

# Hematopoiesis at the onset of metamorphosis: terminal differentiation and dissociation of the *Drosophila* lymph gland

Melina Grigorian · Lolitika Mandal ·  
Volker Hartenstein

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**Abstract** The *Drosophila melanogaster* hematopoietic organ, called lymph gland, proliferates and differentiates throughout the larval period. The lymph gland of the late larva is comprised of a large primary lobe and several smaller secondary lobes. Differentiation into two types of hemocytes, plasmatocytes and crystal cells, is confined to the outer layer (cortical zone) of the primary lobe; the center of the primary lobe (medullary zone), as well as the secondary lobes, contain only proliferating prohemocytes. A small cluster of cells located at the posterior tip of the primary lobe serves as a signaling center (PSC) that inhibits precocious differentiation of the medullary zone. The larval lymph gland is stabilized by layers of extracellular matrix (basement membranes) that surround individual hemocytes, groups of hemocytes, as well as the lymph gland as a whole. In this paper, we investigated the events shaping the lymph gland in the early pupa. The lymph gland dissociates and hemocytes disperse during the first 12 h after puparium formation (APF), leaving behind empty husks of basement membrane. Prior to lymph gland dissociation, cells of the medullary zone differentiate, expressing the early differentiation marker Peroxidase (Pxn), as well as, in part, the late

differentiation marker P1. Cells of the PSC spread throughout the pupal lymph gland prior to their dispersal. Cells of the secondary lobes undergo a rapid phase of proliferation that lasts until 8 h APF, followed by expression of Pxn and dispersal. These hemocytes do not express P1, indicating that they disperse prior to full maturation.

**Keywords** *Drosophila* · Blood · Lymph gland · Metamorphosis

## Introduction

The hemolymph (blood) of *D. melanogaster* contains three types of mature hemocytes, the most prevalent of which is the plasmatocyte. The plasmatocyte carries out a phagocytic function, engulfing dead cells, debris, and pathogens. The second most prevalent cell type is the crystal cell, which aids in the innate immune response as well as in wound healing by carrying out melanization. The third and most rare hemocyte type is the lamellocyte. The lamellocytes are large flat cells, which encapsulate and neutralize objects too large to be phagocytized (Evans et al. 2003; Crozatier and Meister 2007; Krzemien et al. 2010). Lamellocytes are not seen in embryos or adults and are rarely found in larvae. However, upon immune challenge, via parasitization with a wasp egg, the number of lamellocytes increases substantially (Lanot et al. 2001; Sorrentino et al. 2002).

Blood formation in *Drosophila* takes place during two phases, the early embryo, and the larva. In the embryo, a subset of head mesodermal cells become specified as plasmatocytes and crystal cells (Tepass et al. 1994; Lebestky et al. 2000; Alfonso and Jones 2002); these cells

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M. Grigorian · L. Mandal · V. Hartenstein (✉)  
Department of Molecular Cell and Developmental Biology,  
University of California Los Angeles,  
Los Angeles, CA 90095, USA  
e-mail: volkerh@mcdb.ucla.edu

### Present Address:

L. Mandal  
Indian Institute of Science Education and Research (IISER)  
Mohali,  
Sector 26, MGSIPAP Complex,  
Chandigarh 160019, India

play a role during embryonic development, and form the pool of differentiated, motile cells of the larval hemolymph. The second phase of blood formation takes place in the lymph gland, a hematopoietic organ that develops in close association with the cardio-vascular system (dorsal vessel) and that originates from the thoracic mesoderm of the embryo (Poulson 1950; Rugendorff et al. 1994; Mandal et al. 2004). In the late embryo and early larva, the lymph gland forms a small, bilaterally paired cluster of undifferentiated blood progenitors (prohemocytes). Prohemocytes proliferate rapidly during the larval period. In the late larva, the lymph gland is comprised of a pair of large primary lobes, which have developed from the small lymph gland primordium; to this, a series of secondary and tertiary lobes, located posterior to the primary lobes, have been added (Jung et al. 2005). Hemocyte maturation takes place in the primary lobes in a tightly regulated spatio-temporal pattern. The center of the primary lobes, called medullary zone, contains prohemocytes that remain in a slowly proliferating, undifferentiated state throughout the larval period. Cells of the medullary zone are considered “stem cell-like” and are maintained in this state by the posterior signaling center (PSC) via a number of signaling pathways (Mandal et al. 2007; Krzemien et al. 2007). The medullary zone is surrounded by an outer cortical zone, in which cells differentiate, expressing a number of molecular and morphological markers of plasmatocytes and crystal cells (and, in cases of injury or infection, lamellocytes).

Under normal conditions, cells of the lymph gland remain together and do not disperse into the hemolymph. In that regard, *Drosophila* appears to present an exception, since in other insects, including other Dipterans, hemocytes of the lymph gland are constantly given off into the hemolymph (Akai and Sato 1971; Hinks and Arnold 1977; Nakahara et al. 2003). In *Drosophila*, only infection or injury (Rizki and Rizki 1992; Sorrentino et al. 2002; Agaisse et al. 2003) triggers increased proliferation and maturation in the lymph gland and causes the release of hemocytes into circulation. In the absence of infection/injury, the lymph gland of *Drosophila* dissociates during the early pupal stage (Lanot et al. 2001). By 15 h after puparium formation (APF), all lobes of the lymph gland have dissolved. Adult *Drosophila* have no compact blood forming organ. This may be an adaptation to the short life span of fruit flies; it is also possible that undifferentiated, pluripotent hemocyte progenitors remain active in circulation or in the form of small clusters of cells which so far have eluded detection.

