Hematopoietic progenitors and hemocyte lineages in the *Drosophila* lymph gland

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A B S T R A C T

The *Drosophila* lymph gland (LG) is a model system for studying hematopoiesis and blood cell homeostasis. Here, we investigated the patterns of division and differentiation of pro-hemocytes in normal developmental conditions and response to wasp parasitism, by combining lineage analyses and molecular markers for each of the three hemocyte types. Our results show that the embryonic LG contains primordial hematopoietic cells which actively divide to give rise to a pool of pro-hemocytes. We found no evidence for the existence of *bona fide* stem cells and rather suggest that *Drosophila* pro-hemocytes are regulated as a group of cells, rather than individual stem cells. The fate-restriction of plasmatocyte and crystal cell progenitors occurs between the end of embryogenesis and the end of the first larval instar, while Notch activity is required for the differentiation of crystal cells in third instar larvae only. Upon parasitism, lamellocyte differentiation prevents crystal cell differentiation and lowers plasmatocyte production. We also found that a new population of intermediate progenitors appears at the onset of hemocyte differentiation and accounts for the increasing number of differentiated hemocytes in the third larval instar. These findings provide a new framework to identify parameters of developmental plasticity of the *Drosophila* lymph gland and hemocyte homeostasis in physiological conditions and in response to immunological cues.

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Introduction

Drosophila hematopoiesis occurs in two spatially and temporally distinct phases (Crozatier and Meister, 2007). The first, embryonic phase provides circulating hemocytes in the larval hemolymph (Holz et al., 2003). The second phase takes place during larval development in a specialised hematopoietic organ, termed the lymph gland (LG). Hematopoiesis in the LG has become the subject of intensive investigations as a model for the control of hemocyte homeostasis and cellular immune responses and studying conserved mechanisms between insects and vertebrates (Evans et al., 2003; Jung et al., 2005; Krzemien et al., 2010). The LG contains hematopoietic progenitors (pro-hemocytes) which can give rise to three types of hemocytes. Two types, the plasmatocytes which are monocyte-like cells involved in phagocytosis and crystal cells which are required for melanisation, are released in the hemolymph upon dispersal of the LG at the onset of metamorphosis. A third type, lamellocytes which are devoted to the encapsulation of foreign bodies too large to be phagocytosed, differentiate in response to specific immune challenges such as parasitization by wasps, a common threat for many insects (Crozatier and Meister, 2007; Lanot et al., 2001; Rizki and Rizki, 1984).

Lamellocyte differentiation which occurs at the beginning of the third instar larval stage, at the expense of the pool of progenitors, represents an interesting example of specific cellular immune response in invertebrates.

In third instar (L3) larvae, the mature lymph gland is composed of a pair of anterior, primary lobes which form during embryogenesis and a variable number of more posterior, secondary lobes which form during larval development. While secondary lobes likely represent reservoirs of immature pro-hemocytes, the primary lobes comprise a medullary zone (MZ), containing the hematopoietic progenitors, a cortical zone (CZ) containing differentiated hemocytes and the so-called Posterior Signalling Center (PSC). Cell tracing experiments based on GFP expression in the MZ under control of domeless-gal4 showed that the differentiated hemocytes in the primary lobes originate from MZ cells (Jung et al., 2005).

The PSC, initially identified as a small cluster of posterior LG cells expressing the Notch (N) ligand Serrate (Ser) (Lebestky et al., 2003) was recently shown to play a key function in controlling the balance between multipotent pro-hemocytes and differentiating hemocytes in the larval LG (Krzemien et al., 2007; Mandal et al., 2007). In L3 larvae, PSC cells act, in a non-cell autonomous manner, to maintain JAK/STAT signalling activity in pro-hemocytes, thereby preserving the multipotent character necessary for these cells to adopt a lamellocyte fate in response to parasitism (Krzemien et al., 2007; Mandal et al., 2007). PSC cells are specified in the embryo by expression of Antennapedia (Antp) and Collier (Col) (Crozatier et al., 2004; Mandal et al., 2007). The morphogen Hedgehog (Hh) starts to be expressed in

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PSC cells in second instar (L2) larvae and is required for hemocyte homeostasis in L3 larvae (Mandal et al., 2007). Wg and its receptor are also expressed in the PSC where their activity controls the number of PSC cells (Sinenko et al., 2009). How Hh and Wg activity provided by PSC could be connected to JAK-STAT signalling in pro-hemocytes remains, however, unknown.

The key role of the PSC in the maintenance of hematopoietic progenitors is reminiscent of the vertebrate hematopoietic stem cell (HSC) niche, a term coined more than 30 years ago to describe the structural and regulatory micro-environment sustaining long-term renewal of HSC in the bone marrow (Kiel and Morrison, 2008; Schofield, 1978). Cell "stemness" refers to the potential to self renew and at the same time produce daughter cells that can commit to lineage-specific differentiation (Kohlmaier and Edgar, 2008). Mouse HSCs isolated from the bone marrow were operationally defined as able to reconstitute long-term, multilineage hematopoiesis after transplantation in a recipient individual (Dykstra et al., 2007; Spangrude et al., 1988). Unfortunately, a similar reconstitution assay is not available in *Drosophila*.

Here we addressed the question of whether there exist *Drosophila* hematopoietic stem cells in association with the niche (PSC) in the lymph gland. Our data support the conclusion that the small population of primordial hematopoietic cells specified in the embryonic LG actively divide during larval development and give rise to a large pool of hematopoietic progenitors. Hemocyte homeostasis appears to be controlled at the level of this pool rather than the level of individual stem cells. Plasmatocyte versus crystal cells fate restriction is imposed on these progenitors in embryos and first instar larvae. Hemocyte differentiation normally starts in early L3 and we found that Notch signalling is only required during this late phase for crystal cell differentiation. Analyses of the patterns of mitoses, relative to hemocyte differentiation in third instar larvae indicate the existence of a population of mitotic cells which have left the medullary zone. We propose that these cells are intermediate progenitors which undergo differentiating divisions and account for the increasing number of differentiated cells found in the lymph gland in third instar larvae.

Materials and methods

Fly stocks

The following strains were used: *pcol85Gal4>UASmcd8GFP* (*col>GFP*) (Krzemien et al., 2007); *PG125-Gal4* (*dome-Gal4*) (Bourbon et al., 2002), *dome-MESO* (Hombria et al., 2005), N^{ts2} (Shellenbarger and Mohler, 1975), *esg-Gal4* (Micchelli and Perrimon, 2006), *Esg-GFP* (a gift from N. Bausek), *Viking-GFP* (*Vkg-GFP*) (Morin et al., 2001), *UAS-PonGFP* (a gift from F. Schweisguth) (Lu et al., 1998)), *Neur-lacZ* (Phillips et al., 1993), *Lz-LacZ* (Bataille et al., 2005) and *lz-Gal4* (Lebestky et al., 2000). *Oregon R* was used as wt and w^{1118} as a control for Nts experiments.

Immunohistochemistry

Dissections of lymph glands and immunostaining procedures were as described in (Krzemien et al., 2007). Primary antibodies were: rabbit anti-proPO (1/200) (gift from H.M. Müeller), anti-αPS4 1/200 (Krzemien et al., 2007), anti-Wicked (gift from JR Huynh) (Fichelson et al., 2009), anti Brat (gift from J. Knoblich) (Betschinger et al., 2006), anti- Miranda (Matsuzaki et al., 1998), anti-H3P (1:200, Upstate Bioscience), anti-lacZ (Capell, 1/5000). Mouse anti-Col 1/50 (Dubois et al., 2007), anti-Fibrillarin 72B9 (Reimer et al., 1987), anti-BrdU (1:50), anti-P1 (1/30) (gift from I. Ando), antiProPO, 1/100 (gift from T. Trenczek), anti-Prospero (Hybridoma Bank), anti-Talin (1/50) (gift from N. Brown) (Brown et al., 2002). Phalloidin was purchased from Interchim, TO-PRO-3 from Molecular Probes and BrdU (5 Bromo-2'-deoxyuridine) from Sigma-Aldrich. Samples were mounted in Vectashield medium (Vector Laboratories) and analysed by confocal microscopy (Leica SP2 or SP5, Wetzlar, Germany).

