The *Drosophila* lymph gland as a developmental model of hematopoiesis

Seung-Hye Jung*, Cory J. Evans*, Christine Uemura and Utpal Banerjee†

Department of Molecular, Cell and Developmental Biology, Department of Biological Chemistry and Molecular Biology Institute, University of California, Los Angeles, CA 90095, USA

*These authors contributed equally to this work
†Author for correspondence (e-mail: banerjee@mbi.ucla.edu)

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Summary

***Drosophila* hematopoiesis occurs in a specialized organ called the lymph gland. In this systematic analysis of lymph gland structure and gene expression, we define the developmental steps in the maturation of blood cells (hemocytes) from their precursors. In particular, distinct zones of hemocyte maturation, signaling and proliferation in the lymph gland during hematopoietic progression are described. Different stages of hemocyte development have been classified according to marker expression and placed within developmental niches: a medullary zone for quiescent prohemocytes, a cortical zone for maturing hemocytes and a zone called the posterior signaling center for specialized signaling hemocytes. This establishes a framework for the identification of *Drosophila* blood cells, at various stages of maturation, and provides a genetic basis for spatial and temporal events that govern hemocyte development. The cellular events identified in this analysis further establish *Drosophila* as a model system for hematopoiesis.**

Key words: *Drosophila*, Hematopoiesis, Blood, Hemocyte, Lymph gland

Introduction

Blood cell development in *Drosophila* has been shown to employ several genetic mechanisms with similarities to those that operate during vertebrate hematopoiesis (reviewed by Evans et al., 2003). Likewise, significant similarities exist between vertebrates and *Drosophila* in the early developmental strategies employed when vascular and hematopoietic components separate within the cardiogenic mesoderm (Mandal et al., 2004). *Drosophila* is also well established as a model of innate immunity, particularly the mechanisms and pathways regulating humoral immune responses (Brennan and Anderson, 2004; Hoffmann, 2003; Hultmark, 1993). Circulating *Drosophila* blood cells (hemocytes) have been shown to act as sentinels by monitoring the environment and initiating humoral immune responses via a cytokine-like signaling mechanism (Agaisse et al., 2003). These hemocytes also provide cellular immunity by removing microbial pathogens via phagocytosis and through the encapsulation and melanization of larger parasitic pathogens (Lavine and Strand, 2002; Meister, 2004).

In *Drosophila*, mature hemocytes arise from two spatially and temporally distinct phases of hematopoietic development, one early in the embryonic head mesoderm and another at later larval stages in a specialized organ called the lymph gland (Lebestky et al., 2000; Rizki, 1978; Tepass et al., 1994). The embryonic phase of hematopoiesis gives rise to mature, circulating hemocytes of the larval stages, whereas lymph gland hemocytes, under normal, non-immune conditions, do not enter circulation until the onset of metamorphosis (Holz et al., 2003). Once in circulation, these lymph gland-derived hemocytes, along with a subset of hemocytes derived from the embryonic head mesoderm, can persist into the adult stage (Holz et al., 2003). The lymph gland originates in the cardiogenic mesoderm of the embryo and subsequently grows by cellular proliferation during the larval instars. Although the function of the lymph gland as a hematopoietic organ is well established (reviewed by Evans et al., 2003; Lanot et al., 2001; Shrestha and Gateff, 1982; Sorrentino et al., 2002), surprisingly little is known about the spatial and temporal events regulating this process. This paucity of detail is primarily due to a lack of lineage-specific genetic markers, which is in sharp contrast to the level of detail afforded to other *Drosophila* tissues such as the salivary glands, imaginal discs and ovaries, not to mention that seen for vertebrate hematopoietic systems. In this report, we address the spatiotemporal events of lymph gland hematopoiesis through an in-depth characterization of known and novel genetic markers.

In *Drosophila*, there are at least three terminally differentiated hemocyte types: plasmatocytes, crystal cells and lamellocytes (Evans et al., 2003; Rizki, 1956). Each type is thought to be derived from a common precursor that expresses and requires the GATA factor Serpent (Srp) (Rehorn et al., 1996; Tepass et al., 1994). Plasmatocytes represent 90-95% of all mature *Drosophila* hemocytes and function in the phagocytic removal of dead cells and microbial pathogens (Rizki, 1978; Tepass et al., 1994). The specification of plasmatocytes requires the transcription factors Glial cells missing (Gcm) and Gcm2 (Alfonso and Jones, 2002; Bernardoni et al., 1997). Additionally, circulating

plasmatocytes have been shown to express Peroxidasin (Pxn) (Nelson et al., 1994), a component of the extracellular matrix, and an uncharacterized surface marker called P1 antigen (Asha et al., 2003; Vilmos et al., 2004). Crystal cells, which constitute ~5% of the hemocyte population, are non-phagocytic cells that facilitate innate immune and wound-healing responses by mediating the process of melanization (Lanot et al., 2001; Rizki, 1978; Russo et al., 1996). Crystal cell differentiation requires the cell-autonomous expression of the transcription factor Lozenge, a Runt-domain protein that shares homology with mammalian Runx proteins, including Acute Myeloid Leukemia 1 (AML1 or Runx1) (Daga et al., 1996; de Bruijn and Speck, 2004; Lebestky et al., 2000). Additionally, mature crystal cells express Prophenoloxidase A1 (ProPOA1), an oxidoreductase related to hemocyanins and vertebrate tyrosinases that mediates melanization reactions upon activation (Rizki and Rizki, 1985; Soderhall and Cerenius, 1998). Lamellocytes are relatively large (15-40 μm across), flat, adherent cells that primarily function in the encapsulation and neutralization of objects too large to be engulfed by plasmatocytes (Rizki and Rizki, 1992). Lamellocytes have not been found in embryos or adults and are rarely observed during larval stages, although large numbers of these cells can be induced to differentiate in larvae upon challenge with parasitic wasp eggs (Lanot et al., 2001; Sorrentino et al., 2002). Genetically, mature lamellocytes have commonly been identified by their expression of a reporter in the *misshapen* locus (Braun et al., 1997; Lanot et al., 2001; Sorrentino, 2002), which encodes a component of the JUN kinase signaling cascade. Additionally, lamellocytes express an uncharacterized surface marker called L1 antigen (Asha et al., 2003). All *Drosophila* hemocytes specifically express the marker Hemese (He) (Kurucz et al., 2003), while a majority of plasmatocytes and crystal cells express the Collagens Viking and Cg25C (Le Parco et al., 1986; Yasothornsrikul et al., 1997) and the Von Willebrand-like factor Hemolectin (Hml) (Goto et al., 2003; Goto et al., 2001; Sinenko and Mathey-Prevot, 2004).

Several signaling pathways have been associated with the *Drosophila* hematopoietic process. Hyperactivation of the JAK homolog Hopscotch (Hop) causes extensive hemocyte proliferation, lamellocyte differentiation and melanized pseudotumor formation at high frequency (Harrison et al., 1995; Hou et al., 2002; Luo et al., 1995). Despite this apparent role in regulating hemocyte proliferation, *hop* loss-of-function has no overt effect upon the numbers of circulating hemocytes or the growth of the lymph gland (Remillieux-Leschelle et al., 2002; Sorrentino et al., 2004). Loss of function does, however, impair the ability of the lymph gland to mount an effective immune response to parasitization (Sorrentino et al., 2004). In mammals, JAK/STAT signaling is required for various aspects of hematopoiesis and immunity and dysregulation of this pathway has been associated with numerous malignancies including lymphomas and leukemias (Rane and Reddy, 2002; Ward et al., 2000). Misregulation of the *Drosophila* Toll pathway, which is related to vertebrate NFκB/IκB signaling pathways, also causes significant hematopoietic defects that include aberrant proliferation and differentiation (Gerttula et al., 1988; Qiu et al., 1998). Finally, the receptor tyrosine kinase Pvr, which shares homology with vertebrate PDGF and VEGF receptors, is known to function in embryonic hemocytes where it controls migration and cell survival (Bruckner et al., 2004; Cho et al., 2002; Heino et al., 2001; Sears et al., 2003). The Pvr pathway may also have a role in proliferation control because misexpression of the ligand Pvf2 causes significant expansion of the lymph gland (Munier et al., 2002). Taken together, it is clear that the JAK/STAT, Toll and Pvr pathways play significant roles in *Drosophila* hematopoiesis.

A considerable amount is known about the role of the Notch signaling pathway in controlling lymph gland hematopoiesis. In addition to a role in proliferation, Notch signaling is required for the early specification of the lymph gland (Mandal et al., 2004) and the determination of the crystal cell hemocyte lineage, both in the lymph gland and in the embryonic head mesoderm (Duvic et al., 2002; Lebestky et al., 2003). Furthermore, the study of Notch function in hematopoiesis has led to the identification of the first subdomain or compartment in the lymph gland, which was termed the posterior signaling center (PSC) (Lebestky et al., 2003). The PSC consists of a small cluster of cells at the posterior tip of each of the primary (anterior-most) lymph gland lobes and is defined by the expression of the Notch ligand Serrate (Ser). Serrate signaling through Notch mediates the commitment of prohemocytes to the crystal cell lineage via the expression of Lozenge. Recently, the transcription factor Collier, which shares homology with mammalian Early B-cell Factor (EBF), has been shown to be expressed in the PSC earlier than Ser and to control Ser expression in these cells (Crozatier et al., 2004). *collier* mutant larvae fail to produce lamellocytes, even upon parasitization, indicating a role of the PSC in both crystal cell and lamellocyte specification.