The process of lymph gland dissociation in the pupa has been analyzed electron microscopically (Lanot et al. 2001), but many questions have remained unanswered. For example, do cells of the lymph gland all differentiate before moving out into the hemolymph? Can one detect

differences in the rate of maturation/dissociation between primary and secondary lobes? Do the zones of the primary lobe remain intact until the very end, when lymph gland dissociation starts to occur? What happens to the reticular extracellular matrix that surrounds individual hemocyte progenitors of the larval lymph gland? To address these questions, we have employed the use of a combination of hemocyte specific markers, as well as transmission electron microscopy (TEM), in order to analyze the stages of lymph gland dissociation.

## Materials and methods

### Antibodies

Antibodies used for immunohistochemistry include:  $\alpha$ -Mouse-Antennapedia (1:4) (Developmental Hybridoma Bank),  $\alpha$ -rat BrdU (1:100) (Abcam),  $\alpha$ -Mouse-GFP (1:400) (Sigma),  $\alpha$ -Rabbit-GFP (1:2,000) (Molecular Probes),  $\alpha$ -Mouse-L1 (1:100) (Kurucz et al. 2003),  $\alpha$ -Mouse P1 (1:100) (Kurucz et al. 2007),  $\alpha$ -Mouse-Peroxidasin (1:500) (Nelson et al. 1994), and  $\alpha$ -Rabbit-ProPo (1:1,000) (Muller et al. 1999). All secondary antibodies are from Jackson ImmunoResearch and were used at 1:200 (FITC and CY3), 1:100 (CY5), and 1:500 (ALEXA).

### Fly lines

Protein trap line, ZCL1973X (Kelso et al. 2004), which labels Trol expression with GFP was used. G147, a GFP trap line associated with a microtubule protein (Morin et al. 2001) was also used.

### Pupal fixation

Pupae were pinned dorsal side up, and their pupal case removed. Pupal lymph gland was carefully exposed before a fixative (1:3 of 37% formaldehyde: 1 $\times$  PBS) solution was applied to the sample for 20 min. Pupae were then washed three times in 0.1% PBT for 15 min each. Immunohistochemistry was then carried out.

### Immunohistochemistry

Samples were washed with a solution of 0.1% PBT three times for 15 min each at room temperature while rotating. Samples were then blocked for 30 min in a solution of 10% NGS made in 0.1% PBT. Samples were then placed in a 1 $^{\circ}$  antibody solution made in 10% NGS and left to rotate overnight at 4 $^{\circ}$ C. Samples were then washed and blocked as described above and placed in a 2 $^{\circ}$  antibody conjugated

to a fluorescent marker. Samples were then left in 2° antibody to rotate overnight at 4°C. Samples were washed as described above and mounted in vectashield containing a 1:500 concentration of Toto-3-iodide (invitrogen).

#### BrdU incubation, fixation, and staining

Dissected pupal lymph glands were placed in a solution of 75 µg/ml of BrdU. Lymph glands were allowed to incubate in BrdU solution for 40–60 min on ice. Tissue was fixed in a 1:3 solution of 37% formaldehyde: 1× PBS for 20–30 min. Samples were washed four times for 10 min each in 0.3% PBST and blocked in 10% NGS for 30 min. Samples were incubated in primary antibody overnight at 4°C on rotator. Samples were washed four times, for 10 min each, in 0.3% PBST. After blocking for 30 min in 10% NGS, samples were incubated in 2° antibody overnight at 4°C. After washing in 0.3% PBST twice for 10 min, tissue was fixed for a second time in a 1:3 solution of 37% formaldehyde and 1× PBS for 15 min, followed by washing in 0.3% PBST three times for 10 min each. DNA was denatured for 30 min by adding 2 N HCl. After three washes in 0.3% PBST, 10 min each, samples were blocked for 30 min. Samples were incubated in anti-BrdU antibody overnight at 4°C. Samples were washed with 0.3% PBST four times for 10 min each, blocked for 30 min in 10% NGS, and incubated in 2° antibody overnight at 4°C. This was followed by washing samples with 0.3% PBST four times for 10 min. Samples were mounted in Vectashield.

#### EM fixation

Fixative solution contained 0.2 mL EM phosphate buffer A (0.2 M NaH<sub>2</sub>PO<sub>4</sub>), 0.8 mL EM phosphate buffer B (0.2 M Na<sub>2</sub>HPO<sub>4</sub>), 0.4 mL 16% EM grade formaldehyde, 0.125 mL 50% glutaraldehyde, and 0.475 mL DI water; kept on ice. Dissected tissue was fixed for 24 h at 4°C, followed by washing five times for 10 min in phosphate buffer (1.0 mL EM phosphate buffer A, 4.0 mL EM phosphate buffer B, 5.0 mL DI water). Postfixation was carried out in 1% osmium tetroxide (0.5 ml DI water, 0.5 ml 4% osmium tetroxide and 1 ml phosphate buffer 0.2 M pH 7.3) at 4°C, in dark, for 60 min. Washing was done four times for 10 min in DI water. Tissue was dehydrated through acetone series at 4°C (50%, 70%, and 96% acetone for 10 min each; 100% acetone three times for 10 min each). For embedding, tissue was placed in 1:3 epon:acetone mixture for 2 h at room temperature while rotating, placed in 1:1 epon:acetone mixture for 3 h at room temperature while rotating, placed in 3:1 epon:acetone mixture overnight at room temperature, and placed in epon overnight. Blocks were polymerized for 16 h at 60°C in flexible plastic mold.

#### Confocal

BioRad Radiance 2000 confocal microscope with LaserSharp 2000 acquisition software, ×40 oil immersion lens was used.