BrdU pulse-chase experiments

BrdU pulse chase experiments were made as described in (Lee et al., 2006), with the following modifications: *col>GFP* and *dome>GFP* second or third instar larvae were fed for either 1 h, 4 h or 12 h with BrdU (1 mg/ml; Sigma-Aldrich) diluted in fly food. Half of the larvae was immediately processed for BrdU staining (pulse experiments) and the second half was left growing for 24 h or 36 h after removal of BrdU before fixation (chase experiments). Dissected lymph glands were fixed and treated in 2 N HCl for 30 min prior to anti-BrdU staining.

Mitotic index

dome>GFP embryos were collected for periods of 2 h and let to develop for 54 h, 72 h, 96 h and 120 h at 25 °C before LG dissection and H3P antibody staining. The LG were then laser scanned using a Leica SP5 microscope and Z series of images collected. The mitotic index of a LG was measured as the total number of mitotic figures divided by the total number of cells. Cell numbers were estimated as follows: the surface of the lymph gland was divided by the surface for one cell and multiplied by the number of cell layers on the Z axis. The LG surface was measured using the ImageJ software and the number of cell layers by "z" optical sectioning with Imaris software. For calculating the mitotic index after wasp parasitism, second instar larvae were let for 2 h in presence of wasps. The lymph glands were dissected 6 h later, fixed and stained. Results obtained with dome>GFP and Oregon R strains were pooled together since no strain specific differences were observed. The statistical analysis was by Student *t*-test, at $p<0.05$.

Notch[ts] experiment

In order to determine the temporal window of N requirement for crystal cell formation, N^{ts2} larvae were shifted from 22 °C to 29 °C for periods of 24 h or 48 h, starting at different developmental time points. Lymph glands were dissected and stained for crystal cells at late L3 stage.

Clonal analysis

For clonal analyses, we used the *Flp out* inducible system (Golic and Lindquist, 1989). 2 h embryo collections from the *yw,hsFlp; Sp/Cyo yw* x *act-FRT y$^+$ FRT Gal4 UAS GFP* cross were let to develop at 18 °C before a 20-min heat shock at 37 °C at different periods during embryonic/larval development (20 different time points between 17 and 168 h after egg laying (AEL). After heat shock, the larvae were shifted back to 18 °C for 6 h and then shifted again to 25 °C until late L3. The shift to 25 °C was necessary to obtain consistent numbers of crystal cells which do differentiate in lower numbers at low temperature. Lymph glands dissected from late L3 larvae were stained with antibodies against plasmatocyte and crystal cell specific markers and analysed by confocal microscopy. "z" series were collected and analysed in 3D (Imaris software) to determine which type of differentiated cells were present in each clone.

Results and discussion

At the end of embryogenesis, the *Drosophila* LG is composed of two cell types, primordial hematopoietic cells and PSC cells; review by (Krzemien et al., 2010). The lymph gland grows until metamorphosis, from ~30 cells per lobe in the embryo to ~5000 per anterior lobe (Mandal et al., 2007; Sinenko et al., 2009; Sorrentino et al., 2002). Differentiation of larval hemocytes is first observed in the anterior, primary lobes of the LG in early L3 larvae (Lanot et al., 2001; Shrestha and Gateff, 1982). From this stage, a medial region where cells show minimal intercellular space can be distinguished both by Nomarski optics and the accumulation of high level E-cadherin, a surface protein involved in cell-adhesive properties (Jung et al., 2005). This medial region, termed the medullary zone (MZ) contains pro-hemocytes characterised by the expression of Domeless, the *Drosophila* JAK-STAT signalling receptor and dome-MESO, a reporter of JAK-STAT signalling ((Hombria et al., 2005; Krzemien et al., 2007), Fig. 1A, D). Staining for collagen (Viking (Vkg)-GFP) expression revealed the surrounding of clusters of MZ cells by extracellular basement material ((Jung et al., 2005; Lanot et al., 2001); Fig. 1B). It also revealed that PSC cells form a morphologically discrete structure, as a group of cells surrounded by ECM (Fig. 1C). The discovery that the PSC functions as a niche in third instar larvae (Krzemien et al., 2007; Mandal et al., 2007) evoked the possibility that it maintains physical interactions with hematopoietic stem cells, similar to interactions between vertebrate HSC and the endosteal-perivascular niche in the bone marrow or between *Drosophila* GSC and their niches in the testis and ovary. These physical interactions involve accumulation of a transmembrane protein, N-cadherin in vertebrate HSC (Calvi et al., 2003; Lo Celso et al., 2009; Xie et al., 2009) and DE-cadherin in the *Drosophila* gonads (Song et al., 2002). In the *Drosophila* LG, however, all MZ cells express high levels of DE-Cadherin rather than a specific subset (Jung et al., 2005). An alternative possibility could be that the PSC communicates with a
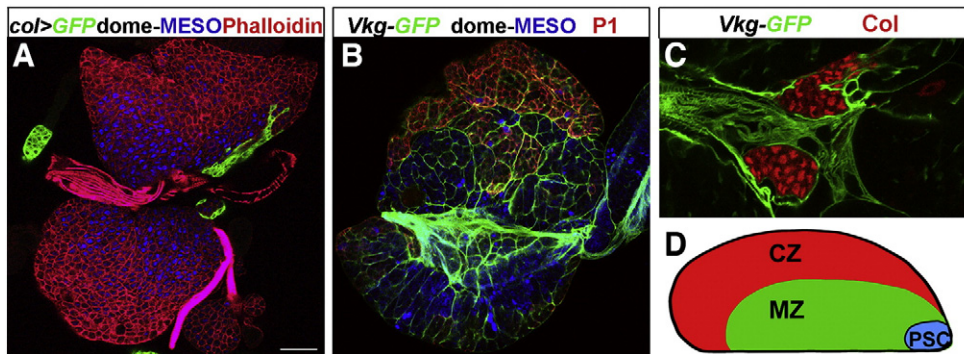
Fig. 1. Differential gene expression and ECM distribution in the third-instar larval lymph gland. (A–C) Single confocal sections of primary lobes of third instar larval lymph glands; anterior is on the left. (A) The medullary zone (MZ) and Posterior Signalling Center (PSC) are marked by the expression of nuclear LacZ (dome-MESO, blue) and GFP (col>mCD8GFP, green), respectively. Phalloidin staining (red) of the F-actin network highlights individual cells in the cortical zone (B) The extracellular matrix marked by Vkg-GFP expression (green) highlights groups of cells forming pocket-like structures in the MZ (dome-MESO, blue) and clusters of differentiated plasmocytes (P1 immunostaining, red) in the CZ. (C) The PSC cells (Col immunostaining, red) are clustered and surrounded by a dense meshwork of ECM (Vkg-GFP, green). Scale bar 20 μm. (D) Schematic representation of an anterior lobe of the LG in third instar larvae. MZ: medullary zone, CZ: cortical zone and PSC: Posterior Signalling Centre.

large population of progenitor cells, either via the long filopodial extensions which characterise PSC cells in third instar larvae or the synthesis of morphogens such as Hh or Wg, or both (Krzemien et al., 2007; Mandal et al., 2007; Sinenko et al., 2009). In order to determine whether there exist HSC in the *Drosophila* LG, we used several criteria, such as slow self-renewal allowing for long half-life and markers of asymmetric division.