In this report, we provide a description of the lymph gland as a developmental system based on the dynamic expression and function of a number of known as well as uncharacterized hematopoietic and pro-hemocytic markers. This molecular genetic and structural analysis reveals novel features and provides a comprehensive picture of, and a mechanistic basis for, the spatial and temporal events that give rise to blood cells from their precursors within the lymph gland.

Materials and methods

Drosophila strains and crosses

The following *Drosophila* strains were used in the described experiments: *odd-lacZ* (J. Lengyel); GFP-trap lines *ZCL0611*, *ZCL2375*, *ZCL2826*, *ZCL2856*, *ZCL2867* and *ZCL2897* (L. Cooley); *G147* and *G454* (X. Morin); *Cg-gal4* (C. Dearolf); *dome-gal4* (S. Noselli); *upd3-gal4* (N. Perrimon); *Dot-gal4* (D. Kimbrell); *Ser-lacZ* (E. Knust); hml^{Δ}*-gal4, UAS-GFP* (S. Sinenko); Pvr^{c2195} *FRT40A/CyO* (Exelixis, D. Montell); and *Ser-gal4*. A 9.5 kb enhancer region of *Serrate* (Bachmann and Knust, 1998) was used to generate *Ser-gal4* transgenic flies (Joyce Stamm and U.B., unpublished). The following stocks were obtained from the Bloomington Stock Center: *hml-gal4; UAS-GFP,* msn^{06946}*/TM3 (msn-lacZ), w*; In(2LR)* noc^{4L} Sco^{rv9R} b^{1}*/CyO, P{w[+mC]=ActGFP}JMR1, hsFLP* and *Ubi-GFP FRT40A, twist-gal4, P{ry[+t7.2]=Act5C(FRT.polyA)lacZ.nls1}3 ry[506], y[1] w[*]; P{UAS-FLP1.D}JD1*. Oregon R was used for wild type. To generate somatic clones, *hsFLP;* Pvr^{c2195} *FRT40A/Ubi-GFP, FRT40A* larvae were heat-shocked at 37°C for 1 hour at the first instar.

Immunohistochemistry

Lymph glands were stained as previously described (Lebestky et al., 2000). The following antibodies were used in the described experiments: rabbit α-Srp (D. Hoshizaki), rabbit α-βgal (Cappell), mouse α-βgal (Promega), mouse α-Lz, mouse α-Pxn (J. Fessler and L. Fessler), mouse α-Hemese, mouse α-P1 and mouse α-L1 (I. Ando),

mouse α-Cut (Developmental Studies Hybridoma Bank), rat α-DE-cadherin (V. Hartenstein), rabbit α-U-shaped (R. Schulz), rat α-Pvr (B. Shilo and P. Garrity) and rat α-ProPO (H. Müller). Alexa Fluor 488-, Alexa Fluor 546- (Molecular Probes) and Cy3- (Jackson Laboratory) conjugated secondary antibodies were used. Lymph glands were mounted in Vectashield (Vector Laboratories) alone or Vectashield with To-Pro-3 (Molecular Probes) for nuclear staining. Unless otherwise stated, late third instar larvae were used. For second instar lymph gland staining, first instar larvae were collected upon hatching at 1 hour intervals, maintained at 25°C, and dissected between 40 and 44 hours post-hatching.

BrdU analysis

Dissected lymph glands were incubated in the 75 μg/ml BrdU in PBS for 1 hour, fixed immediately in 4% formaldehyde/PBS for 30 minutes, washed three times for 10 minutes each in PBS, blocked in 10% normal goat serum/PBS, then incubated in mouse α-GFP antibody (Molecular Probes) or α-Pxn overnight. Lymph glands were washed four times in PBS, fixed again in 2% formaldehyde/PBS for 15 minutes, washed three times for 5 minutes, and then incubated in 2 M HCl for 30 minutes to denature BrdU-labeled DNA. Lymph glands were then washed four times for 10 minutes each and stained with rat α-BrdU (Abcam) antibody, followed by standard secondary antibody staining and mounting.

Results

Gross structure of the larval lymph gland

In the late embryo, the lymph gland consists of a single pair of lobes containing ~20 cells each. These express the transcription factors Srp and Odd skipped (Odd) (Fig. 1A,B), and each cluster of hemocyte precursors is followed by a string of Odd-expressing pericardial cells that are proposed to have nephrocyte function. These lymph gland lobes are arranged bilaterally such that they flank the dorsal vessel, the simple aorta/heart tube of the open circulatory system, at the midline (Fig. 1B). By the second larval instar, lymph gland morphology is distinctly different in that two or three new pairs of posterior lobes have formed and the primary lobes have increased in size approximately tenfold (to ~200 cells, Fig. 1C,D). By the late third instar, the lymph gland has grown significantly in size (approximately another tenfold) but the arrangement of the lobes and pericardial cells has remained the same. The cells of the third instar lymph gland continue to express Srp (Fig. 1E) and Odd (not shown).

The third instar lymph gland also exhibits a strong, branching network of extracellular matrix (ECM) throughout the primary lobe (Fig. 1F,G). This network was visualized using several GFP-trap lines in which GFP is fused to endogenous proteins (Kelso et al., 2004; Morin et al., 2001). For example, line *G454* represents an insertion into the *viking* locus, which encodes a Collagen IV component of the extracellular matrix (Yasothornsrikul et al., 1997). The hemocytes in the primary lobes of *G454* (expressing Viking-GFP) appear to be clustered into small populations within pockets or chambers bounded by GFP-labeled branches of

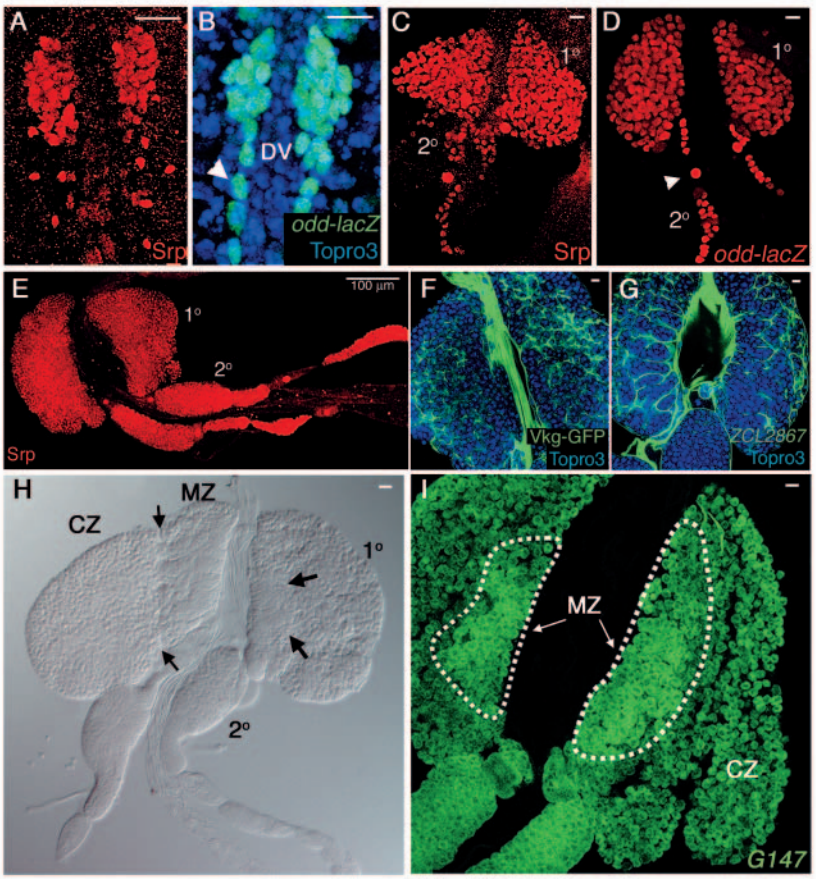

Fig. 1. Morphological features of the developing lymph gland. (A,B) Lymph glands at embryonic stage 16. Cells of the embryonic lymph gland (shown in whole mount) express the hemocyte marker Srp (A, red) and Odd (*odd-lacZ* expression in B, green; the bluish-green appearance is due to overlap with nuclear marker To-pro-3 in blue). Pericardial cells (B, arrowhead), also express Odd. Cells between the two symmetric lymph gland clusters represent dorsal vessel (DV) cells (B). Scale bar: 10 μm. (C,D) Second instar larval lymph gland. Expression of Srp (C) and *odd-lacZ* (D) is maintained in the second instar. By this stage, the lymph gland consists of a pair of primary (1°) lobes and smaller, posterior secondary (2°) lobes separated by pericardial cells (arrowhead in D). Scale bar: 10 μm. Expression of *odd-lacZ* is slightly higher in the region of the posterior signaling center (PSC) of the primary lobes. (E) Multi-lobed structure of the third instar lymph gland. Srp (red) is ubiquitously expressed. Pericardial cells separate lymph gland lobes as in the second instar. Scale bar: 100 μm. (F,G) Expression pattern of Viking-GFP fusion protein (F, green) reveals chamber-like structures within the lymph gland. Similar structures are seen in lymph glands of the uncharacterized GFP-trap line *ZCL2867* (G, green). Scale bar: 10 μm. To-pro-3 (blue) shows nuclei. (H,I) Morphological features of the third instar lymph gland. (H) A DIC image of the third instar lymph gland shows two distinct regions within the primary lobe that we call the cortical zone (CZ) and the medullary zone (MZ) (see text for details). Arrows indicate the boundary between the zones. The secondary (2°) lobe is similar in appearance to the medullary zone. (I) Confocal section through a lymph gland from the GFP-trap line *G147*. Cells in the medullary zone (MZ, outlined) are compactly arranged, whereas cells in the cortical zone (CZ) exhibit voids and gaps. Scale bar: 10 μm.