## Results

### Lymph gland hemocytes express differentiation markers prior to dissociation

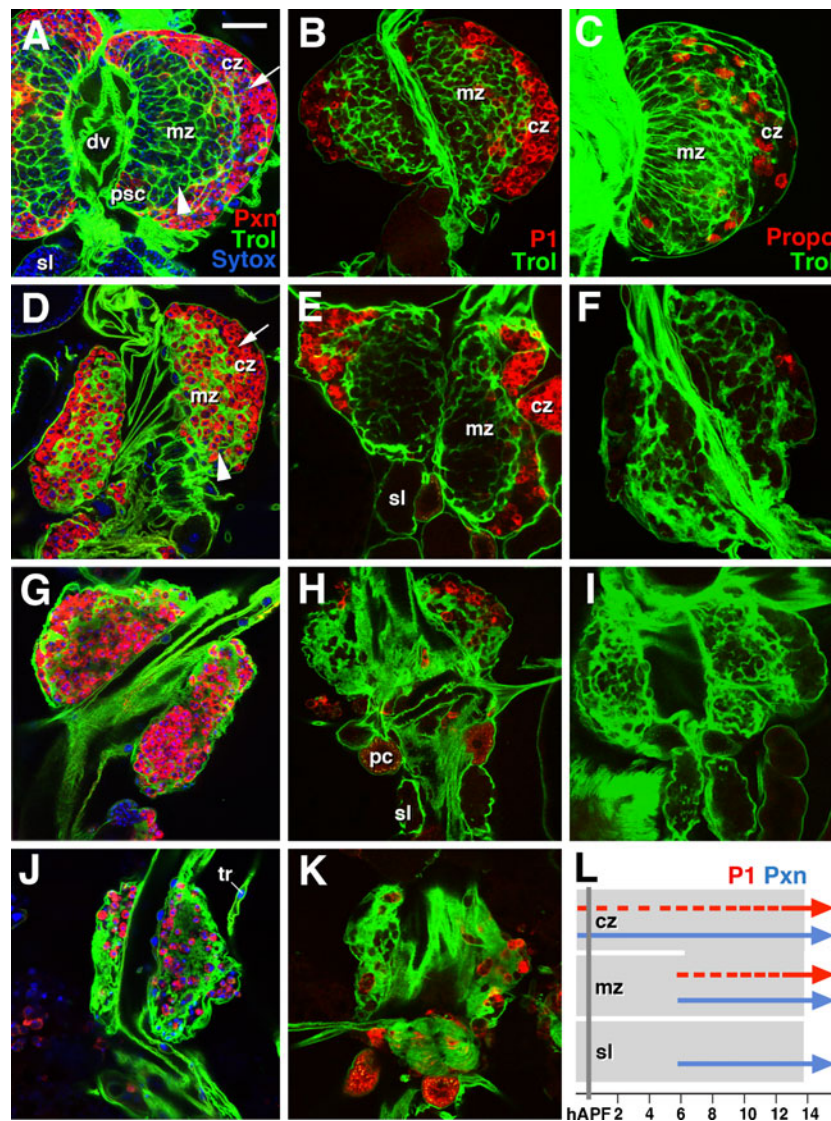
In order to determine whether all the cells of the lymph gland differentiated before their release and dispersal, and at what time point this occurred, we analyzed animals at white prepupal stage and 2, 4, 8, and 10 h APF, using the ZCL1973X (Trol-GFP protein trap) line that expresses GFP in the lymph gland (Fig. 1a–k). As a differentiation marker for plamatoocytes, we labeled dissected lymph glands with anti-peroxidasin (Pxn; early differentiation marker) and P1 (late differentiation marker); in addition, Propo, a marker for differentiated crystal cells, was also used (Jung et al. 2005).

In late larvae, expression of Pxn is restricted to the cortical zone (Fig. 1a). Around 4 h APF, we observe sporadic Pxn signal in the medullary zone (Fig. 1d), and by 8 h, most, if not all cells of the pupal lymph gland are Pxn-positive (Fig. 1g). At the same time, the structural distinction between a medullary and cortical zone breaks down. Thus, in late larvae, the medullary zone is distinguished from the cortical zone by a denser Trol-positive ECM reticulum, as well as a higher packing density of hemocytes (Fig. 1a). By 8 h APF, the strong expression of Trol in the medullary zone has become reduced and Trol expression looks more homogeneous throughout what remains of the primary lymph gland lobes (Fig. 1g).

The expression of P1 is temporally offset relative to Pxn. By 4 h APF, when many central (medullary) cells are already Pxn-positive, P1 is still restricted to the cortical zone (Fig. 1e). However, by 8 and 10 h APF, P1-positive cells are clustered throughout the lymph gland (Fig. 1h, k, respectively).

Crystal cells, labeled by Propo, form a small population of hemocytes that are evenly scattered throughout the cortical zone of late larvae (Fig. 1c). In the pupal lymph gland, crystal cells are almost entirely absent; we only saw small groups of 1–3 crystal cells in the periphery of lymph gland lobes of pupae aging up to 2 h APF (Fig. 1f). Our findings indicate that hemocytes differentiate before they move out of the lymph gland; crystal cells appear to be the first cells to leave.