Long term incorporation of 5-bromo-2 deoxyuridine (BrdU) has been used to identify quiescent stem cells (Arai et al., 2004; Cotsarelis et al., 1990; Wilson et al., 2008). In BrdU pulse-chase assays, slowly cycling cells retain BrdU while it is diluted in cells that divide often. We used this approach to determine whether a specific population of slower cycling cells exists in the lymph gland in L2 and L3 larvae, focusing on cells in contact with the niche. Either long (12 h) or short (between 1 and 4 h) BrdU incorporations were performed (see M and M). Long incorporation periods were intended to increase the probability of labelling rarely dividing stem cells. Both early L2 and mid-L2 larvae were fed for 12 h on BrdU-containing media, such that together, the incorporation period covered the entire span of the second instar. We found that, while lymph glands dissected directly after the pulse were strongly BrdU positive, no BrdU-retaining cells could be detected after a 36 h chase (not shown). The same result was observed when 4 h BrdU incorporation was done in early L3 larvae, followed by a 24-h chase period (not shown). In contrast, 1 h or 4 h BrdU incorporation in midL3 lymph gland followed by 24 h chase showed many cells retaining high level of BrdU (Fig. S1). Interestingly, whereas we could observe many BrdU positive cells in the MZ immediately following BrdU incorporation, cells which retained BrdU after a 24 h chase were mostly found in the CZ. It likely corresponds to the fraction of cells in S-phase undergoing a terminal division before differentiating (Fig. S1). BrdU labelling experiments did not provide evidence, however, for the existence of slowly cycling cell in contact with the PSC.

Stem or stem-like cell renewal via "asymmetric division" has been described in several *Drosophila* tissues, including the gonads, CNS and intestinal stem cells (ISCs). We therefore investigated whether markers of stem cells or asymmetric division in these tissues were expressed in the LG. These included the Prospero and Escargot transcription factors (Hirata et al., 1995; Kiger et al., 2000; Knoblich et al., 1995; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006), Wicked (a component of sno-RNP) (Fichelson et al., 2009) and markers of Notch-mediated asymmetric division such as Partner of Numb (Pon)-GFP (Lu et al., 1998), Neuralized (Neur)-LacZ (Phillips et al., 1993), Miranda (Ikeshima-Kataoka et al., 1997; Shen et al., 1997) and Brat (Betschinger et al., 2006; Lee et al., 2006). Unlike in control tissues, we could detect neither Pros nor Esc-Gal4 or Esc-GFP

expression in the MZ. Likewise expression of Pon-GFP and Neu-lacZ did not reveal evidence for N-controlled asymmetric divisions in the lymph gland (data not shown). Finally, some *Drosophila* stem cells such as ISCs and RNSCs (renal and nephric stem cells) are characterised by a small nuclear size, compared to their progeny (Micchelli and Perrimon, 2006; Singh et al., 2007). Conversely, neuroblasts which are considered as stem-like cells can be distinguished by their large size and large nucleolus, correlating with sustained ribosome biogenesis and growth (Betschinger et al., 2006). In either case, stem cells can be distinguished from their progeny by their different nucleolar size. Immuno-staining of LG dissected at different times throughout the third instar for the nucleolus marker Fibrillarin (Reimer et al., 1987) failed to reveal a sub-population of pro-hemocytes which could be distinguished by a different nucleolus size. In summary, we found no evidence that a subset of LG cells in direct contact with the PSC cells in the lymph gland of L2 or L3 larvae could display characteristics of stem cells, on criteria of slow cycling or expression of specific markers for other types of *Drosophila* stem cells or asymmetric divisions.

Mitosis and differentiation in the maturing lymph gland: evidence for a population of intermediate progenitors

It was previously shown that differentiating hemocytes in the CZ are derived from pro-hemocytes of the MZ (Jung et al., 2005). Based on BrdU incorporation patterns, the same study suggested that cells in the MZ become quiescent in L3 larvae, whereas proliferation of differentiated cells would be responsible for the expansion of the CZ. This conclusion was somewhat puzzling in view of previous observations that dividing cells in circulation mostly correspond to undifferentiated cells (Lanot et al., 2001) and that cells in the posterior lobes divide until pupariation without sign of differentiation (Jung et al., 2005), see below). We therefore re-examined the patterns of cell divisions in the LG of L2 and L3 larvae, using immunostaining for the mitotic cell marker H3P (phosphorylated Histone H3). By counterstaining of all nuclei with TO-PRO-3 we found that the mitotic index (calculated as the number of H3P-positive cells/total number of cells) is highest in L2 and early L3 larvae when the MZ develops and contributes most of the LG tissue (see also (Sorrentino et al., 2002). This index drops significantly between mid and late L3 when massive hemocyte differentiation takes place and the CZ expands (Fig. 2A). Co-staining for H3P and the MZ marker Dome>GFP showed that a fraction of cell divisions still occur in the MZ as defined by Dome>GFP expression (Fig. 2B, C, F), showing that some progenitors in the MZ remain mitotically active throughout the third larval stage (Fig. 2B). A significant fraction of mitotic events is, however, observed outside the
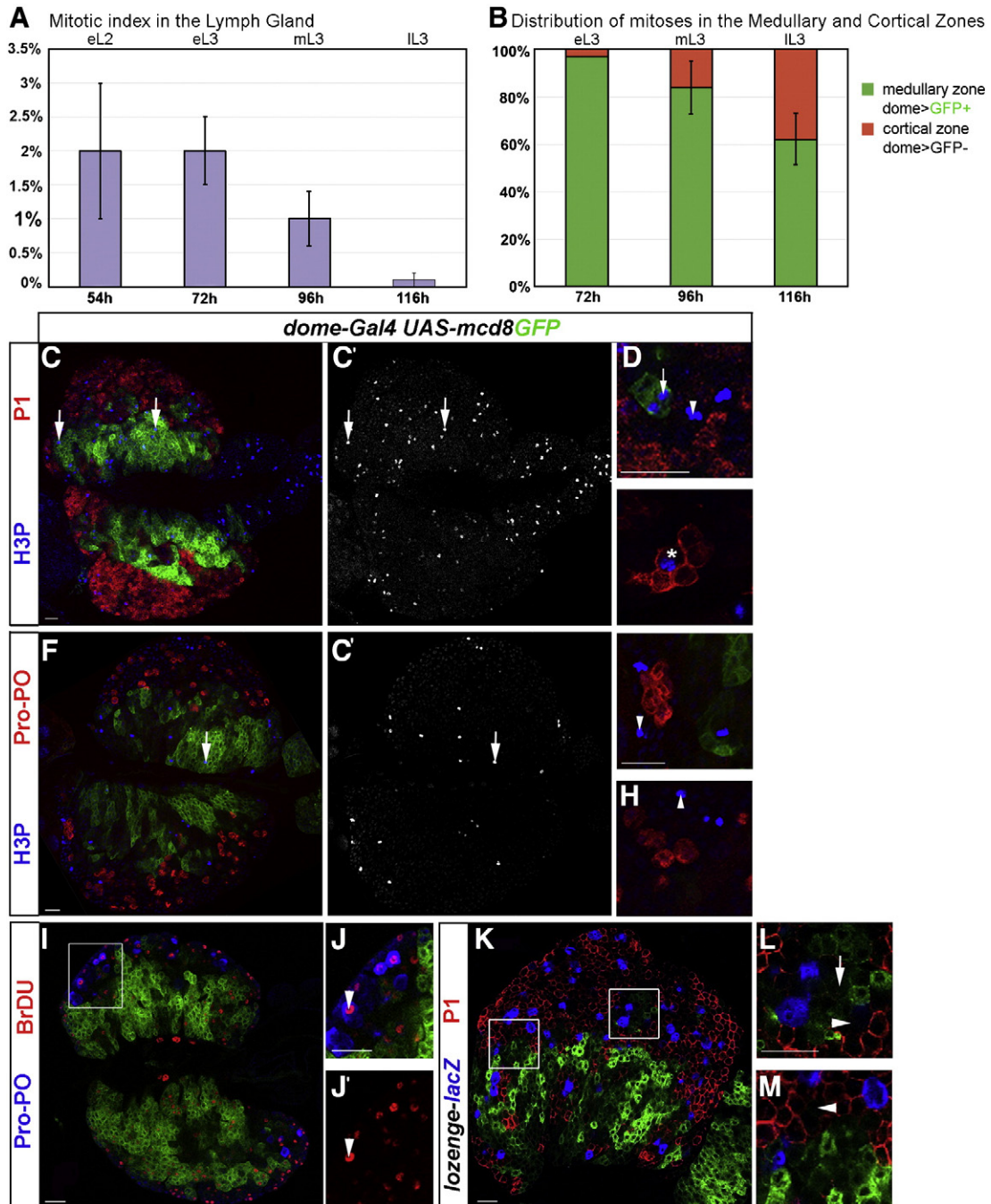
Fig. 2. Identification of intermediate progenitors in the third instar lymph gland. (A) Graphic representation of the mitotic index (number of mitotic figures/total number of cells) in the lymph gland at 54, 72, 96 and 116 h of development at 25 °C, corresponding to the L2, early (e), mid (m) and late (l) L3 periods, respectively. Numbers of LG counted were: 8 (54 h), 15 (72 h), 21 (96 h) and 10 (116 h). (B) Distribution of mitotic cells, visualised by H3P staining, in third instar larval lymph glands. Starting in mid-L3, a significant number of mitotic figures is observed outside the MZ, as defined by dome>GFP expression. (C–H) Distribution of mitotic divisions in mid-L3 LG (blue in C–H, white in C', F'). Examples of mitotic divisions within the MZ, as visualised by dome>GFP expression (green), are indicated by arrows. A significant fraction of mitoses lies outside the MZ (arrowheads in D–H). Rare P1-positive plasmatocytes (red) divide (asterisk in E) and no crystal cell (red in G, H). (I–J') An example of crystal cell (ProPO staining, blue) which incorporates BrdU (red) is indicated by an arrowhead. (K–M). There exists a cell population outside the MZ which expresses neither plasmatocyte (P1, red) nor crystal cell (lozenge-lacZ, blue) differentiation markers (arrowheads in L, M). The framed regions in I and K are enlarged in J, and L and M, respectively. Scale bar, 20 μm.

