*-positive cells (arrow) is observed.
 (H–I') Expressions of *Srp* (gray) and *Su(H)LacZ* (red) in the cluster. (H and H') A cell (arrow) that has high levels of *Srp* expression also expresses *Su(H)LacZ*. (I and I') With stabilization of *Su(H)LacZ* (red) expression, the *Srp* expression declines (I and I').
 (J–L) Crystal cell number compared with control (J) drastically reduces on knocking down Notch expression (K), while its overexpression causes huge increment. (M) Quantitative analysis of (J)–(L).
 (N) *hml* expression in differentiated cells of primary (1°) lobe of the lymph gland.
 (O) Loss of *Pvr* in *hml* cells leads to complete differentiation of 1° lobe and while tertiary (3°) and quaternary (4°) lobe cells (arrows) remain undifferentiated.
 (P) *hml-Gal4*, *UAS-GFP* hematopoietic hub houses *Srp* (red) *hml* (green) and *Hnt* (cyan) expressing cells.
 (Q) Only few *hml* (green) cells and *Hnt* (cyan) cells are seen in *UAS-PvrRNAi*; *hml-Gal4*, *UAS-GFP* hub, which has plenty of *Srp* (red, arrow) only cells.
 (R) *collier* expresses (green) in PSC, 2° lobe and the 3° and 4° lobes of late third instar larval lymph gland.
 (S–S'') *col-Gal4* lineage traced cells (green) and *Srp* (gray) expression in the cluster. (S') and (S'') are zoomed in region of S showing *collier* lineage traced cells that are either high (arrowhead) or low (arrow) in *Srp* expression.
 (T and T') *collier* lineage traced cells (EGFP) in *w*; *UAS-GTRACE/+;kn-Gal4/+ hub* are either P1 (gray, membrane) or *Hnt* (gray, nucleus) or lack both *Hnt* and P1 expression (arrows in T–T').
 (U–U'') A crystal cell (*Hnt*) or plasmacyte (P1) can arise from *collier* lineage traced cells (EGFP). (U') and (U'') are zoomed in image of U.
 (V) Potential of *Srp* positive *col* lineage traced cells in the hematopoietic hub.
 Oenocyte (Oe) Fb, fat body, PC, pericardial cell. Scale bar represents 20 μm. Error bar denotes SE. See also Figure S2.



is a decrease in the number of plasmacytes in circulation from 2 to 5 dpe (Figure 4G), there is almost comparable increase in their number in the hub. We, therefore, presume that homing of circulating hemocytes also contributes in the initial increase of plasmacytes in the hub. While the gradual loss in resident plasmacytes suggests that the adult is regularly using the hemocytes from the hub as it ages.

Next, we addressed whether these plasmacytes has the potential to mount response when immune challenged, thereby aiding the fly in combating infections. We observed that when challenged with RFP labeled *E. coli*, the plasmacyte in the hub are able to phagocytose the bacteria (Figures 4H–4H’), confirming the fact that they are primed and ready for challenges imposed during adulthood. This observation of ours is in tune with the results of a previous study that demonstrated the existence of heart associated macrophages that can phagocytose (Horn et al., 2014).

Drosophila larval plasmacytes are endowed with the capacity to divide in order to increase their population (Makhijani et al., 2011). However, it is believed that adult plasmacytes lack the ability to proliferate and therefore considered to be in a state of senescence (Honti et al., 2014). Our data also endorse this finding, as no proliferating plasmacytes are found in hematopoietic hub even after continuous 5 days of 5-bromo-2-deoxyuridine (BrdU) feeding (Figures 4I–4I’ and S4B1–S4B5). However, analysis of the hub in flies infected with *E. coli* reveals BrdU incorporation in several plasmacytes, suggesting that they are in the S phase of cell cycle (Figures 4J–4J’ and S4A1–S4A5). Quantitative analysis reveals that at least 50% of the infected flies show BrdU incorporation (Figure 4K) compared with none of the uninfected ones. These data establish that the adult plasmacytes are not in complete senescence and do have the potential to re-enter into proliferative stage when need arises. Furthermore, our observation that soon after infection there is a quick release of a large fraction of hemocytes from the hub (Figures S4C–S4F) highlights the contribution of the hub to fight out infection.