The expression of differentiation markers presented above is paralleled by morphological signs of differentia-



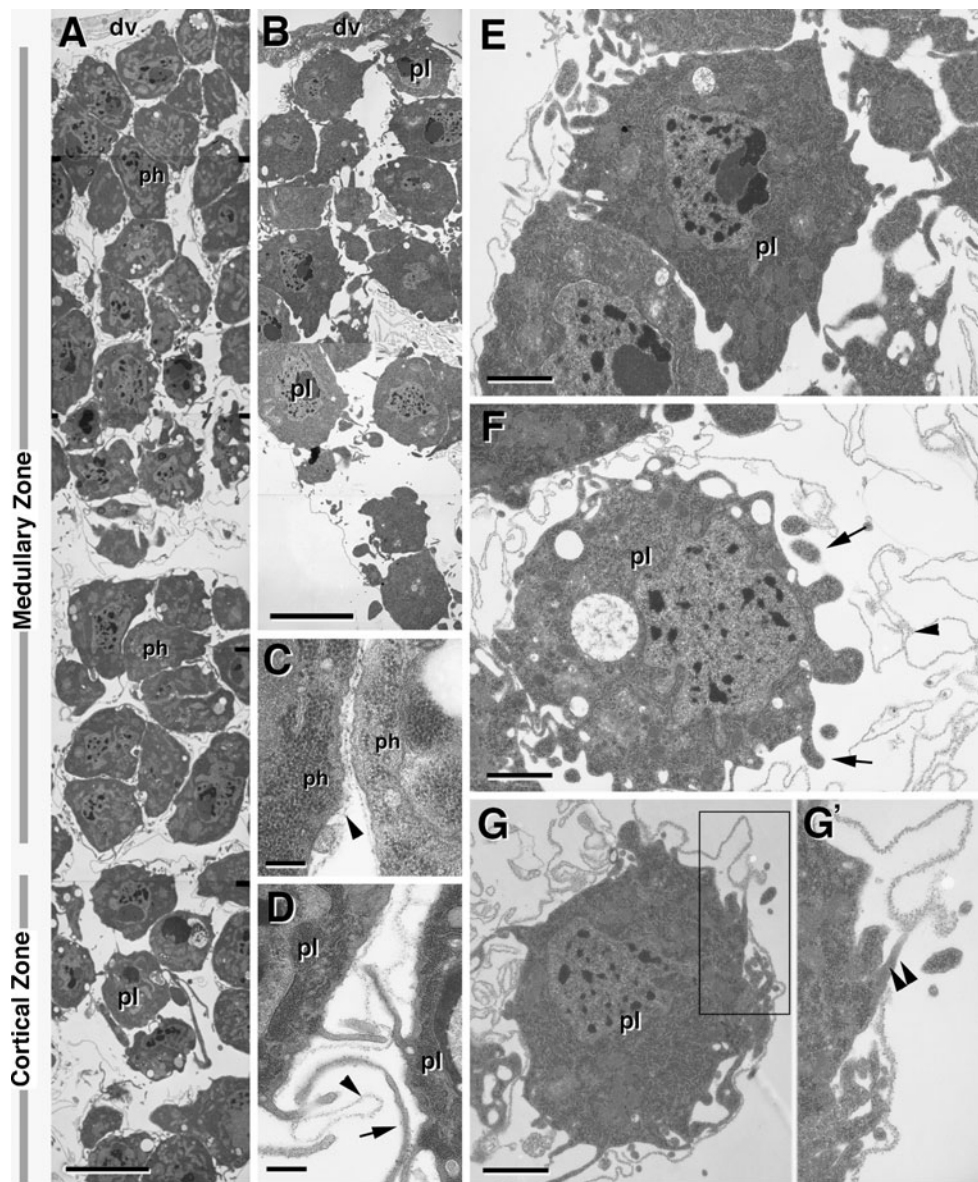
**Fig. 1** Expression of hemocyte differentiation markers in the pupal lymph gland. Panels show Z-projections of confocal sections of central part of primary lymph gland lobe. Expression of Trol-GFP (Protein Trap line: ZCL1973X) in extracellular matrix outlines the lymph gland (green). Panels of left column (a, d, g, j) show labeling with early plasmatocyte marker anti-Peroxidase (Pxn, red). Second column (b, e, h, k) represents labeling with late differentiation marker P1 (red); in right column (c, f, i), crystal cell marker Propo was used (red). Upper row (a, b, c) represents late third instar to white prepupa (0–2 h APF), second row (d–f) 2–4 h pupa, third row (g–i) 8 h pupa,

and fourth row (j, k) 10 h pupa. Panel L provides a timeline (in hours after puparium formation) of the expression pattern of P1 (red) and Peroxidase (blue) in different domains of pupal lymph gland. The top portion of the figure represents the cortical zone (cz). Below this is seen the medullary zone (mz) and finally the secondary lobes (sl). White arrowheads in a and d indicate the medullary zone which, until about 6 h APF, can be clearly distinguished from cortical zone (cz; white arrows in a and d). Other abbreviations: dv dorsal vessel; sl secondary lobe; psc posterior signaling center; pc pericardial cell; tr trachea; APF after puparium formation. Bar 20  $\mu$ m (a–k)

tion, as documented by TEM. Undifferentiated blood progenitors of the larval medullary zone are characterized by a higher nucleus–cytoplasm ratio than hemocytes in the cortical zone (Fig. 2a). They also lack the filiform processes and membrane ruffles typical for mature hemocytes. In the 4 h pupa, some cells with the characteristics of undifferentiated blood progenitors still persist (Fig. 2b), but mature plasmatocytes predominate throughout the lymph gland (Fig. 2e–f).