MZ as defined by Dome>GFP expression at 96 h (mid-L3) and 116 h of development (late L3) (Fig. 2B–H). Three-dimensional reconstructions, based on serial optical sections, of lymph glands double-stained for H3P and either a plasmatocyte (P1) or crystal cell (ProPO) markers (Fig. 2C–H) indicated that few P1 positive cells were mitotically active in the LG (Fig. 2D–E). Furthermore, we could not find examples of cell double-positive for H3P and ProPO (Fig. 2F–H), showing that differentiation of crystal cells is post-mitotic. This observation is consistent with the absence of crystal cell division in circulation

(Lanot et al., 2001) and indicates that BrdU incorporation which is observed in crystal cells in the LG (Fig. 2I, J) does not reflect cell proliferation but rather reveals endoreplication (C. Polleselo, personal communication).

Our data suggested the existence of population of intermediate progenitor cells leaving the MZ. To verify this, we performed triple-staining of third instar LG for MZ cells (dome>GFP), plasmatocytes and crystal cells, using in this case Lz-LacZ, a reporter line which mimics the expression of Lozenge, a transcription factor controlling

crystal differentiation (Bataille et al., 2005). This triple staining revealed a population of cells which are neither differentiated cells nor MZ cells (Fig. 2K) and are intermingled with differentiated hemocytes. Correlated with H3P stainings (Fig. 2C', F'), we propose that these cells are intermediate progenitors which undergo terminal divisions before entering differentiation.

Hemocyte progenitors are committed to a plasmatocyte or crystal cell fate, early during lymph gland development

Analysis of GFP-labeled clones generated by MARCM at different times during LG development suggested the existence of hematopoietic stem cells in the embryonic and early larval LG, while precursors able to undergo a limited number of divisions would also be present at later stages (Minakhina and Steward, 2010). The absence of evidence for stem cells provided by our above experiments was in favour of the specification of a small pool of primordial hematopoietic cells in the embryo, followed by an expansion phase in developing L2 and early L3 LG giving rise to the large population of pro-hemocytes observed in mid-late L3. When these progenitors become committed to adopt either a plasmatocyte or a crystal cell fate remained, however, unknown. One possibility was that pro-hemocytes remain multipotent up to differentiation. In this case, lineage commitment could be concomitant with differentiating mitoses in L3 LG. Another possibility was that restriction of cell fate is imposed earlier on progenitors, during the amplification phase.

To discriminate between these possibilities, we performed lineage analyses using a Flp-out strategy (hs-flp; actinFRTstopFRT Gal4, UAS-mcd8GFP) that allows cell labelling by inducing GFP expression at a given time during development and following their progeny (Golic and Lindquist, 1989). Clones were induced at different time points between mid-embryogenesis and early third instar (Fig. 3A, B). Larvae were then left to develop at 25 °C until late L3 before dissection and immunostaining for crystal cell (ProPO) and plasmatocyte (P1) markers. When heat-shock treatment was applied between 17 h and 28 h of development, about 40% of lymph glands (n = 41) showed a single clone of GFP-labelled cells, making likely that all GFP-positive cells in a lobe originate from a single event of recombination. Interestingly, and apart from PSC clones, the early clones showed variation in size and shape, suggesting that the small pool of primordial hematopoietic cells present in the embryonic LG is already heterogeneous. When both a PSC and non-PSC clone were found in the same lobe, they were not always adjacent (Fig. 3K), further suggesting that only a subset of primordial cells maintain direct contact with the PSC. This is consistent with the topology of the LG in stage 15–17 embryos (Crozatier et al., 2004). As also reported by (Minakhina and Steward, 2010), we noted that labelled cells tend to disperse when they leave the MZ (Fig. 3C, E). We also observed a strong decrease of GFP expression when cells differentiate, possibly linked to exit from the proliferation phase (Fig. 3D, F). Lobes with two or three physically separated patches of GFP-labelled cells per lobe were observed with progressively increasing frequency when heat shock was applied later during embryogenesis and in 1st instar larvae. In this case, a fraction of clones contained both crystal cells and plasmatocytes (mixed clones) (Fig. 3B, E, H). Such mixed clones were no longer observed, however, when clones were induced later than 96 h AEL, which corresponds to the L1–L2 transition at 18 °C (Fig. 3B, K). Past this transition, only monotypic clones were observed, i.e., clones containing non-differentiated cells and either plasmatocytes or crystal cells, but not both (Fig. 3H–L). We conclude that in L2 larvae, the lymph gland contains a mixture of progenitors already fated to give either plasmatocyte or crystal cell progeny (see Fig. 3B). The onset of expression of the tyrosine kinase receptor Pvr, a gene specifically required for plasmatocyte differentiation and only partly overlapping expression of two other proteins expressed in pro-hemocytes, Peroxydasin and Hemolectin at that stage (Jung et al., 2005), strengthens our conclusion

of some heterogeneity among the pro-hemocytes in the LG in L2 larvae at that stage.