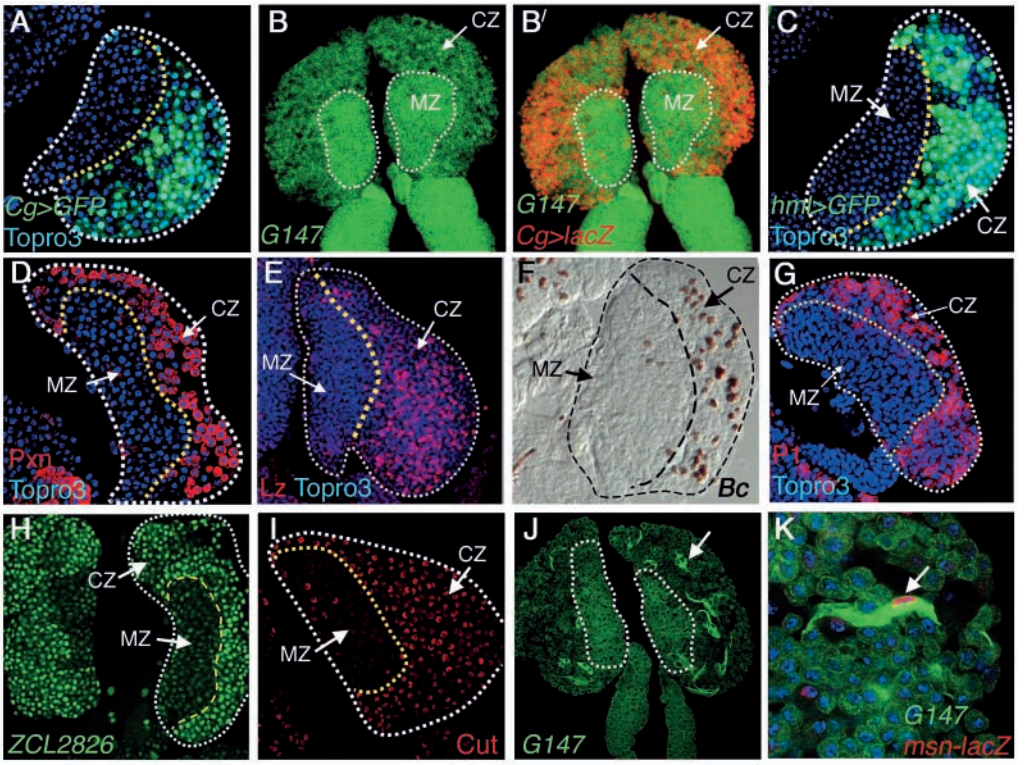

Fig. 2. Maturing hemocytes are found in the cortical zone. (A) *Cg-gal4/UAS-GFP*-expressing cells (green) are found in the periphery of the primary lobe. (B,B′) Flattened confocal image of a third instar *G147* lymph gland. GFP expression (green) identifies the cortical and medullary zones. *Cg-gal4/UAS-lacZ* (B′, red) expression is restricted to the cortical zone (CZ) and is excluded from the medullary zone (MZ). (C-G) Mature hemocyte markers are restricted to the cortical zone. Hemocyte markers *hml-gal4/UAS-GFP* (C, green) and Pxn (D, red), crystal cell markers Lz (E; red) and *Black cells* (*Bc,* F), and plasmatocyte marker P1 (G, red) are limited to the cortical zone. (H,I) The uncharacterized GFP-trap line *ZCL2826* (H, green) and the transcription factor Cut (I, red) are preferentially expressed in the cortical zone. (J,K) Lamellocytes, when present, are found in the cortical zone. Lamellocytes can be distinguished by morphology in *G147* background (J,K, green, arrows) and by co-localization with *msn-lacZ* (K, red, arrow). To-pro-3 (blue) marks nuclei. All lymph glands presented are of the third larval instar.

various sizes (Fig. 1F). Other lines, such as the uncharacterized GFP-trap line *ZCL2867* (Fig. 1G), also highlight this branching pattern. What role this intricate ECM network plays in hematopoiesis, as well as why multiple cells cluster within these ECM chambers, remains to be determined.

Careful examination of dissected, late third-instar lymph glands by differential interference contrast (DIC) microscopy revealed the presence of two structurally distinct regions within the primary lymph gland lobes that have not been previously described (Fig. 1H). The periphery of the primary lobe generally exhibits a granular appearance, whereas the medial region looks smooth and compact. These characteristics were examined further with confocal microscopy using a GFP-trap line *G147*, in which GFP is fused to a microtubule-associated protein (Morin et al., 2001). The *G147* line is expressed throughout the lymph gland but, in contrast to nuclear markers such as Srp and Odd, distinguishes morphological differences among cells because the GFP-fusion protein is expressed in the cytoplasm in association with the microtubule network (Fig. 1I). Cells in the periphery of the lymph gland make relatively few cell-cell contacts, thereby giving rise to gaps and voids among the cells within this region. This cellular individualization is consistent with the granularity of the peripheral region observed by DIC microscopy (Fig. 1H). By contrast, cells in the medial region were relatively compact with minimal intercellular space, which is also consistent with the smoother appearance of this region by DIC microscopy. Thus, in the late third instar, the lymph gland primary lobes consist of two physically distinct regions: a medial region consisting of compactly arranged cells, which we term the medullary zone; and a peripheral region of loosely arranged cells that we term the cortical zone (Fig. 1H,I).

Maturing hemocytes are restricted to the cortical zone of primary lymph gland lobes

Mature hemocytes have been shown to express several markers, including collagens (Asha et al., 2003; Fessler et al., 1994; Yasothornsrikul et al., 1997), Hemolectin (Goto et al., 2003; Goto et al., 2001), Lozenge (Lebestky et al., 2000), Peroxidasin (Nelson et al., 1994) and P1 antigen (Asha et al., 2003; Vilmos et al., 2004). We found that the expression of the reporter *Collagen-gal4* (*Cg-gal4*) (Asha et al., 2003), which is expressed by both plasmatocytes and crystal cells, is restricted to the periphery of the primary lymph gland lobe (Fig. 2A). Comparison of *Cg-gal4* expression in *G147* lymph glands, in which the medullary zone and cortical zone can be distinguished, revealed that maturing hemocytes are restricted to the cortical zone (Fig. 2B,B′). In fact, the expression of each of the maturation markers mentioned above is found to be restricted to the cortical zone. The reporter *hml-gal4* (Goto et al., 2003) and Pxn, which are expressed by the plasmatocyte and crystal cell lineages, are extensively expressed in this region (Fig. 2C,D). Likewise, the expression of the crystal cell lineage marker Lozenge is restricted in this manner (Fig. 2E). The spatial restriction of maturing crystal cells to the cortical zone was verified by several means, including the distribution of melanized lymph gland crystal cells in the *Black cells* background (Fig. 2F) and analysis of the terminal marker ProPOA1 (not shown). The cortical zone is also the site of P1 antigen expression (Fig. 2G), a marker of the plasmatocyte