An Antennapedia-positive PSC persists in the early pupa

The PSC of the late larva forms a bilateral cluster of cells located at the posterior margin of the primary lobes (Fig. 3a). Antennapedia (Antp)-positive cells at numbers comparable to those typical for the larval PSC persisted until 10 h APF, immediately before dissociation of the lymph gland. After that, Antp expression is no longer associated with the dorsal vessel, indicating that



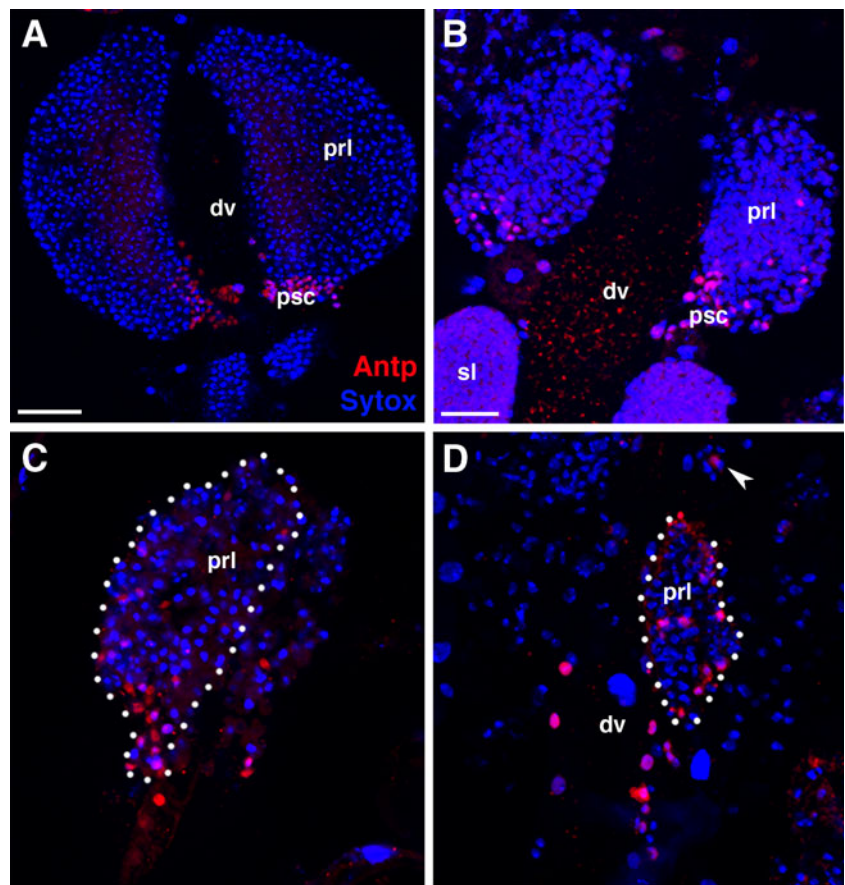
**Fig. 2** Ultrastructure of the lymph gland of the third instar larva and 4 h pupa. **a, b** Low magnification electron micrographs of primary lymph gland lobe of third instar larva (**a**) and 4 h pupa (**b**). Medial at top; note wall of dorsal vessel (*dv*) visible in both panels. At larval stage, cells in the medial two thirds of the lymph gland form part of the medullary zone, as indicated to the left of panel **a**. These cells are more densely packed and exhibit the ultrastructural features of prohemocytes (*ph*). In the periphery of the larval lymph gland (cortical zone), cells are spaced further apart and are mostly differentiated plasmacytes (*pl*). In the early pupa (**b**), the lymph gland has significantly decreased in diameter, and a clear distinction between medullary zone and cortical zone is no longer visible. Most

cells represent differentiated plasmacytes. **c, d** Basement membranes deposited in the cleft between two adjacent prohemocytes (*arrowhead* in **c**) and plasmacytes (*arrowhead* in **d**; *arrow* points at extension of plasmacyte membrane). **e–g** Plasmacytes in 4 h pupal lymph gland. Many plasmacytes have already moved out of the lymph gland, leaving behind the basement membranes that originally surrounded them (*arrowhead*; *arrow* points at extensions of plasmacyte membrane). Plasmacyte shown in **g** appears to be in the process of leaving. The boxed area in **g** is shown in higher magnification in **g'**. In **g'**, the area where the hemocyte is breaking through the ECM is pointed out by a *double arrowhead*. Bars 5  $\mu$ m (**a, b**); 1  $\mu$ m (**e, f, g**); 0.1  $\mu$ m (**c, d**)

PSC cells, along with other hemocytes, disperse into the hemolymph. Interestingly, in 8, 10, and 12 h pupae, Antp-positive cells appeared more widely distributed over the small remainder of the lymph gland (Fig. 3b–d, respec-

tively), rather than forming a tight cluster as in the larva. This could indicate that hemocytes in the hours immediately prior to dispersal mix more freely than at the larval stage.

**Fig. 3** Fate of the posterior signaling center (PSC) in the dissociating pupal lymph gland. Expression of PSC marker *Antennapedia (Antp)*. Panels show confocal sections of late larval (a), 8 h pupal (b), 10 h pupal (c), and 12 h pupal (d) lymph gland lobes labeled with anti-*Antp* (red) and Sytox (nuclear marker, blue). Lymph gland lobes are demarcated with a dashed outline in panels c and d. The primary lobe outlined in panel c abuts the contralateral primary lobe. In panel d, a group of cells that originate from the primary lobe but appear disconnected from the remaining portion of the lobe are pointed out with an arrowhead. In pupa, the *Antp*-positive cells appear to migrate away from the PSC and spread out throughout the lymph gland (b–d). Other abbreviations: *prl* primary lobe; *sl* secondary lobe; *dv* dorsal vessel. Bar 30  $\mu\text{m}$  (a); 20  $\mu\text{m}$  (b–d)