Summing up, this study unravels the presence of active hematopoietic hubs in *Drosophila* adults (Figure 4L). Refuting the existing notion that adults rely on long-lived hemocytes originating from embryonic and larval stages, we are successful in establishing that a surge of hematopoiesis happens in these hubs as the precursors present within differentiate into both crystal cells and plasmacytes. The functionality of the hub gets further validated as we observed that besides exhibiting phagocytic activity the otherwise quiescent cells re-enter into

proliferative mode in response to bacterial infection. These findings bring about a paradigm shift in our understanding of the process of hematopoiesis in *Drosophila*. With its well-characterized embryonic and larval hematopoietic activities, *Drosophila* has been serving as a powerful model for hematopoietic studies. In spite of that, the system seemed to be incomplete due to lack of detailed developmental analysis of hematopoiesis in adults. Our effort in establishing that the process of definitive hematopoiesis extends to adults expands the scope of exploiting this model system.

In vertebrates, the hematopoietic stem cells (HSCs) originate from hemangioblast and undergo maturation and expansion by a complex developmental process that requires the involvement of the yolk sac, the aorta-gonad-mesonephros (AGM) region, the placenta and the fetal liver before finally colonizing into bone marrow (Mikkola and Orkin, 2006). HSCs present within the bone marrow are however, not identical. Primarily based on their potential they are classified into three types: (1) myeloid biased, (2) lymphoid biased, and (3) balanced HSCs that are bipotent in nature and thus can give rise to both myeloid and lymphoid lineages (Müller-Sieburg et al., 2002). In a strikingly similar manner, the larval lymph gland houses blood cell precursors originating from hemangioblasts that arise from the embryonic cardiogenic mesoderm, a region analogous to the developing AGM of vertebrates (Mandal et al., 2004). Upon undergoing expansion within the lymph gland, some of these precursors actually home into the adult hematopoietic hub. Importantly, we found that the precursor cells within the hub are not homogeneous. While some of them are capable of differentiating into plasmacytes independent of N signaling, there exist few bipotent precursors. Based on the results of N lineage trace experiments we speculate that these precursors initially turn on N and those that subsequently maintain N signaling adopts crystal cell fate. In contrast, failure to maintain N signaling triggers them to become plasmacytes. This mirrors a striking resemblance to the different types of HSCs present in the vertebrate bone marrow. However, there being only myeloid lineage specification in flies, it seems that in this case the precursors are of two types: (1) biased for plasmacyte lineage and (2) balanced bipotent precursors that can give birth to both plasmacytes and crystal cells.

Conservation of hematopoietic events across the two taxa mentioned above along with the fact that the cells of the hub are nested in a functional network Laminin A and Pericardin projects this hematopoietic hub as a simple rendering of the vertebrate bone marrow. Given the fact that the vertebrate bone marrow is not easily accessible, we believe that our finding will

Figure 3. Evidence of Post-larval Hematopoiesis in *Drosophila*

(A) Hematopoietic hub of adult fly. Box indicates the area of imaging.

(B and B’) *hml* lineage traced (EGFP) and live (RFP) expression in the hub at 5 dpe of *w; hml-Gal4/UAS-GTRACE; +/- flies*.

(C–D’) Zoomed-in regions of B showing the plasmacytes that are either lineage traced or lineage traced as well as actively expressing *hml* or only have live *hml* expression (only RFP, arrows).

(E–F’) Activation of G-TRACE construct was prevented by following the timeline (E’) in *w; UAS-GTRACE/+; notch-Gal4/ P{tubP-GAL80^{ts}}2 fly*. (F) and (F’) are higher magnification of (E).

(G–H’) N lineage trace and live expression in crystal cells (Hnt) of adult hub (G) where activation of G-TRACE by *notch-Gal4* following timeline (G’) was done.