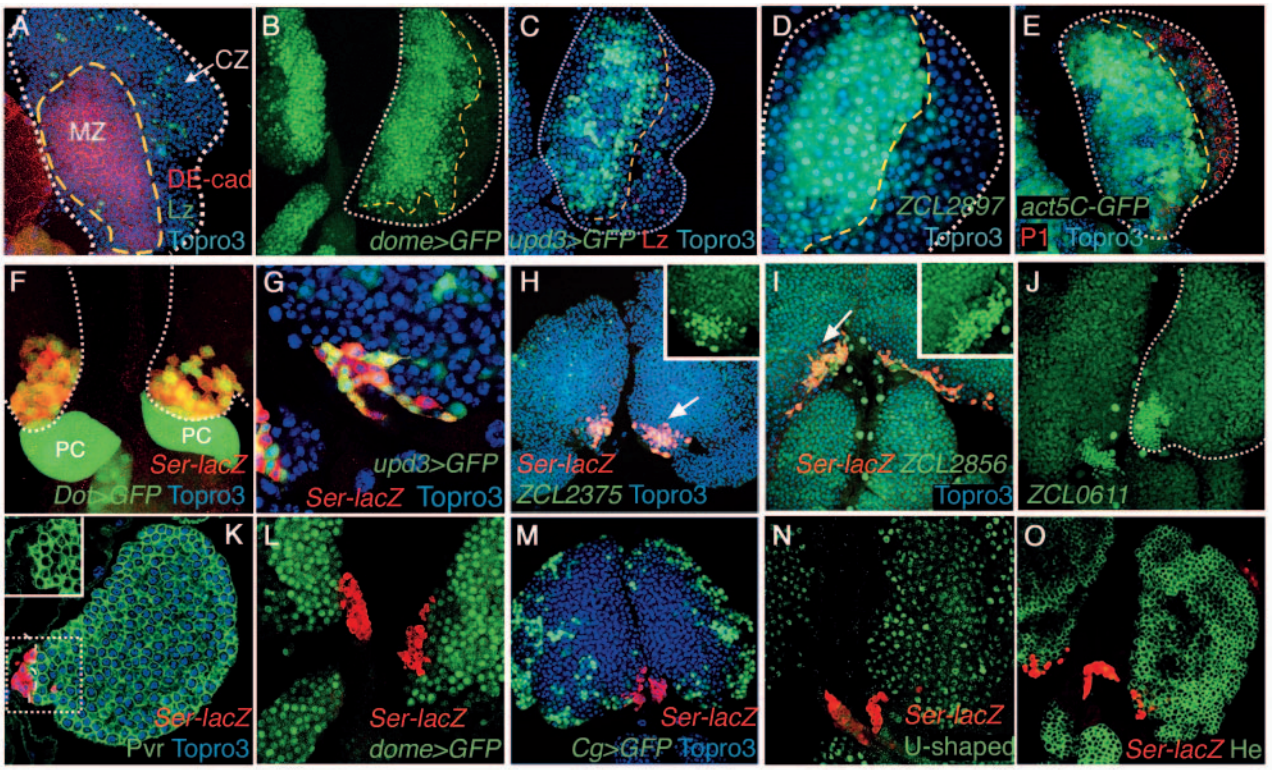

Fig. 3. Expression profile of the medullary zone and the PSC. (A-E) Marker expression in the medullary zone. Medullary zone cells are characterized by their expression of DE-cadherin (A, red), *dome-gal4/UAS-GFP* (B, green), *upd3-gal4/UAS-GFP* (C, green), the uncharacterized GFP fusion line *ZCL2897* (D, green), and *act5C-GFP* (E, green). The cortical zone is marked by Lz staining (A, green; C, red) as well as by P1-antigen staining (E, red). (F-J) Markers expressed in the posterior signaling center (PSC) cells. PSC cells are marked by *Ser-lacZ* (F-I, red) and show high levels of expression of *Dorothy-gal4/UAS-GFP* (F, green), *upd3-gal4/UAS-GFP* (G, green), uncharacterized GFP-trap lines *ZCL2375* (H, green), *ZCL2856* (I, green) and *ZCL0611* (J, green). In H and I, insets show only the GFP expression in the PSC regions indicated by the arrows. *upd3-gal4/UAS*-GFP (G) is expressed in only a subset of PSC cells. PC, pericardial cell. (K-O) Lack of expression of specific markers also defines the PSC. PSC cells are positively marked by *Ser-lacZ* (red) and characterized by absence of expression of Pvr (K, green; inset shows the PSC region without *Ser-lacZ* for clarity), *dome-gal4/UAS-GFP* (L, green), *Cg-gal4/UAS-GFP* (M, green), U-shaped (N, green) and Hemese (O, green). To-pro-3 (blue) marks nuclei.

lineage. The uncharacterized GFP fusion line *ZCL2826* also exhibits preferential expression in the cortical zone (Fig. 2H). Last, we found that the homeobox transcription factor Cut (Blochlinger et al., 1993) is preferentially expressed in the cortical zone of the primary lobe (Fig. 2I). Although the role of Cut in *Drosophila* hematopoiesis is currently unknown, homologs of Cut are known to be regulators of the myeloid hematopoietic lineage in both mice and humans (Bjerregaard et al., 2003; Sinclair et al., 2001). Cells of the rare third cell type, lamellocytes, are also restricted to the cortical zone (Fig. 2J,K), based upon cell morphology and the expression of a *msn-lacZ* reporter (*msn*^{06946}). In summary, based on the expression patterns of several genetic markers that identify the three major blood cell lineages, we propose that the cortical zone is a specific site for hemocyte maturation.

Differential gene expression in the medullary zone

The medullary zone was initially defined by structural characteristics (Fig. 1H,I) and subsequently by the lack of expression of mature hemocyte markers (Fig. 2). However, we have also identified several markers that are exclusively expressed in the medullary zone at high levels but not the cortical zone. Consistent with the compact arrangement of cells in the medullary zone, we found that *Drosophila* E-cadherin (DE-cadherin or Shotgun) is highly expressed in this region (Fig. 3A). No significant expression of DE-cadherin was observed among maturing cells in the cortical zone. E-cadherin, in both vertebrates and *Drosophila*, is a Ca^{2+}-dependent, homotypic adhesion molecule often expressed by epithelial cells and is a crucial component of adherens junctions (Perez-Moreno et al., 2003). Attempts to study *DE-cadherin* mutant clones in the medullary zone where the protein is expressed were unsuccessful as no clones were recoverable. We also found that the reporter lines *domeless-gal4* (Bourbon et al., 2002) and *unpaired3-gal4* (Agaisse et al., 2003) are preferentially expressed in the medullary zone (Fig. 3B,C). The gene *domeless* (*dome*) encodes a receptor molecule known to mediate the activation of the JAK/STAT pathway upon binding of the ligand Unpaired (Brown et al., 2001; Chen et al., 2002). The *unpaired3* (*upd3*) gene encodes a protein with homology to Unpaired and has been associated with innate immune function (Agaisse et al., 2003). These *gal4* lines are used here only as markers that correlate with the medullary zone and, at the present time, we have no evidence that their associated proteins have a role in lymph gland hematopoiesis. Other markers of interest with preferential expression in the

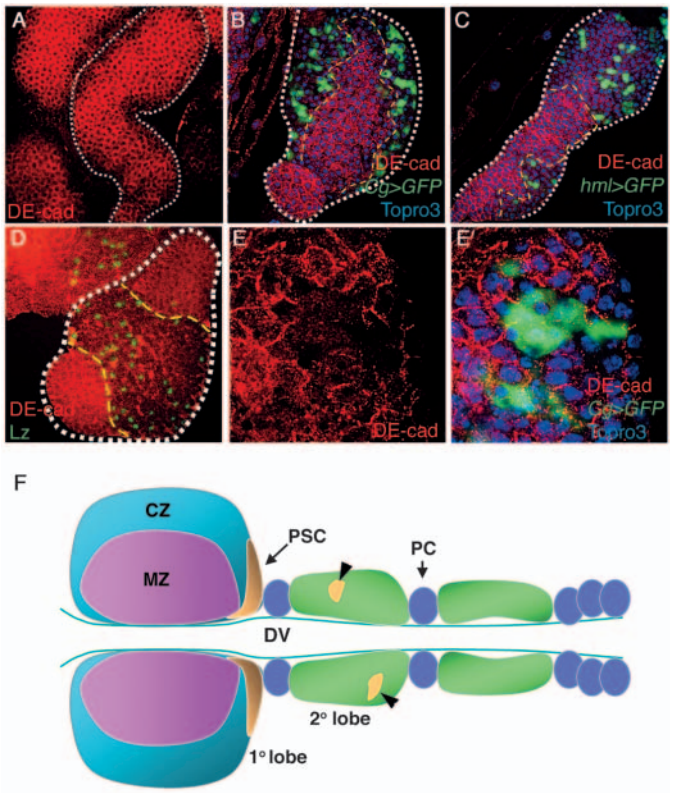

Fig. 4. Hemocyte development in the secondary lymph gland lobe of third instar larva. (A) The secondary lobe expresses high levels of DE-cadherin (red). (B-E′) Hemocyte formation in larger secondary lobes. *Cg-gal4/UAS-GFP* (B,E′, green), *hml-gal4/UAS-GFP* (C, green) and Lz (D, green) are seen in patches where DE-cadherin (B-E′, red) expression is downregulated. This is particularly apparent in high-magnification images (compare E with E′). To-pro-3 (blue) marks nuclei. (F) Schematic diagram of the 3rd instar lymph gland based on our analysis of morphology and hemocyte markers. The lymph gland flanks the dorsal vessel (DV) and each lobe is separated by pericardial cells (PC). The primary (1°) lobe consists of three distinct zones: (1) the cortical zone (CZ), where maturing hemocytes are found; (2) the medullary zone (MZ), with prohemocytes lacking differentiation markers; and (3) the posterior signaling center (PSC), which is a unique population of specialized hemocytes. Secondary (2°) lobes usually contain immature hemocytes except in random sites of maturation (arrowheads).

medullary zone include the molecularly uncharacterized GFP-trap line *ZCL2897* (Fig. 3D) and *actin5C-GFP* (Fig. 3E). Cells expressing hemocyte maturation markers are not seen in the medullary zone. It is therefore reasonable to propose that this zone is largely populated by prohemocytes that will later mature in the cortical zone. Prohemocytes are characterized by their lack of maturation markers, as well as their expression of several markers described in this section.