The frequency of mixed clones was significantly smaller in larvae submitted to heat-shock between 24 and 48 h AEL (19%, n = 32) than between 17 and 24 h, (33%, n = 30), indicating that fate restriction is progressively established between mid-embryogenesis and the end of L1 (Fig. 3B). Together, the absence of multipotent progenitors after the L1–L2 transition and high mitotic index in L2 and early L3 LG (Fig. 2) indicate that the MZ is a transient amplification zone of fate-restricted progenitors. The maintenance of a small population of multipotent stem cells could have escaped our clonal analysis, because of a low number and possibly slow-cycling character. We, however, retrieved clones comprising only PSC cells at different Flp-out times, indicating that our analysis could detect a small population of rarely dividing cells. PSC-clones were identified, based on clustering, localisation, shape of the cells and presence of filopodia. The retrieval of PSC-only clones when the Flp-out was induced before 24 h AEL (Fig. 3C) confirmed that PSC cells are clonally related and do not mix with the pool of primordial hematopoietic progenitors and their progeny during larval development.

We further noticed that "plasmatocyte clones" showed patches of P1 positive cells, indicating that plasmatocytes differentiate more or less concertedly (Fig. 3F, L). On the contrary, "crystal cell clones" (either mixed or monotypic) contained only one or a few ProPO positive crystal cells (Fig. 3G–J). Whether this results from N-mediated selection of individual cells from equivalence groups ((Lebestky et al., 2003) and see below) is an interesting possibility.

When heat shock treatment was applied in L2 larvae or early L3 larvae, many small clones could be observed, consistent with the small number of divisions between the time of heat-shock and LG dissection. When induced in L2, many clones contained only non-differentiated cells while other contained a mixture of non-differentiated and differentiated hemocytes. When induced in early L3, we observed many pairs, or groups of 4 undifferentiated cells likely resulting from a single or two successive division events. Those clones of undifferentiated cells were interspersed with differentiated hemocytes (Fig. 3M, N), confirming that a population of mitotically active intermediate progenitors have left the MZ.

Notch activity is necessary in third instar larvae for crystal cell differentiation

In the absence of N signalling, the number of circulating crystal cells in the hemolymph, and differentiating crystal cells in the LG is drastically reduced (Duvic et al., 2002). N signalling activates Lz expression, thereby orienting pro-hemocytes towards a crystal cell fate, with Serrate acting as the N ligand in this process (Lebestky et al., 2003). However, whereas crystal cell differentiation in the LG takes place in the third instar, we found that progenitors are fated to become either plasmatocytes or crystals cell earlier during development (Fig. 3). This raised the question of whether, in addition to being instructive for crystal cell differentiation in third instar larvae, N signalling was also acting in lineage restriction. In order to address this question, we used N^{ts}, a temperature-sensitive mutant allele of N and shifted larvae to restrictive temperature at different time intervals during larval development. A shift during the third instar prevented crystal cell differentiation (Fig. 4A, B), confirming that N signalling is critically required in L3 for crystal cell differentiation in the LG (Lebestky et al., 2003). Crystal cell differentiation was not affected, however, when N was inactivated for periods of 24 h or 48 h, between 24 h and 96 h AEL at 22 °C, i.e., from the end of embryogenesis to mid-L2 when fate restriction of progenitors takes place (Fig. 3). To further verify that N signalling was not required earlier than the crystal cell differentiation period, N^{ts} mutant embryos were shifted at the non-permissive temperature (29 °C) and the developing larvae were maintained at non-permissive temperature until L3. Crystal cells

A Time of development

| | 21h / 41h | 48h / 94h | 72h / 144h | 96h / 188h | 120h / 234h | AEL at 25°C / AEL at 18°C |

egg laying (EL)

EMBRYO | L1 | L2 | eL3 | lL3

B

Time-window of heat shock treatment (AEL at 18°C)	17-24h	24-48h	48-72h	72-96h	96-120h	> 120h
Developmental stage at 18°C	Embryonic		1st instar larval		2nd instar larval	
Number of clones with differentiated cells	30	32	26	21	19	16
Number of mixed clones	10	6	3	1	0	0
Percentage of mixed clones	33%	19%	12%	5%	0%	0%