(H–H’) Zoomed in images of (G).

(I–K’) A subset of plasmacytes (P1) in hub was N lineage traced (I) where G-TRACE was activated by *notch-Gal4* following timeline (I’). (J) and (J’) are zoomed in of (I). (K and K’) Another example.

(L) Scheme depicting the surge of hematopoiesis based on our observation.

Scale bar represents 20 μ m. See also Figure S3.

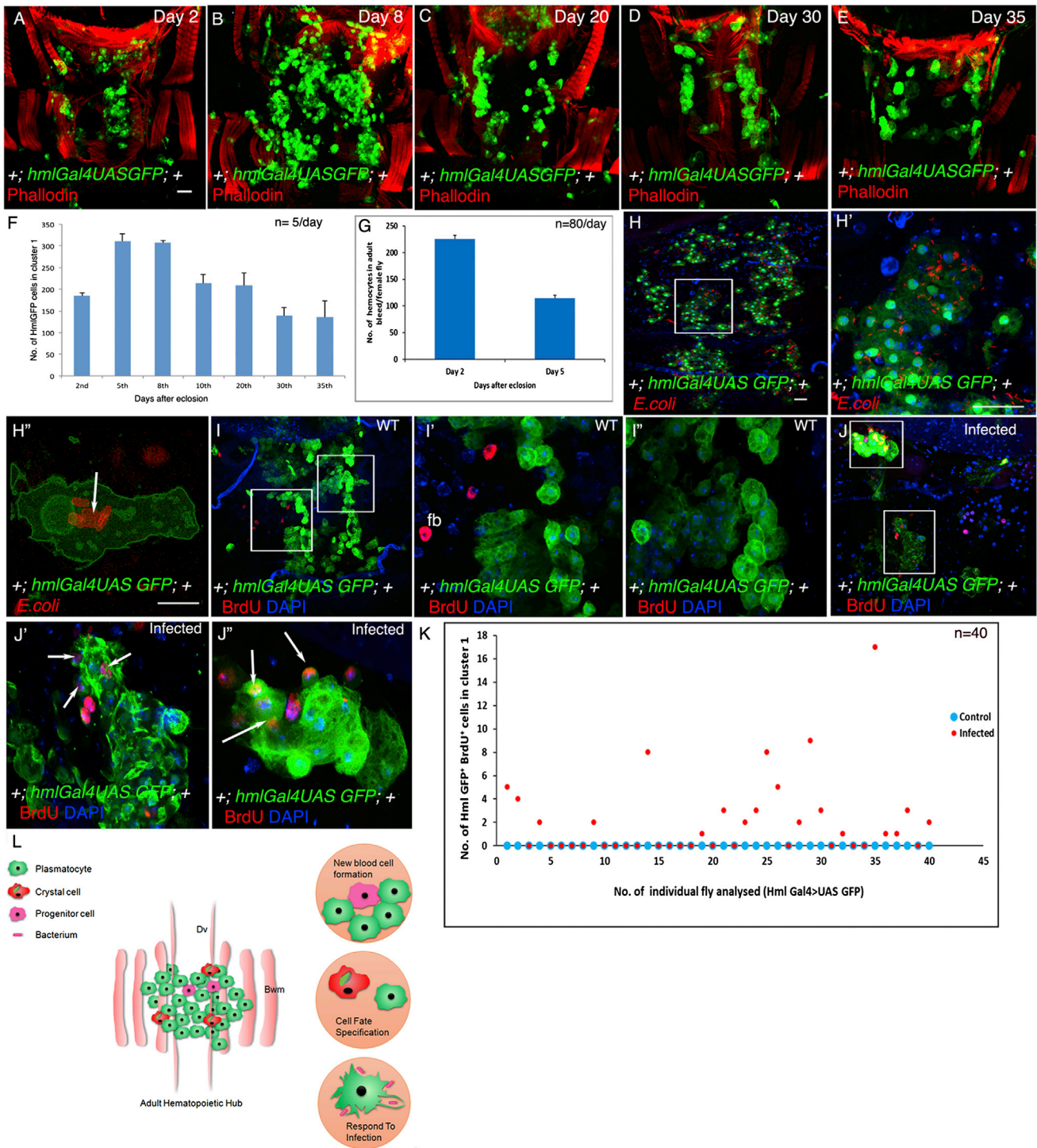


Figure 4. The Hematopoietic Hub Is Functionally Active

(A–E) Plasmatocytes number in the hub at 2–35 dpe.