The PSC represents a distinct lymph gland zone

The PSC is defined by its expression of the Notch ligand Serrate (Lebestky et al., 2003) and the transcription factor Collier (Crozatier et al., 2004). During our analysis, several additional markers were identified that exhibit specific or preferential expression in the PSC region. For example, we found that the reporter *Dorothy-gal4* (Kimbrell et al., 2002) is strongly expressed in this zone (Fig. 3F). The *Dorothy* gene encodes a UDP-glycosyltransferase (Zhou et al., 2001), which belongs to a class of enzymes that function in the detoxification of metabolites. The *upd3-gal4* reporter, which has preferential expression in the medullary zone, is also strongly expressed among cells of the PSC (Fig. 3G). Last, we found three uncharacterized GFP-gene trap lines, *ZCL2375*, *ZCL2856* and *ZCL0611* (Kelso et al., 2004), which are preferentially expressed in the PSC (Fig. 3H-J). This analysis has made it clear that the PSC is a distinct zone of cells that can be defined by the expression of multiple gene products.

We have also found that the PSC can be defined just as definitively by the characteristic absence of several markers. For example, the RTK receptor Pvr, which is expressed throughout the lymph gland, is notably absent from the PSC (Fig. 3K). Likewise, *dome-gal4* is not expressed in the PSC (Fig. 3L), further suggesting that this population of cells is biased toward the production of ligands rather than receptor proteins. Maturation markers such as *Cg-gal4*, which are expressed throughout the cortical zone, were never found to be expressed by PSC cells (Fig. 3M). Additionally, we found that the expression levels of the hemocyte marker Hemese (Kurucz et al., 2003) and the Friend-of-GATA protein U-shaped (Fossett et al., 2001) are dramatically reduced in the PSC when compared with other hemocytes of the lymph gland (Fig. 3N,O). Taken together, both the expression and lack of expression of a number of genetic markers defines the cells of the PSC as a unique hemocyte population.

Hematopoietic differentiation in secondary lymph gland lobes

In contrast to primary lobes of the third instar, maturing hemocytes are generally not seen in the secondary lobes. Correspondingly, secondary lobes often have a smooth and compact appearance (Fig. 1H), much like the medullary zone of the primary lobe. Consistent with this appearance, secondary lymph gland lobes also express high levels of DE-cadherin (Fig. 4A). The size of the secondary lobe, however, varies from animal to animal and this correlates with the presence or absence of maturation markers. Smaller secondary lobes contain a few or no cells expressing maturation markers, whereas larger secondary lobes usually exhibit groups of differentiating cells. Direct comparison of DE-cadherin expression in secondary lobes with that of *Cg-gal4*, *hml-gal4* or Lz revealed that the expression of these maturation markers occurs only in areas in which DE-cadherin is downregulated (Fig. 4B-E′). Therefore, although there is no apparent distinction between cortical and medullary zones in differentiating secondary lobes, there is a significant correlation between the expression of maturation markers and the downregulation of DE-cadherin, as is observed in primary lobes.

Temporal analysis of lymph gland hematopoietic development

The relatively late ‘snapshot’ of lymph gland development in the third larval instar establishes the existence of spatial zones within the lymph gland that are characterized by differences in structure as well as gene expression (Fig. 4F). In order to understand how these zones form over time, lymph glands of second instar larvae, the earliest time at which we are able to