The secondary lobes undergo a growth spurt prior to lymph gland dissociation

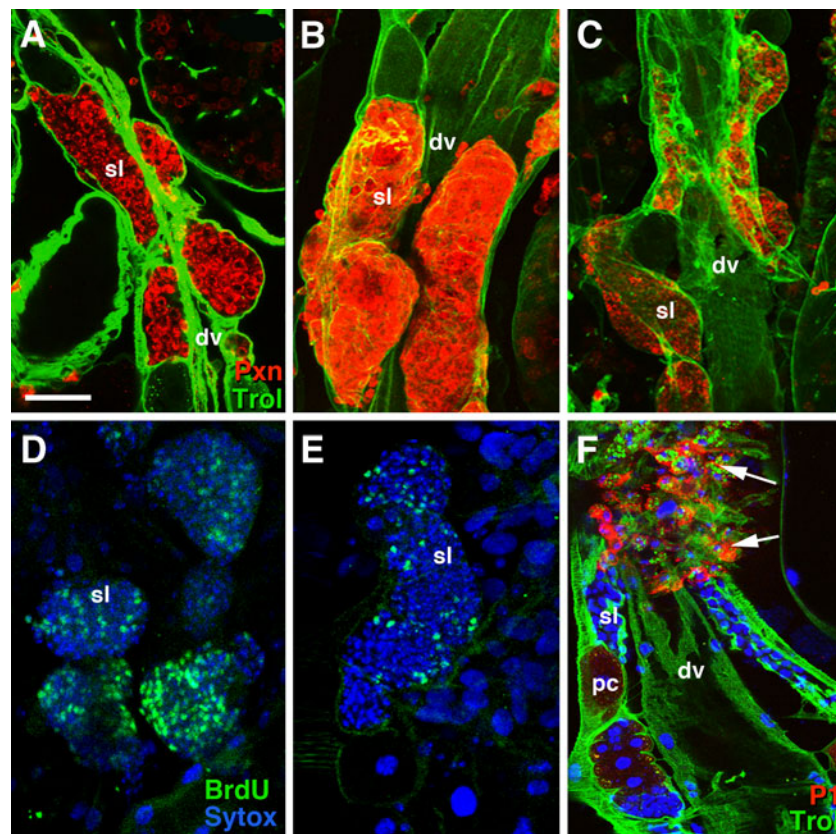
The secondary and higher order lobes of the larva do not generally express any of the differentiation markers found in the cortical zone of the primary lobes, as most of the cells are undifferentiated (Jung et al. 2005). This prompted the question whether hemocytes of the secondary lobes (which dissociate at the same time as the primary lobes) disperse as undifferentiated blood progenitors or go through an accelerated phase of maturation prior to dispersal. Using the markers Pxn and P1, we observed that secondary lobe hemocytes do initiate differentiation during a short time interval: from 4 h APF onward, most, if not all, cells of the secondary lobes are Pxn-positive (Fig. 4a–c). However, expression of the differentiation marker P1 is never initiated (Fig. 4f), indicating that dispersal of the secondary lobe cells takes place before they have terminally differentiated. It should be noted that, as in the larva, expression of the crystal cell marker Propo is never observed in the secondary lobes (data not shown).

We noted that the secondary lobes transiently increase in size between the 4 and 8 h pupal time points, before diminishing by 10 h (Fig. 4a–c). To assess whether cell

division was in fact taking place, 4 and 8 h pupal lymph glands were incubated with BrdU. For both time points, high numbers of BrdU-positive cells were seen in the secondary lobes (Fig. 4d–e, respectively); by contrast, no BrdU incorporation takes place in the primary lobes during this time interval (data not shown).

The reticular ECM during hemocyte dispersal

During the larval period, blood progenitors surround themselves with a thin lamella of extracellular matrix. It contains collagen and laminin (Jung et al. 2005; Krzemien et al. 2010), as well as perlecan (in *Drosophila*: Trol; Fig. 1a). Electron microscopically, the ECM lamella is approximately 30–50 nm thick and has a granulated appearance (Fig. 2c). Characteristically, ECM lamellae do not adhere tightly to the cell surface, but leave an open space of variable diameter (Fig. 2d). Trol-driven GFP visualizes impressively the ECM of the larval lymph gland. In the medullary zone, each cell or small groups of cells are surrounded by ECM lamellae; in the cortical zone, many lamellae have disappeared, and larger clusters of (differentiated) hemocytes occupy a common “chamber” which is bound by ECM (Fig. 1a–c).



**Fig. 4** Pupal development of the secondary lymph gland lobes. **a–c** Confocal sections of secondary lobes (*sl*) of 4 h (**a**), 8 h (**b**), and 10 h pupa (**c**), labeled with ZCL1973X (Trol; *green*) and anti-Peroxidasin (Pxn, *red*). **d, e** Labeling of proliferating cells in secondary lobes of 4 h (**d**) and 8 h (**e**) lymph gland with anti-BrdU (*green*), following 45 min incubation of dissected lymph glands in BrdU-containing

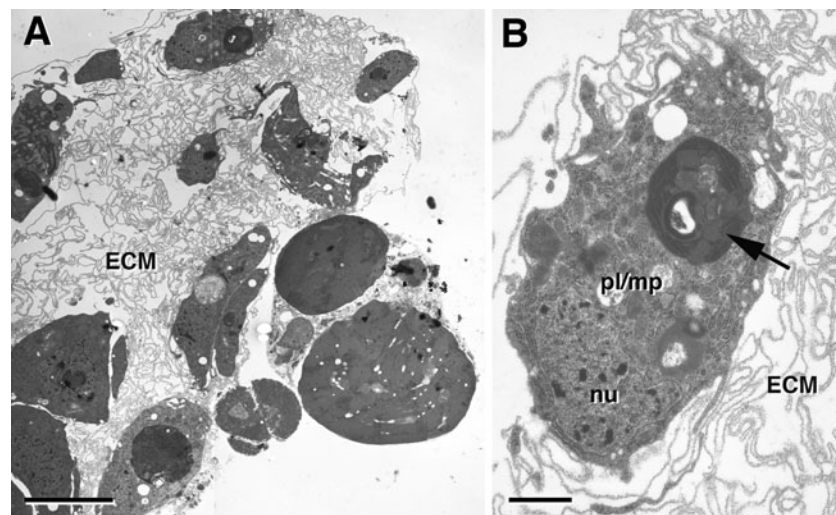
solution. **f** Labeling of 10 h secondary lobe with anti-P1 (*red*). P1-positive cells (*arrows*) are dispersed hemocytes originating from the primary lobes; cells within the secondary lobes (*sl*) are P1-negative. Other abbreviations: *dv* dorsal vessel; *pc* pericardial cell. Bar 20  $\mu$ m (**a–d**)