(F) Quantitative analysis of the data in (A)–(E).

(G) Comparative account of plasmatocytes number present in adult circulation at 2 and 5 dpe.

(H–H'') Plasmatocytes in the hub can phagocytose *E. coli* (H). (H') and (H'') are further zoomed in images of (H).

(I–I'') While fat body (Fb) cells incorporate BrdU (red, I'') in adult fly, hemocytes of hub do not.

(J) Only on infection BrdU incorporation is seen.

(legend continued on next page)

establish *Drosophila* adult hematopoiesis as a simpler yet genetically amenable model to tease out normal and aberrant hematopoiesis and questions related to development, immunity, wound healing, and aging.

EXPERIMENTAL PROCEDURES

Genetics

hml-Gal4ΔUAS-2xEGFP, dot-Gal4, crq-Gal4, lz-Gal4, kn-Gal4, notch Gal4, mef2-Gal4, UAS-N full, UAS-N RNAi, UAS-lanA RNAi, UAS-2xEGFP, UAS-nlsGFP, and loh^{MB05750} flies were obtained from Bloomington Stock Center. pxn-Gal4, gcm-Gal4, ZCL2897, 12xSu(H)lacZ, G-TRACE, HHLT Gal4, and UAS-PvrRNAi and he-Gal4UAS-nlsGFP were provided by U. Banerjee, B Shilo, and I. Ando. For temporal control on Gal4 activity, temperature-sensitive allele of gal80 was used.

Immunohistochemistry

Antibodies P1, Srp, anti-proPO, and Laminin A were kind gifts from I. Ando, D. Hoshizaki, M. Kanost and S. Baumgartner. Hnt(1G9), Prc(EC11), anti-BrdU, and β-galactosidase were from Developmental Studies Hybridoma Bank, Abcam, and Promega, respectively. Secondary antibodies were from Jackson ImmunoResearch Laboratories. For all of the experiments, 5–8 dpe (unless mentioned) female flies were used. Tissues were fixed in 4% formaldehyde, blocked in 10% bovine serum albumin (BSA) before primary antibody incubation at 4°C. Secondary antibody incubation was done at 4°C. Imaging was done in Zeiss LSM 780 confocal microscope. Fly cuticle was imaged by autofluorescence at 633 nm. Images were processed by Image J and Photoshop CS3.

Infection Studies

Glass capillary was used to pierce the thorax of fly with *E. coli* expressing RFP. Infected flies were reared in normal food until dissection.

BrdU Assay

Flies 3 dpe were grown in corn meal yeast fly food supplemented with 200 μl of 6-mg/ml BrdU in PBS for 2 days before infecting with *E. coli*. They were then reared in BrdU food for 5 days, with two intermittent flips until dissection.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three movies and can be found with this article online at <http://dx.doi.org/10.1016/j.devcel.2015.03.014>.

AUTHOR CONTRIBUTIONS

S.G. did bulk of the experiments, imaging, and data analysis. A.S. helped in initial characterization. S.M. helped in interpretation and manuscript writing. Conception, designing, analysis, manuscript writing, and research supervision were by L.M.

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(J' and J'') Zoomed in region of (J).

(K) Quantification of BrdU incorporation in the hub of control (blue) and infected flies (red).

(L) Scheme depicting potential of the hematopoietic hub.

Scale bar represents 20 μm; 5 μm (H''). Error bar denotes SE. See also Figure S4.

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