hs-Flp; act -FRTstopFRT-Gal4 UAS-GFP   P1   Pro-PO
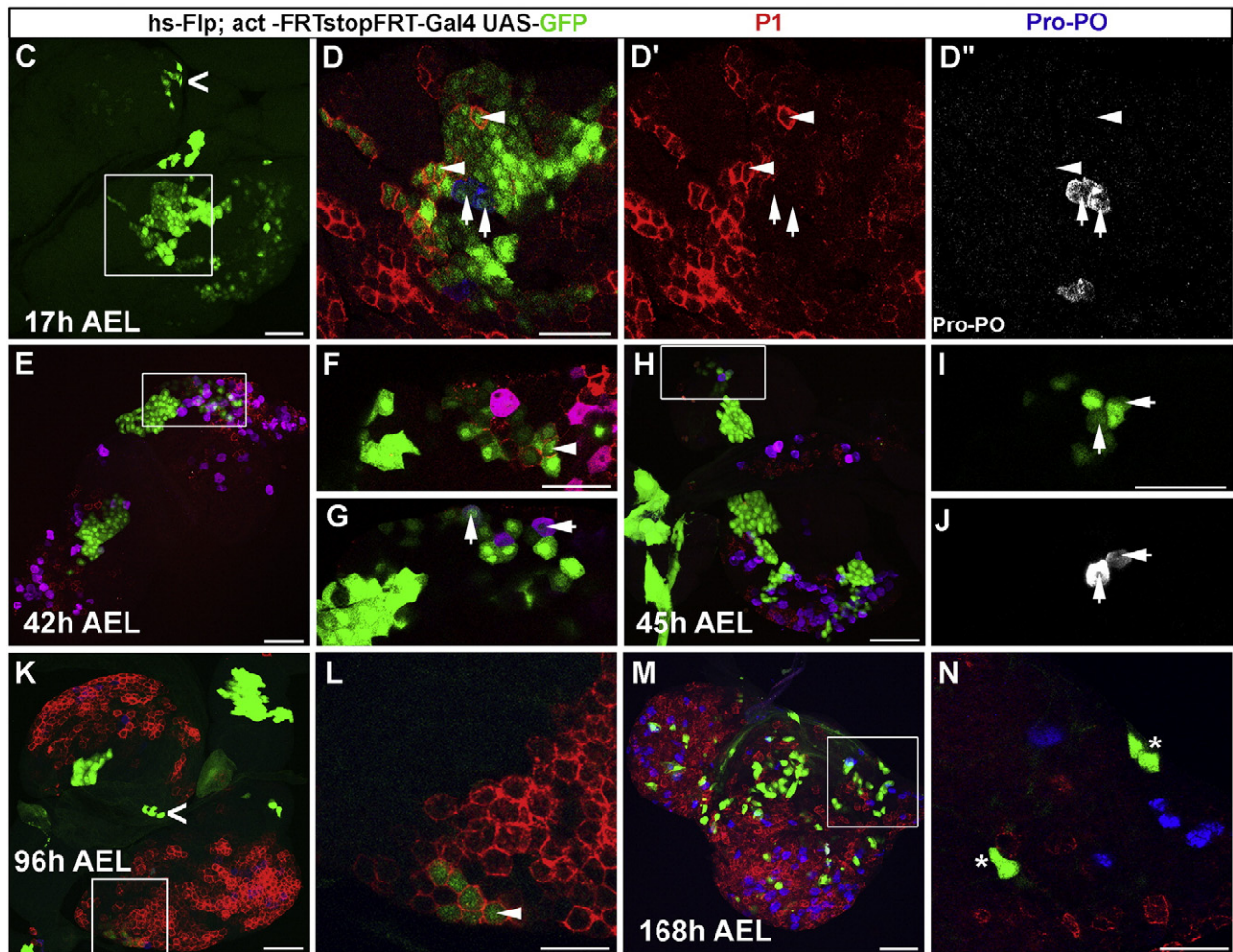
Fig. 3. Segregation of plasmatocyte and crystal cell fates occurs early during lymph gland development. (A) Time scale of larval development, indicating the times of each developmental stage at 18 °C and 25 °C. Time is given in hours after egg-laying (AEL). (B) Table recapitulating the number of clones induced at a given time and analysed. The proportion of *hs-FLP; actGal4>UAS-GFP* clones containing both types of hemocytes (mixed clones) is indicated relative to the developmental period when the clones were induced. Beyond 96 h AEL at 18 °C, which corresponds to the L1–L2 transition, no mixed clones were observed. (C–N) Representative examples of clones induced at different development times, as indicated in each panel. In all cases, dissected mature lymph glands were immunostained for plasmatocytes (P1, red) and crystal cells (Pro-PO, blue). Single confocal sections are shown in D, F, G, I, J, L and N; anterior is left. (C) A PSC clone (open arrowhead) is present in one lobe and a large clone in the other. (D–D") higher magnification of the framed region in C shows the presence of both undifferentiated cells, P1 (arrowhead) and ProPO (arrows) positive cells. (E) Two independent clones of similar size are observed. The bottom clone contains only undifferentiated cells. (F–G) Higher magnifications of different portions of the other clone (framed in E) shows that it contains both P1 (arrowhead in F) and ProPO (arrows in G) positive cells. (H) A crystal cell clone is observed in one lobe. Clones containing only undifferentiated cells are observed in the other; (I–J) higher magnification of the framed region in H (arrows: crystal cells). (K–L) Two clones, one containing undifferentiated cells and the other only PSC cells (open arrowhead), are observed in one lobe. A P1 clone (framed) is observed in the other lobe (arrowhead; magnification shown in L). (M–N) Clones induced in early-L3 are small and often made of only pairs of undifferentiated cells (asterisk, magnification shown in N) interspersed with differentiated hemocytes. (C, E, H, K, M) Scale bar 50 μm. (D, F, G, I, J, L, N) Scale bar 30 μm.
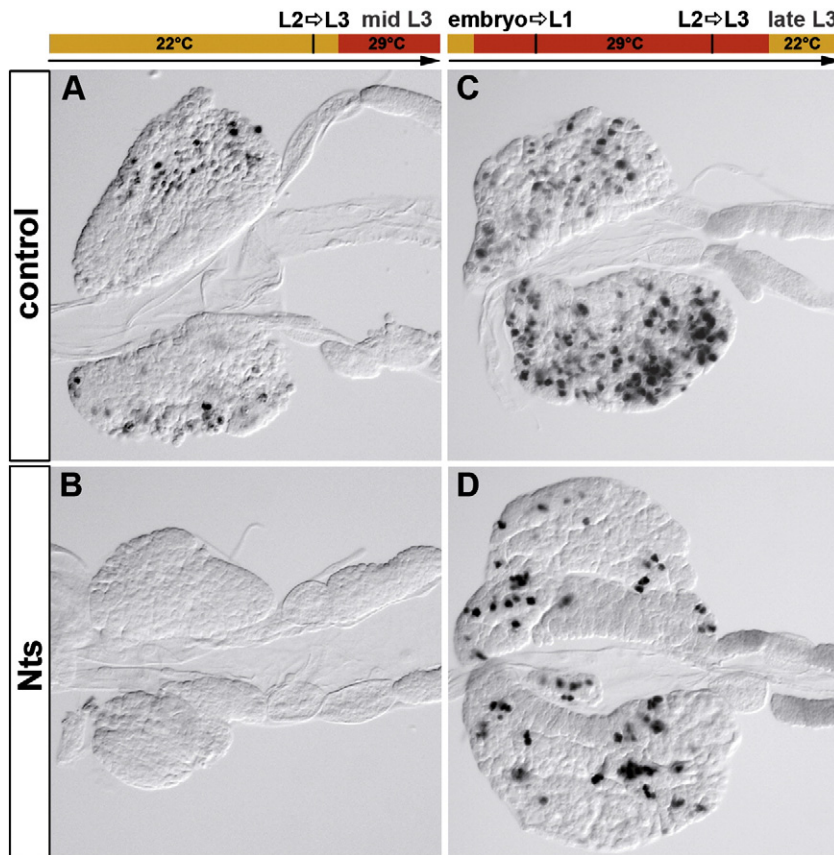
Fig. 4. Notch signalling is required for crystal cell differentiation only in third instar larvae. (A) *white (w, control)* or (B) N^{ts} larvae shifted to non-permissive temperature after 120 h of development at 22 °C (early to mid L3 stage). Notch inactivation at the third instar larval stage prevents crystal cell differentiation (ProPO antibody staining, black). Crystal cell differentiation in both (C) *w* and (D) N^{ts} larvae shifted to 29 °C during embryonic development and kept at non-permissive temperature until mid-L3.

differentiated in N^{ts} lymph glands in these conditions (Fig. 4C, D), confirming that the only time point when N signalling is required for crystal cell differentiation in lymph glands is L3. Higher numbers of crystal cells were observed in control lymph glands raised at 29 °C, due to the heat-sensitive w^{1118} background.

Lamellocytes differentiate at the expense of crystal cells

Massive differentiation of pro-hemocytes into lamellocytes occurs as a specific immune response to parasitization by wasps such as *Leptopilina boulardi*. A prerequisite for lamellocyte differentiation is complete switching off JAK/STAT signalling in the MZ. This takes place as early as 4–6 h after infestation, thereby preceding by several hours the first signs of lamellocyte differentiation (Makki et al., 2010). By comparing the mitotic index of third instar LG in either non-infested or infested larvae (6 h after egg laying) (Fig. 5A), we confirmed that lymph glands of parasitized larvae show an increased mitotic index (Sorrentino et al., 2002). We further showed that this mitotic burst is a rapid and transient response to parasitization since only few mitotic figures are observed 24 h after wasp egg-laying, while differentiation of lamellocytes takes place (Fig. 5B). On the contrary, proliferation is still actively going in LG of non-parasitized larvae at the same stage (Fig. 2A). In order to verify that the burst of mitoses observed in response to parasitism is a transient response which precedes lamellocyte differentiation, we performed double stainings for H3P and Talin (D-Rhea) an actin-binding protein specifically expressed in lamellocytes ((Luo et al., 2002; Shrestha and Gateff, 1982) and data not shown). The complete absence of overlap indicated that the differentiating lamellocytes in the lymph gland are post-mitotic cells (Fig. 5B). We therefore conclude that the premature wave of mitoses occurring following parasitization

corresponds to a wave of terminal differentiating mitoses allowing the massive production of lamellocytes.