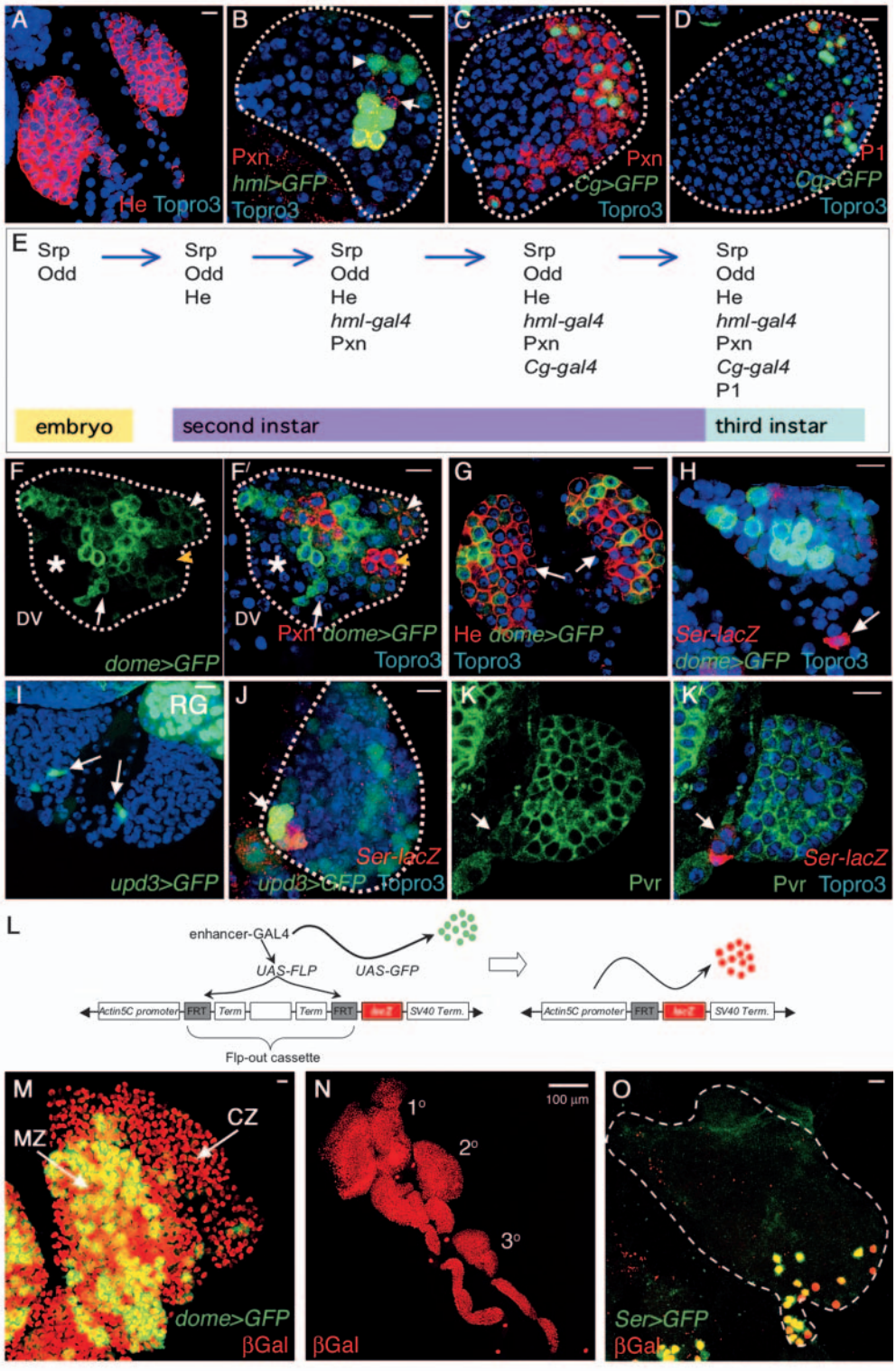

Fig. 5. Temporal analysis of hemocyte maturation. (A) Hemese (He, red) is expressed by all hemocytes in the primary lymph gland of the second instar larva. (B,C) Maturation marker expression in second instar lymph glands. (B) Expression of Pxn (red) and *hml$^{\Delta}$-gal4/UAS-GFP* (green) commence at approximately the same time during development. Although most cells express both markers (yellow), some express only *hml$^{\Delta}$-gal4/UAS-GFP* (arrowhead), and others express only Pxn (arrow). (C) *Cg-gal4/UAS-GFP* (green) is expressed in a small subset of cells expressing Pxn (red). (D) P1 antigen (red) is first expressed in the early third instar lymph gland. Fewer cells are seen expressing P1-antigen than *Cg-gal4/UAS-GFP* (green). (E) Schematic representation of the order of appearance of plasmatocyte maturation markers. (F-G) Maturing hemocytes, prohemocytes and pre-prohemocytes in the second instar lymph gland. (F,F′) Maturing hemocytes are marked by Pxn expression (F′, red). These cells either express low levels of *dome-gal4/UAS-GFP* (green, white arrowheads) or no *dome-gal4/UAS-GFP* (yellow arrowheads). Prohemocytes do not express Pxn, but are marked by *dome-gal4/UAS-GFP* (F,F′, arrows). Finally, pre-prohemocytes are located medially, close to the dorsal vessel (DV) and are characterized by the absence of both *dome-gal4/UAS-GFP* and Pxn expression (asterisks, blue only). These pre-prohemocytes do express Hemese (G; red, arrows). (H-K′) The second instar PSC (arrows) are marked by *Ser-lacZ* (H,J,K′; red). Expression of *upd3-gal4/UAS-GFP* (I,J, green; RG, ring gland) initiates in the PSC (I, arrows) and expands later to other cells of the lymph gland (J, green). *dome-gal4/UAS-GFP* (H, green) and Pvr (K,K′; green) are not expressed in the PSC. (L-O) Gal4-based cell-lineage tracing in the larval lymph gland. (L) Schematic diagram of the cell-lineage marking system. Test stock flies of the genotype *UAS-FLP, actin5C-FRT-STOP-FRT-lacZ* is crossed to various Gal4-expressing lines (*enhancer-gal4, UAS-GFP*). Gal4 activates GFP (green) and FLP recombinase expression, which then removes the 'FLP-out' cassette such that the constitutive *actin5C* promoter then drives *lacZ* expression (red) permanently within all subsequent daughter cells. (M) Cortical zone cells are derived from *dome-gal4*-expressing cells. The *dome-gal4* reporter causes cortical zone (CZ) cells in the third instar lymph gland to be permanently marked with β-gal expression (red) despite the lack of *dome-gal4* expression (as assessed by GFP) in this zone. (N) As a positive control, third instar lymph gland cells were found to be permanently marked by β-gal expression (red) because of *twist-gal4* activation in the embryonic mesoderm, from which the lymph gland is derived. No GFP expression is detectable, indicating that *twist-gal4* is no longer expressed in the third larval instar. (O) As a negative control, we show that cells of the PSC do not contribute to cortical zone cells. Lineage tracing using *Serrate-gal4* revealed that PSC cells remain few in number and do not give rise to cortical zone cells. The majority of the Serrate-expressing cells appear yellow because of simultaneous expression of GFP (green) and β-gal (red). To-pro-3 (blue) marks nuclei. Scale bars: 10 μm in A-M,O; 100 μm in N.

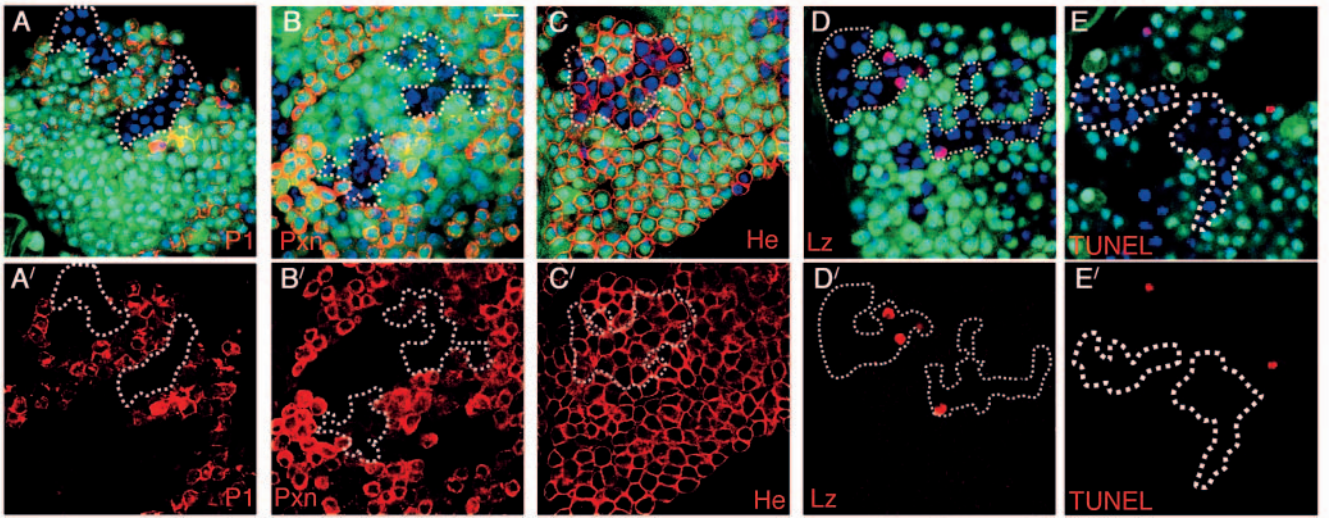

Fig. 6. Role of Pvr in plasmatocyte differentiation. (A-E′) *Pvr⁻/Pvr⁻* mutant clones marked by the absence of GFP (A-E, green) expression. To-pro-3 marks all nuclei (blue). Representative clones are outlined and analyzed for marker expression in A′-E′. P1 antigen (A,A′, red) and Pxn (B,B′, red) are missing in *Pvr⁻/Pvr⁻* clones, but Hemese (C,C′, red) and Lz (D,D′, red) expression remain unchanged. TUNEL (E,E′, red) staining shows that cell death is not enhanced in the mutant clones.

dissect and stain, were examined for the expression of hematopoietic markers. As expected, Srp and Odd are expressed throughout the lymph gland during the second instar (Fig. 1C,D) as they are in the late embryo and third instar lymph gland (Fig. 1A,B,E). Likewise, the hemocyte-specific marker Hemese is expressed throughout the lymph gland at this stage (Fig. 5A), although it is not present in the embryonic lymph gland (not shown).

To determine whether the cortical zone is already formed or forming in second instar lymph glands, we examined the expression of various maturation markers in a pair-wise manner to establish their temporal order. Of the markers examined, we found that *hml-gal4* and Pxn are the earliest to be expressed. The majority of maturing cells were found to be double-positive for *hml-gal4* and Pxn expression, although a few cells were found to express either *hml-gal4* or Pxn alone (Fig. 5B). This indicates that the expression of these markers is initiated at approximately the same time, although probably independently, during lymph gland development. The marker *Cg-gal4* is next to be expressed as it was found among a subpopulation of Pxn-expressing cells (Fig. 5C). Finally, P1 antigen expression is initiated late, usually in the early third instar (Fig. 5D). Interestingly, the early expression of each of these maturation markers is restricted to the periphery of the primary lymph gland lobe, indicating that the cortical zone begins to form in this position in the second instar. Whenever possible, each genetic marker was directly compared with other pertinent markers in double-labeling experiments, except in cases such as the comparison of two different *gal4* reporter lines or when available antibodies were generated in the same animal. In such cases, the relationship between the two markers, for example *dome-gal4* and *hml-gal4*, was inferred from independent comparison with a third marker such as Pxn.

By studying the temporal sequence of expression of hemocyte-specific markers, one can describe stages in the maturation of a hemocyte. As an example, the maturation steps of a typical plasmatocyte are indicated in Fig. 5E. It should be noted, however, that not all hemocytes of a particular lineage are identical. For example, in the late third instar lymph gland, the large majority of mature plasmatocytes (~80%) expresses both Pxn and *hml-gal4*, but the remainder expresses only Pxn (~15%) or *hml-gal4* (~5%) alone. Thus, while plasmatocytes as a group can be characterized by the expression of representative markers (shown in Fig. 5E), populations expressing subsets of these markers indeed exist. It remains unclear at this time whether this heterogeneity in the hemocyte population is reflective of specific functional differences.

In the third instar, Pxn is a prototypical hemocyte maturation marker, while immature cells of the medullary zone express *dome-gal4*. Comparing the expression of these two markers in the second instar revealed an interesting developmental progression. As mentioned above, a group of cells along the peripheral edge of these early lymph glands already express Pxn. These developing hemocytes downregulate the expression of *dome-gal4* (Fig. 5F,F′), as they do in the third instar. Next to these developing hemocytes is a group of cells that expresses *dome-gal4* but not Pxn; these cells are most similar to medullary zone cells of the third instar and are therefore prohemocytes. Interestingly, there also exists a group of cells in the second instar that expresses neither Pxn nor *dome-gal4*. This population is most easily seen in the medial parts of the gland, close to the centrally placed dorsal vessel (Fig. 5F′). These cells resemble earlier precursors in the embryo, except they express the marker Hemese (Fig. 5G). We call these cells pre-prohemocytes. Our interpretation of the expression data is that pre-prohemocytes upregulate *dome-gal4* to become prohemocytes. As prohemocytes begin to mature into hemocytes, *dome-gal4* expression is downregulated, while the expression of maturation markers is initiated. The prohemocyte and hemocyte populations continue to be represented in the third instar as components of the medullary and cortical zones, respectively.

The cells of the PSC are already distinguishable in the late embryo by their expression of *collier* (Crozatier et al., 2004).