As the lymph gland dissociates in the pupa and hemocytes disperse in the hemolymph, they leave behind the ECM lamellae. As shown in Fig. 5a, b, the lymph gland of a 10-h pupa consists of empty shells of folded ECM lamellae, almost devoid of hemocytes. What this implies is that hemocytes, rather than digesting the ECM, push through gaps or only digest small, localized areas in the ECM lamellae as they move out of the lymph gland. A figure suggesting a hemocyte in the process of “escaping” is shown in Fig. 2g–g’. We were not able to determine for how much longer, after 12 h APF, the lymph gland-associated ECM remains. The finding that ECM stays behind after the cells that produced it have left is unexpected, since in other tissues that have been studied, cell migration or dispersal is preceded by the disappearance (digestion) of the ECM. For example, in case of the wing, a thick, multilayered membrane underlies the epithelium in the 6-h pupa (Srivastava et al. 2007). During eversion of the wing, which is accompanied by large scale cell movement, this ECM membrane all but disappears. The lymph gland associated ECM may have special properties that render it

particularly stable, so that it is able to persist in the absence of cells. Another difference between lymph gland and wing metamorphosis, that may be relevant, is that hemocytes merely have to break through the surrounding ECM lamella, after which they become freely motile cells, whereas the wing epithelium moves as an intact layer, and remnants of ECM (if they existed) would impede this movement.

## Discussion

The time course of production of hemocytes and their release into circulation shows considerable differences between insect groups. In most cases studied (e.g., Akai and Sato 1971; Nakahara et al. 2003), bursts of hemocyte proliferation and release are coupled to the molting cycle of the larva. On top of this cyclic pattern, the ratio of differentiated versus undifferentiated cells generally increases with larval age. With the onset of metamorphosis, compact hematopoietic organs, which have been described



**Fig. 5** Ultrastructure of the 10 h pupal lymph gland. **a** Low power electron micrograph showing remnant of primary lymph gland lobe in the 10 h pupa. Few cells, typically phagocytotically active plasmatocytes, are left. Aside from these cells, the lymph gland forms a “husk” of folded basement membranes (ECM) that at earlier stages contained

hemocytes. **b** Phagocytotically active plasmatocyte/macrophage (*pl/mp*) found within a 10 h pupal lymph gland, containing lysosomes with electron dense cellular debris (*arrow*). Other Abbreviations: *nu* nucleus Bars 5  $\mu\text{m}$  (**a**); 1  $\mu\text{m}$  (**b**)

for many insects (Han et al. 1998; Crossley 1964; Monpeysson and Beaulaton 1978; Hoffmann et al. 1979), typically disappear after a final burst of hemocyte release. In other words, adults of most insect species, lack compact hematopoietic organs. The latter is true also for *Drosophila*, but in terms of earlier production and release of hemocytes during the larval stage, one notes that hemocyte proliferation does not appear to be coupled as strictly to the molting cycle and release does not occur at all prior to metamorphosis in *Drosophila*. Only in cases of infection or wounding, is the production of hemocytes stimulated, and their release into circulation seen. The same applies to a number of mutations [e.g., *Hopscotch* (*Hop*), encoding a *Drosophila* Janus Kinase]] in which hemocyte proliferation and/or differentiation is increased (Harrison et al. 1995; Minakhina and Steward 2006). The absence of hemocyte release during larval stages has been experimentally confirmed in the past by the analysis of labeled clones of hemocyte progenitors that were induced in embryos (Holz et al. 2003). These clones came to occupy the lymph glands of larvae but did not spread into circulation until immediately before pupation.

In the current study, we investigated the differentiative processes occurring in the *Drosophila* lymph gland prior to its dissociation. We found that one of the differentiation markers, Pxn, appears to be expressed in all hemocytes before they dissociate. Thus, both the medullary zone of the primary lobes, as well as the secondary lobes of the lymph gland, both of which are domains which only contain undifferentiated prohemocytes in the larva, show labeling with anti-Pxn in the early pupa. Pxn, an ECM protein, is

synthesized by hemocytes among other cell types (Nelson et al. 1994) and is considered an early differentiation marker (Jung et al. 2005). A later differentiation marker, P1, appears only in a fraction of cells of the cortical zone in the larvae. In the 4 h pupa, P1 is still not expressed in the primary lobe medullary zone; only around 10 h APF, when a majority of the lymph gland has already been lost, do we see a majority of the (remaining) cells start to display expression of P1. Cells of the secondary lobes, on the other hand, remained negative for P1 expression till the very end, demonstrating that these cells dissociated as incompletely differentiated hemocytes.

As described previously by Lanot et al. (2001), crystal cells were quickly lost after the onset of metamorphosis. We found the persistence of small numbers of crystal cells until 2 h following the onset of pupation; by 6 h, crystal cells appeared to be completely absent from the lymph gland. Furthermore, as shown previously (Lanot et al. 2001), lamellocytes, the third type of *Drosophila* hemocytes, are never found in the pupal lymph gland. Lamellocytes are only rarely seen under normal conditions in either larva or adult (Lanot et al. 2001); they are induced by infection or wounding (Markus et al. 2005; Rizki and Rizki 1992).