Next we investigated whether lamellocyte differentiation takes place at the expense of plasmatocyte or crystal cell differentiation, or both types of cells. We dissected LG taken out from larvae 24 h after infestation and stained for markers of two cell types simultaneously: plasmatocytes (P1) and lamellocytes (αPS4) or crystal cells (ProPO) and lamellocytes (αPS4). Double staining for lamellocytes and plasmatocytes indicated that both types were present in LG of wasp-infested larvae although the number of plasmatocytes was reduced compared to physiological conditions. Plasmatocytes in infected larvae also displayed more numerous cytoplasmic extensions than in healthy larvae (Fig. 5C, D), suggesting that, upon wasp infestation, plasmatocytes get "activated," a term which is used here by reference to the activation of circulating plasmatocytes in response to injury and their transformation towards podocytes in prepupae (Luo et al., 2002). The ability of "pro-plasmatocytes" in the LG to form numerous cytoplasmic processes was also noticed in early ultra-structural studies (Shrestha and Gateff, 1982). Whether these pro-plasmatocytes can differentiate into lamellocytes, as suggested for a fraction of plasmatocyte precursors in circulation (Avet-Rochex et al., 2010; Honti et al., 2010), remains to be established. Double staining of lymph glands for αPS4 and proPO confirmed the presence of numerous crystal cells and absence of lamellocytes in normal conditions (Fig. 5E). Conversely, very few crystal or, most often no crystal cells at all, were detected following wasp parasitism (Fig. 5F), indicating that lamellocyte differentiation takes place at the expense of crystal cell differentiation. In order to strengthen this conclusion, we used another marker of crystal cells, lzGal4>GFP, an enhancer trap line which recapitulates Lz expression (Lebestky et al., 2000). The complete absence of GFP-positive cells in LG of *lzgal4;UASmCD8GFP*
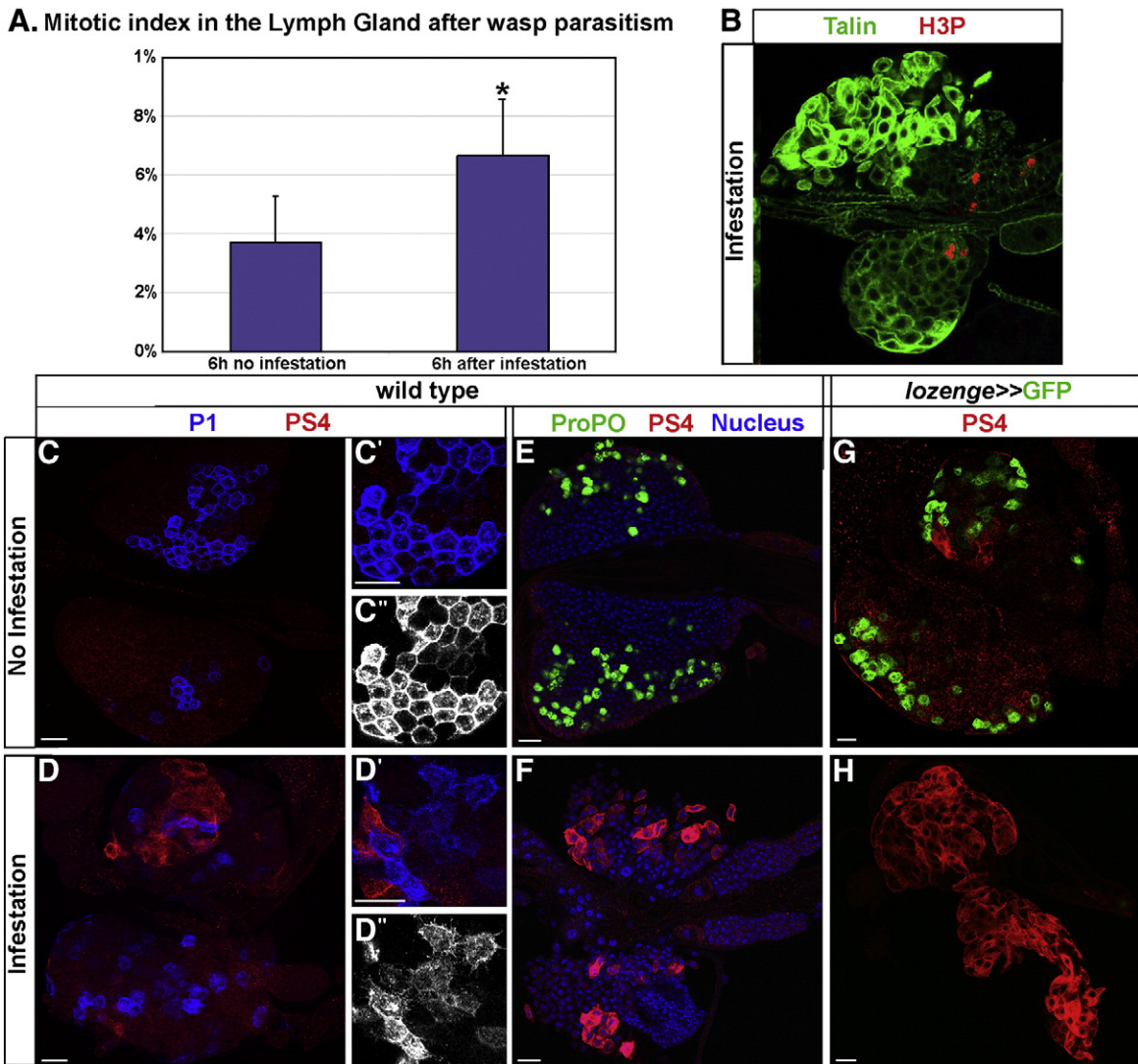
Fig. 5. Wasp infestation-induced lamellocyte production overrides the normal hemocyte differentiation program. (A) The mitotic index (number of mitotic figures/total number of cells) is increased about two-fold 6 h after wasp infestation. Numbers of counted LG: 15 (control) and 20 (infested) larvae. (B–H) Wild-type lymph glands either not parasitized (C, E, G) or 24 h after parasitic wasp infestation (B, D, F); C'–C" and D'–D" are higher magnification views of the LG shown in C and D, respectively. (B) Lamellocytes are post mitotic cells as shown by the absence of overlap between H3P (red) and Talin (lamellocyte, green) immunostaining. (C–D) Lamellocytes (α-PS4, red) and plasmatocytes (P1, blue) differentiate after infestation. (D'–D") Plasmatocytes show a different morphology in infested lymph glands, with a web of cytoplasmic extensions which are not observed in absence of infestation (C'–C"). (E–F) Lamellocytes (α-PS4, red) but no crystal cell (ProPO, green) differentiate after infestation. (G–H) The complete absence of crystal cells and presence of numerous lamellocytes (α-PS4, red) 30 h after infestation is confirmed by the absence of lz-GFP expression (green). Scale bar 20 μm.