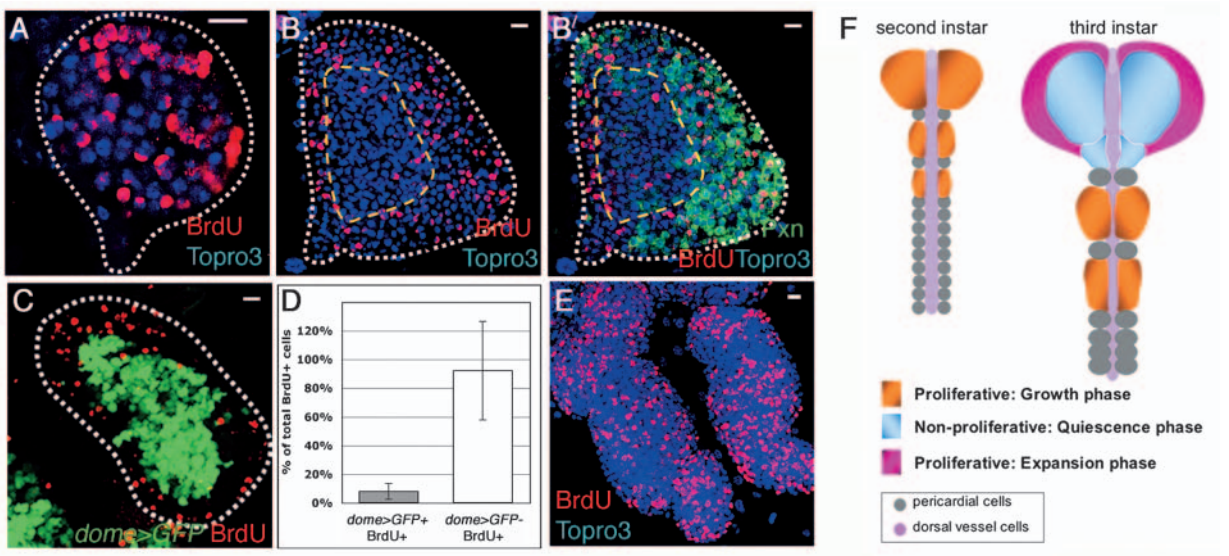

Fig. 7. Proliferation profile of the lymph gland. (A) Second instar primary lobe stained for BrdU incorporation. A single confocal section from the middle of the lymph gland shows BrdU (red) incorporation in randomly positioned cells. (B,B′) Third instar primary lobe stained for BrdU incorporation. A single confocal section from the middle of the lymph gland shows that $BrdU^+$ cells (red) are restricted to the cortical zone marked by Pxn expression (B′, green). (C) Cells incorporating BrdU (red) are excluded from the medullary zone marked by *dome-gal4/UAS-GFP* (green). (D) Quantitation of data represented in C. Twelve third instar lymph gland lobes were counted. The *y*-axis represents the percentage of total BrdU-positive cells. (E) In secondary lobes of the third instar lymph gland, $BrdU^+$ cells (red) are randomly distributed. To-pro-3 (blue) marks nuclei. Scale bars: 10 μm. (F) Three phases of proliferation in lymph gland development. Phase 1: growth phase. Lymph gland cells expand in number from the embryonic stage through the second instar (left panel). Cells in the secondary lobes of the third instar also belong to this phase. Phase 2: quiescence phase. This comprises the prohemocyte population of the third instar medullary zone. Cells of the PSC also rarely divide. Phase 3: expansion phase. Maturing and mature hemocytes divide to increase their numbers.

We found that the canonical PSC marker *Ser-lacZ* is not expressed in the embryonic lymph gland (not shown) and is only expressed in a small number of cells in the second instar (Fig. 5H). This relatively late onset of expression is consistent with *collier* acting genetically upstream of *Ser* (Crozatier et al., 2004). Another finding was that the earliest expression of *upd3-gal4* parallels the expression of *Ser-lacZ* and is restricted to the PSC region (Fig. 5I,J). Finally, Pvr and *dome-gal4* are excluded from the PSC in the second instar (Fig. 5H,K,K′), similar to that seen in the third instar.

To determine whether maturing cortical zone cells are indeed derived from medullary zone prohemocytes, a lineage-tracing experiment was performed in which *dome-gal4* was used to initiate the permanent marking of all daughter cell lineages (Fig. 5L). In this system, the *dome-gal4* reporter expresses both UAS-GFP and UAS-FLP. The FLP recombinase excises an intervening FRT-flanked 'STOP cassette', allowing constitutive expression of *lacZ* under the control of the *actin5C* promoter (Struhl and Basler, 1993). At any developmental time point, GFP is expressed in cells where *dome-gal4* is active, while *lacZ* is expressed in all subsequent daughter cells regardless of whether they continue to express *dome-gal4*. In this experiment (Fig. 5M), cortical zone cells are permanently marked with β-galactosidase despite not expressing *dome-gal4* (as assessed by GFP), indicating that these cells are derived from a *dome-gal4*-positive precursor. This result is consistent with and further supports our independent marker analysis that showed that *dome-gal4*-positive prohemocytes downregulate *dome-gal4* expression as they initiate expression of maturation markers representative of cortical zone cells. As controls to the above experiment, we determined the expression patterns of two other *gal4* lines, *twist-gal4* and *Serrate-gal4*. The reporter *twist-gal4* is expressed throughout the embryonic mesoderm from which the lymph gland is derived. Accordingly, the entire lymph gland is permanently marked by β-galactosidase (Fig. 5N) despite a lack of *twist-gal4* expression (GFP) in the third instar lymph gland. Analysis of *Ser-gal4* revealed that PSC cells remain a distinct population of signaling cells that do not contribute to the cortical zone (Fig. 5O).

Role of Pvr in hemocyte maturation

Genetic manipulation of Pvr function provided valuable insight into its involvement in the regulation of temporal events of lymph gland development. To analyze Pvr function, FLP/FRT-based *Pvr*-mutant clones were generated in the lymph gland early in the first instar and then examined during the third instar for the expression of maturation markers. We found that loss of Pvr function abolishes P1 antigen and Pxn expression (Fig. 6A,A′,B,B′), but not Hemese expression (Fig. 6C,C′). The crystal cell markers Lz (Fig. 6D,D′) and ProPOA1 (not shown) are also expressed normally in *Pvr*-mutant clones, consistent with the observation that mature crystal cells lack or downregulate Pvr (not shown). The fact that *Pvr*-mutant cells express Hemese and can differentiate into crystal cells suggests that Pvr specifically controls plasmatocyte differentiation. *Pvr*-mutant cells do not become TUNEL positive (Fig. 6E,E′) but do express the hemocyte marker Hemese and can differentiate into crystal cells, all suggesting that the observed block in plasmatocyte differentiation within the mutant clone is not due to cell death. Additionally, Pvr-mutant clones were large (Fig. 6) and not significantly different in size from their wild-type