Past studies had demonstrated the presence of mitotic cells (1–2%) in all lymph gland lobes of mid-third and wandering larva (Lanot et al. 2001). For the pupa, we observed a high level of cell division in the secondary lobes at 4 h APF and slightly less at 8 h APF; the primary lobes were virtually devoid of any proliferation until dissociation of the lymph gland occurred around 12 h APF. This finding



supports the view that secondary lobes (which also appear later in development) are always at an earlier state of differentiation than primary lobes: cells remain mitotically active to the very end of lymph gland life, and concomitantly do not complete differentiation, as evidenced by the lack of P1 expression (see above).

How does the release of blood cells from the insect lymph gland compare to the corresponding process in vertebrates? Can one detect similarities that extend to the mechanisms controlling blood cell release across phylum boundaries? The blood forming tissue in most vertebrates is the bone marrow. The bone marrow is formed by blood vessels, capillaries and wide, endothelium bound venous sinuses. The spaces in between this vascular tissue are filled with stromal cells, blood stem and progenitor cells, and blood cells at successive stages of maturation (Lichtman 1981; Petrides and Dittmann 1990). Fully differentiated, postmitotic blood cells form a pool (“postmitotic pool”) of cells that is released in a controlled manner into the sinuses; here, blood cells can stay for extended period of time before moving out into circulation.

The process of releasing blood cells from the stroma into the sinuses (marrow egress) involves dynamic changes in both the blood cells themselves, as well as the cellular elements that form the physical boundary between bone marrow stroma and the blood. This boundary is formed by three layers: the endothelium lining the sinuses, specialized stromal cells called “adventitial cells” (Weiss 1980; Lichtman 1981; Petrides and Dittmann 1990; Inoue and Osmond 2001), and a basement membrane secreted by both cell types and deposited in the space between them. Blood cells and their progenitors themselves are not individually surrounded by a basement membrane, which constitutes a marked difference to the condition found in the insect hematopoietic tissues. When crossing from the stroma into the lumen of the sinuses, blood cells move in between clefts of adventitial cells and create pores in the basement membrane and the endothelial cells. This multistep process is orchestrated by multiple signaling interactions that are incompletely understood. A variety of signals derived from both the bone marrow stroma, as well as from peripheral blood cells and endothelial cells act on receptors on the to-be-released-blood cells, and cause changes in their adhesiveness, motility, and the secretion of extracellular proteins required to pave the way from stroma into the lumen of sinuses (Lapidot and Petit 2002; Velders and Fibbe 2005; Christopher and Link 2007). A well-known signal that inhibits blood cell egress from the marrow is stromal derived factor 1 (SDF1); opposing signals, such as granulocyte colony-stimulating factor (G-CSF) act by degrading SDF1 and thereby allowing blood cells to pass into circulation. G-CSF and other signals also increase the release of metalloproteases from egressing blood cells,

which may be important to locally remove the basement membranes that these blood cells have to traverse.

Localized digestion of basement membranes, possibly combined with an increased motility, appear to be the main processes that play a role in release of blood cells from the lymph gland of *Drosophila*. The underlying molecular mechanisms and its control by signaling pathways is currently not known. Extracellular matrix proteins assembled as basement membranes play a fundamental role in morphogenesis and organogenesis (Ekblom et al. 1985; Doihara et al. 2009; Mitsunaga-Nakatsubo et al. 2009; Tsang et al. 2010). Hemocytes are known to produce and secrete proteins used for stabilizing basement membranes around other tissues. For example, the highly conserved ECM protein SPARC is secreted by hemocytes and acts to stabilize collagen IV in the basement membranes surrounding muscles and other tissues (Martinek et al. 2008; Koehler et al. 2009). However, no hemocyte-derived protein has been described yet that would degrade extracellular matrix, including the basement membranes that captivate hemocytes within the lymph gland. It is possible that an increase in motility could be sufficient for hemocytes to tear and break through the encapsulating basement membranes. Thus, during normal development, a signal triggered by the ecdysone peak that promotes metamorphosis could induce both the proliferative/differentiative events that happen rapidly in the few hours between pupation and lymph gland dissociation, as well as an increased motility of hemocytes. A similar signal could act as part of the immune response that is able to trigger premature lymph gland dissociation in larvae infected with various parasites (Lanot et al. 2001; Sorrentino et al. 2002; Chiu and Govind 2002).

A signaling pathway known to be involved in hemocyte motility in the embryo is the PVR (PDGF-VEGF-Related) pathway. Hemocytes derived from the head mesoderm spread out along stereotyped pathways (Tepass et al. 1994) throughout the embryo. One should note that this phase of hemocyte dispersal, which is formally comparable to the dissociation of the lymph gland considered here, takes place in the absence of basement membranes around hemocytes. Thus, the population of head mesoderm cells that adopts the fate of prohemocytes in the stage 11 embryo does so before basement membranes have formed (Tepass and Hartenstein 1994). Prohemocytes initially form a loose mass of cells in the head of the embryo, lacking a capsule similar to the one that surrounds the prohemocytes of the larval lymph gland. Subsequently, in response to PVR signals, presented by various sources, prohemocytes migrate away from the head mesoderm and, at the same time, differentiate into plasmatocytes with phagocytotic activity (Cho et al. 2002; Bruckner et al. 2004). Lack of PVR, aside from causing an increase in cell death, also has

an effect on hemocyte spreading. PVR signaling is also required for directed migration of larval hemocytes towards wounded tissue (Wood et al. 2006). A role of this pathway, or any other mechanism controlling motility, in hemocyte dispersal during early metamorphosis remains to be seen.

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