larvae 24 h after infestation confirmed the absence of crystal cell differentiation in lymph glands otherwise filled with differentiating lamellocytes (Fig. 5G, H). A different conclusion, namely that parasitism leads to an increase in the number of crystal cells in lymph glands was previously based on the *Black cells* melanisation mutant phenotype (Sorrentino et al., 2002). This apparent contradiction could be explained if lamellocytes expressed at least one of the proPO enzymes which are responsible for the blackening of melanotic corpses. Indeed, it was recently shown that proPO59/PPO3/CG2952 is expressed in lamellocytes and that PPO3 plays a major role in the lamellocyte-mediated spontaneous melanization process (Irving et al., 2005; Nam et al., 2008). To study this possibility, we stained LG for P1 and proPO, using polyclonal proPO antibodies which recognise all proPO isoforms (Asano and Takebuchi, 2009). Whereas in non-parasitisized larvae, proPO positive cells displayed distinctive crystal cell morphology, including the presence of visible crystals under light microscopy, proPO positive cells of parasitised larvae showed a lamellocyte shape (Fig. S2). This shape suggests that proPO positive cells in LG of infested larvae are differentiating lamellocytes. Together,

our data show that lamellocytes differentiate at the expense of crystal cells. This suggests two possibilities: either a fraction of lamellocytes derive from crystal cell progenitors, which are re-fated to become lamellocytes upon wasp infestation, or wasp parasitism interferes with crystal cell differentiation without implying a lineage relationship. The reduced number and modified morphology of P1 positive cells does indeed suggest that lamellocytes also derive from plasmatocyte progenitors. A third possibility could be the existence of a specific pool of "dormant pro-lamellocytes" activated in response to wasp parasitism. Since the premature differentiation of plasmatocytes and crystal cells that is observed in absence of a functional PSC (Krzemien et al., 2007; Mandal et al., 2007) prevents lamellocyte differentiation (Crozatier et al., 2004), we do not favour this third possibility. One recent report proposed that a subepidermal population of sessile blood cells which is released into circulation in response to wasp parasitism is the main source of lamellocytes (Markus et al., 2009). The authors came to this conclusion, based on morphological examination of circulating cells at different times after wasp-egg laying and the encapsulation of wasp eggs in larvae where the LG was
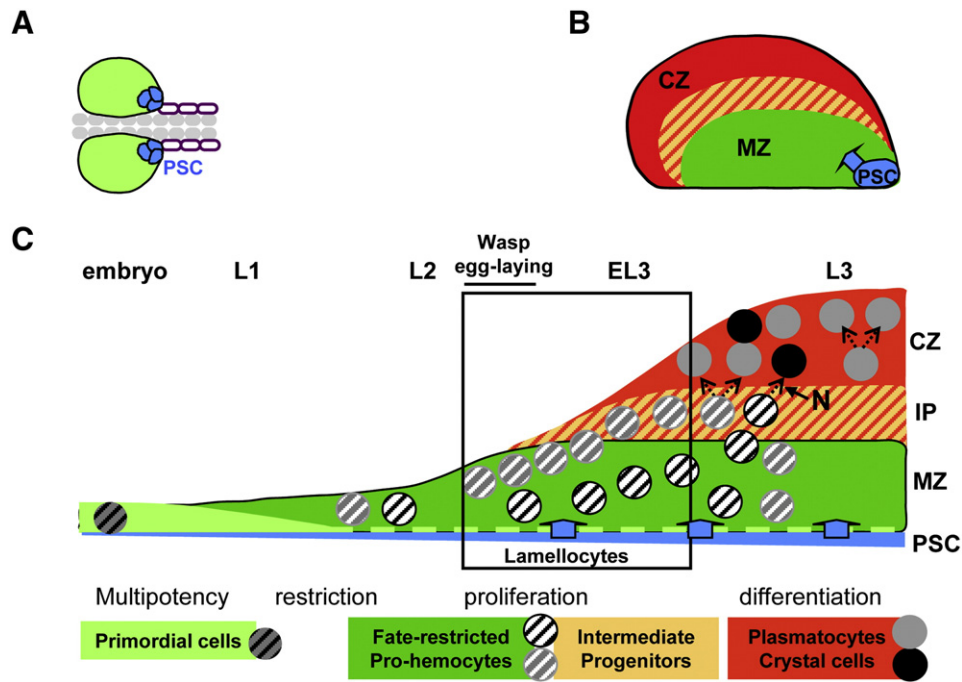
Fig. 6. Schematic view of hematopoiesis in the *Drosophila* lymph gland. (A) Only two types of cells are found in the embryonic lymph gland: Primordial hematopoietic progenitors (pale green) and PSC cells (blue). (B) In third instar larvae, the primary lobe of the lymph gland comprises the PSC, the Medullary zone (MZ), intermediate progenitors (IP) and cortical zone (CZ). (C) A schematic view of larval hematopoiesis: Primordial hematopoietic cells (pale green) undergo a few rounds of division in the 1st instar larval stage, during which fate restriction is established, giving rise to fate-restricted pro-hemocytes. These pro-hemocytes proliferate in the medullary zone (dark green). Starting in early third instar larvae, pro-hemocytes exit the medullary zone and form a pool of actively dividing intermediate progenitor (orange). These intermediate progenitors likely undergo terminal differentiating divisions (dashed arrows from the IP to CZ) since they are intermingled with differentiated hemocytes, of which very few, and exclusively P1-positive cells, divide in the CZ (red). Based on *Nts* experiments, we postulate that it is at the intermediate progenitor stage that N signalling instructs crystal cell differentiation (arrow). Cells in the MZ can be instructed to undergo terminal divisions and differentiate into lamellocytes in response to wasp infestation (framed area). Some plasmatocytes but no crystal cells differentiate in parallel to lamellocytes.

physically separated from the laid egg by ligature. This conclusion, which has been since moderated (Honti et al., 2010), contradicted our initial data showing that the communication between PSC cells and pro-hemocytes in the LG was required for lamellocyte release into circulation (Crozatier et al., 2004). More specifically, character-isation of two different mutants, *col* and *latran (lat)*, has shown that the PSC maintains JAK/STAT signalling activity in pro-hemocytes, thereby preserving their multipotent character and that switching off JAK/STAT signalling is a prerequisite to massive differentiation of lamellocytes upon wasp parasitism (Krzemien et al., 2010; Makki et al., 2010). A correlation between the re-programming of pro-hemocytes in the LG and the number of circulating lamellocytes was independently documented by studies of the Zfrp8 mutant (Minakhina et al., 2007). This does not exclude that some lamellocytes can differentiate in circulation, as first documented by (Shrestha and Gateff, 1982) and more recently by (Avet-Rochex et al., 2010; Honti et al., 2010), suggesting a contribution of both embryonic and larval hematopoiesis to the lamellocyte lineage. Taken together, these recent reports demonstrated not only the key role of the LG in controlling the cellular response to wasp parasitism but also an unanticipated plasticity of embryonic and larval hematopoietic lineages.

In summary (Fig. 6), *Drosophila* larval hematopoiesis relies upon the early specification of two cell lineages in the lymph gland, primordial hematopoietic cells at the origin of the three types of hemocytes and PSC cells (Fig. 6A). PSC cells divide rarely, remain clustered and act as a niche in third instar larvae to control hemocyte homeostasis. Primordial hematopoietic cells actively divide to generate a large pool of progenitors before hemocytes start to differentiate in early L3 larvae. We found no evidence for the existence of "classical" stem cells. Based on our and other's clonal

analyses (Minakhina and Steward, 2010), we propose that larval hematopoietic pro-hemocytes are regulated as a population, rather than as individual stem cells. Lamellocyte differentiation in response to wasp parasitism is preceded by a wave of mitosis and takes place at the complete expense of crystal cell differentiation and part of plasmatocyte differentiation. Finally, we found evidence for a pool of mitotic undifferentiated cells interspersed with differentiated hemo-cytes which we designate as intermediate progenitors and account for the increase in hemocyte numbers observed throughout the 3rd instar (Fig.6B, C). Overall, the *Drosophila* hematopoietic organ shows a striking developmental plasticity since the size and number of LG lobes and the extent of hemocyte differentiation may vary from one larva to the other. Our findings provide a useful framework to identify the parameters of this plasticity and more broadly how the communications between the niche and hematopoietic progenitors integrate physiological and immunological cues.

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