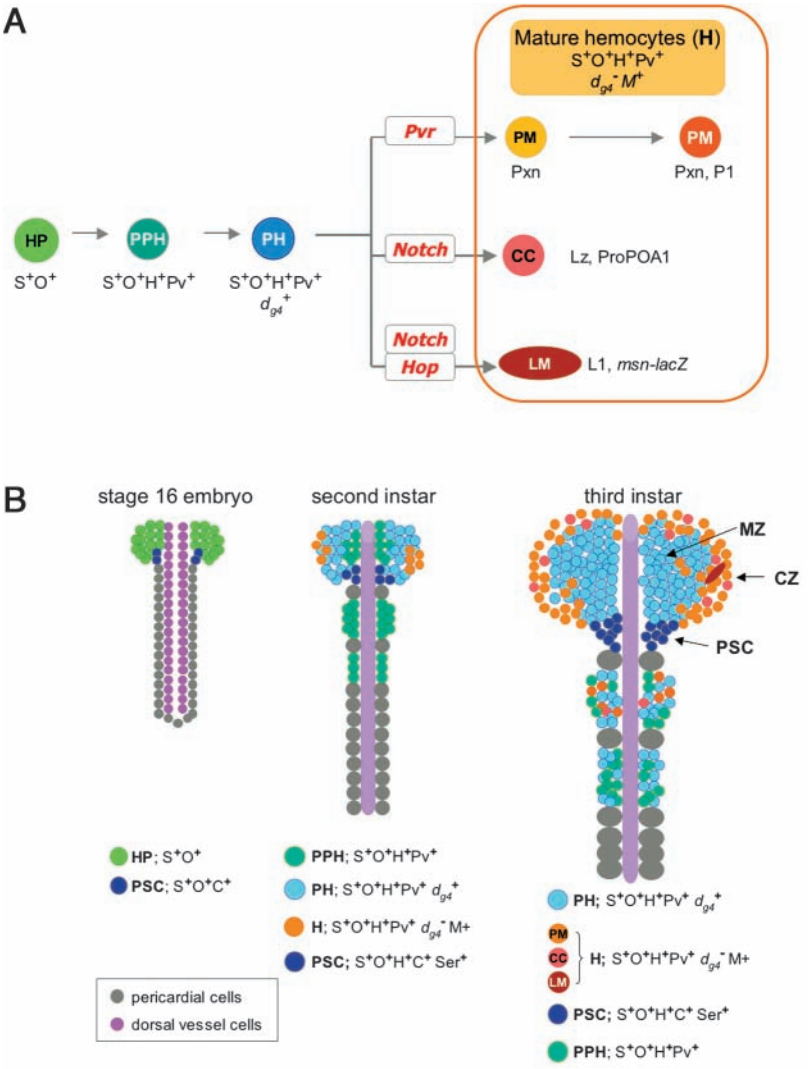

Fig. 8. Schematic diagram of hemocyte maturation in the lymph gland. (A) The earliest lymph gland cells, the hemocyte precursors (HP), express Srp (S^+) and Odd (O^+). As these cells transition into pre-prohemocyte (PPH) fate, they initiate the expression of Hemese (H^+) and Pvr (Pv^+). Prohemocytes (PH) initiate *dome-gal4* ($d_{g4}{}^+$) expression. Maturation to the various hemocyte (H) fates requires downregulation of *dome-gal4* ($d_{g4}{}^-$), upregulation of different maturation markers (M^+) and the involvement of the indicated signaling pathways. (B) Spatial and temporal sequence of hemocyte development in the lymph gland (see text for details). The PSC cells are marked by Collier (C^+) and Serrate (Ser^+) expression. Ubiquitous cell markers such as Srp, Odd, Pvr and Hemese are expressed in all the lymph gland cells marked by the nuclear marker To-pro-3, thus they are expressed in all the cell types and the zones. Mature hemocytes markers such as Pxn, P1 and Lz were compared with *dome-gal4* expression. Lamellocyte marker *msn-lacZ* was compared with *G147*. All the markers used in the diagram except Collier were directly compared with *Ser-lacZ*. PM, plasmatocyte; CC, crystal cell; LM, lamellocyte; CZ, cortical zone; MZ, medullary zone; PSC, posterior signaling center.

twin spots (not shown). Thus, the primary role of Pvr is not in the control of cell proliferation. Targeting Pvr by RNA interference (RNAi) revealed the same phenotypic features (not shown), confirming that Pvr controls the transition of Hemese-positive cells to plasmatocyte fate.

Proliferation profile of the developing lymph gland

We monitored entry into S phase using BrdU incorporation and identified distinct proliferative phases that occur during lymph gland hematopoiesis. In the second instar, proliferating cells are evenly distributed throughout the lymph gland (Fig. 7A). By the third instar, however, the distribution of proliferating cells is no longer uniform; S-phase cells are largely restricted to the cortical zone (Fig. 7B-D). This is particularly evident when BrdU-labeled lymph glands are co-stained with Pxn (Fig. 7B′). Medullary zone cells, which can be identified by the expression of *dome-gal4*, rarely incorporate BrdU (Fig. 7C,D). Therefore, the rapidly cycling prohemocytes of the second instar lymph gland quiesce as they populate the medullary zone of the third instar. As prohemocytes transition into hemocyte fates in the cortical zone, they once again begin to expand in number. This is supported by the observation that the medullary zone in white pre-pupae does not appear diminished in size (not shown), suggesting that the primary mechanism for the expansion of the cortical zone prior to this stage is through cell division within the zone. Proliferating cells in the secondary lobes continue to be distributed uniformly in the third instar (Fig. 7E), suggesting that secondary-lobe prohemocytes do not reach a state of quiescence as do the cells of the medullary zone. These results indicate that cells of the lymph gland go through distinct proliferative phases as hematopoietic development proceeds (Fig. 7F, also see discussion below).

Discussion

The *Drosophila* lymph gland has long been known to be a hematopoietic organ but was largely perceived as a mass of cells that differentiate into the major hemocyte types. The mechanisms by which this occurs were relatively uncharacterized and the structural features of the lymph gland had not been correlated with the cellular events underlying hematopoiesis. We set out to systematically describe lymph gland development in the context of hemocyte maturation. Using genetic markers known to be expressed by hematopoietic cells as a starting point, we examined when and where these markers are expressed in the lymph gland. Furthermore, we identified and defined structural features of the lymph gland not previously described and then determined how these features correlate with hemocyte maturation and patterns of cell proliferation.

Our analysis of the lymph gland revealed three key features that arise during development. The first feature is the presence of three distinct zones in the primary lymph gland lobe of third instar larvae. Two of these zones, which we term the cortical and medullary zones, exhibit structural characteristics that make them morphologically distinct. These zones, as well as

the third zone, the PSC, are also distinguishable by the expression of specific markers. The second key feature is the finding that cells expressing maturation markers such as Lz, ProPOA1, Pxn, *hml-gal4* and *Cg-gal4* are restricted to the cortical zone. The medullary zone is consistently devoid of maturation marker expression and is therefore defined as a region composed of immature hemocytes (prohemocytes). The finding of different developmental populations within the lymph gland (prohemoctyes and their derived hemocytes) is similar to the situation in vertebrates where it is known that hematopoietic stem cells and other blood precursors give rise to various mature cell types. Additionally, *Drosophila* hemocyte maturation is akin to the progressive maturation of myeloid and lymphoid lineages in vertebrate hematopoiesis. The third key feature of lymph gland hematopoiesis is the dynamic pattern of cellular proliferation observed in the third instar. At this stage, the vast majority of S-phase cells in the primary lobe are located in the cortical zone, suggesting a strong correlation between proliferation and hemocyte differentiation. Compared with earlier developmental stages, cell proliferation in the medullary zone actually decreases by the late third instar, suggesting that these cells have entered a quiescent state. Thus, proliferation in the lymph gland appears to be regulated such that growth, quiescence and expansion phases are evident throughout its development (Fig. 7F).

Drosophila blood cell precursors, prohemocytes and maturing hemocytes each exhibit extensive phases of proliferation. The competence of these cells to proliferate seems to be a distinct cellular characteristic that is superimposed upon the intrinsic maturation program. Based on the patterns of BrdU incorporation in developing primary and secondary lymph gland lobes, it is possible to envision at least two levels of proliferation control during hematopoiesis. We propose that the widespread cell proliferation observed in second instar lymph glands and in secondary lobes of third instar lymph glands occurs in response to a growth requirement that provides a sufficient number of prohemocytes for subsequent differentiation. The mechanisms promoting differentiation in the cortical zone also trigger cell proliferation, which accounts for the observed BrdU incorporation in this zone and serves to expand the effector hemocyte population. The quiescent cells of the medullary zone represent a pluripotent precursor population because they, similar to vertebrate hematopoietic precursors, rarely divide and give rise to multiple lineages and cell types.

Based on the analysis described above, we can propose a model by which hemocytes mature in the lymph gland (Fig. 8). Hematopoietic precursors that populate the early lymph gland are first distinguishable as Srp+, Odd+ (S^+O^+, Fig. 8A) cells. These will eventually give rise to a primary lymph gland lobe where the steps of hemocyte maturation are most apparent. During the first or early second instar, these S^+O^+ cells begin to express the hemocyte-specific marker Hemese (He) and the tyrosine kinase receptor Pvr. Such cells can be called pre-prohemocytes and, in the second instar, cells expressing only these markers occupy a narrow region near the dorsal vessel. Subsequently, a subset of these Srp+, Odd+, He+, Pvr+ ($S^+O^+H^+Pv^+$) pre-prohemocytes initiate the expression of *dome-gal4* (d_{g4}), thereby maturing into prohemocytes. The prohemocyte population ($S^+O^+H^+Pv^+d_{g4}{}^+$) can be subdivided into two developmental stages. Stage 1 prohemocytes, which are abundantly seen in the second instar, are proliferative, whereas stage 2 prohemocytes, exemplified by the cells of the medullary zone, are quiescent. As development continues, prohemocytes begin to downregulate *dome-gal4* and express maturation markers (M; becoming $S^+O^+H^+Pv^+d_{g4}{}^{low}M^+$). Eventually, *dome-gal4* expression is lost entirely in these cells (becoming $S^+O^+H^+Pv^+d_{g4}{}^-M^+$), found generally in the cortical zone. Thus, the maturing hemocytes of the cortical zone are derived from prohemocytes previously belonging to the medullary zone. This is supported by lineage-tracing experiments that showed cells expressing medullary zone markers can indeed give rise to cells of the cortical zone. In turn, the medullary zone is derived from the earlier, pre-prohemocytes. Early cortical zone cells continue to express successive maturation markers (M) as they proceed towards terminal differentiation (Fig. 8B). Depending on the hemocyte type, examples of expressed maturation markers are Pxn, P1, Lz, L1, *msn-lacZ*, etc. Our studies have shown that differentiation of the plasmatocyte lineage requires Pvr, while previous work has shown that the Notch pathway is crucial for the crystal cell fate (Duvic et al., 2002; Lebestky et al., 2003). Both the JAK/STAT and Notch pathways have been implicated in lamellocyte production (Duvic et al., 2002; Sorrentino et al., 2004).

Previous investigations have demonstrated that similar transcription factors and signal transduction pathways are used in the specification of blood lineages in both vertebrates and *Drosophila*. Given this relationship, *Drosophila* represents a powerful system for identifying genes crucial to the hematopoietic process that are conserved in the vertebrate system. The work presented here provides an analysis of hematopoietic development in the *Drosophila* lymph gland that not only identifies stage-specific markers, but also reveals developmental mechanisms underlying hemocyte specification and maturation. The prohemocyte population in *Drosophila* becomes mitotically quiescent, much as their multipotent precursor counterparts in mammalian systems. These conserved mechanisms further establish *Drosophila* as an excellent genetic model for the study of hematopoiesis.

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