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Ontogeny of the *Drosophila* larval hematopoietic organ, hemocyte homeostasis and the dedicated cellular immune response to parasitism

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ABSTRACT Over the years, the fruit fly *Drosophila melanogaster* has become a major invertebrate model to study developmental and evolutionary aspects of both humoral and cellular aspects of innate immunity. *Drosophila* hematopoiesis which supplies three types of circulating hemocytes, occurs in two spatially and temporally distinct phases during development. The first embryonic phase is described in detail in accompanying reviews in this *Int. J. Dev. Biol.* Special Issue. The second phase takes place at the end of larval development in a specialised hematopoietic organ, termed the lymph gland. We review here recent studies on the ontogeny of the lymph gland, focusing on the formation and role of the Posterior Signalling Center which acts as a niche for hematopoietic progenitors. We then report recent progress in understanding the dedicated cellular immune response of *Drosophila* larvae against parasitization by Hymenopterae, a common threat for many *Dipterae*. This response involves the differentiation of lamellocytes, a cryptic cell fate, revealing the high degree of plasticity of *Drosophila* hematopoiesis. We end up by integrating studies in *Drosophila* within a more general picture of insect hematopoiesis and hemocyte homeostasis.

KEY WORDS: *Drosophila, hematopoiesis, lymph gland, immunity, niche, signalling*

Introduction to the *Drosophila* cellular immune system, the hemocytes

Drosophila is a holometabolous insect which spends most of its life cycle in decaying organic matter such as rotting fruit, an environment enriched in microorganisms that constantly challenges the immune system. Innate responses can be schematically divided into humoral and cellular components mediated by secreted factors like antimicrobial peptides (AMP) and specific cells, the hemocytes, respectively (Lemaitre and Hoffmann, 2007). *Drosophila* hematopoiesis which provides circulating hemocytes, occurs in two spatially and temporally distinct phases (Crozatier and Meister, 2007; Evans *et al.,* 2003; Holz *et al.*, 2003). The first, embryonic phase is described in detail in an accompanying review (Waltzer *et al.*, 2010). The second phase takes place during larval development in a specialised hematopoietic organ, termed the lymph gland (LG). The LG starts to form at the end of embryogenesis and growths during larval stages (see below) before dispersal at metamorphosis and the releasing of hemocytes in the hemolymph (Crozatier *et al.*, 2007; Crozatier and Meister, 2007). Three different types of hemocytes can be found in *Drosophila*. The first and most abundant type is the plasmatocytes that are functionnally compared to vertebrate macrophages. They are responsible for the removal of apoptotic cells and microorganisms during the entire life fly cycle (Wood and Jacinto, 2007). Plasmocytes are highly motile cells, owing to well developed actin rich filopodia and lamellopodia which allow them to explore their environment (Tepass *et al.*, 1994; Wood *et al.*, 2006; Zanet *et al.*, 2009). In the embryo, plasmatocytes migrate to and scavenge damage tissue using chemotactic cues (Stramer *et al.*, 2005). In the larva, plasmatocytes are recruited to tissue wound to participate in damaged healing through direct, adhesive capture from

Abbreviations used in this paper: AMP, antimicrobial peptide; LG, lymph gland; PSC, posterior signalling center.

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circulation, similar to blood cells in vertebrates (Babcock *et al.*, 2008). This behavior is consistent with the hypothesis that the ability to recognise and adhere to damaged or "non self" tissue is an ancestral feature of immune cells. According to the "danger-hypothesis", the immune system would be alerted by endogeneous stress signals released from injured tissues (Matzinger, 2002; Seong and Matzinger, 2004; Pastor-Pareja *et al.*, 2008). Recent studies have also brought to light possible links between the phagocytic activity of hemocytes and activation of the systemic humoral response. Mutants for *psidin,* which encodes a protein associated with lysosomes expressed in hemocytes, fail to lyse phagocytosed bacteriae and do not synthesize the antimicrobial peptide defensin upon bacterial infection (Berman *et al.*, 2004). A similar phenotype was reported for mutants of *eater*, a gene encoding a phagocytosis receptor expressed by hemocytes (Kocks *et al.*, 2005; Kurucz *et al.*, 2007; Somogyi *et al.*, 2008). Recent genetic ablation of. plasmatocytes has revealed the essential contribution of phagocytes, both to embryonic development, including morphogenesis of the central nervous system and the resistance of *Drosophila* adults upon systemic infection with specific bacteriae. It confirmed that phagocytosis is a critical effector mechanism of the cellular immune response (Charroux and Royet, 2009; Defaye *et al.*, 2009). Interestingly, plasmatocyte-mediated phagocytosis could also participate in a specific and durable, induced protection against specific pathogens, a kind of immune memory. Flies primed with a sublethal dose of *Streptococcus pneumoniae* are protected against a lethal dose of the same bacteria when challenged one week later and this specific memory depends on phagocytosis by plasmatocytes (Pham *et al.*, 2007). Evidence for a memory effect has also been obtained in the social insect bumblebee *Bombus terrestris* (Sadd and Schmid-Hempel, 2006). The molecular basis of this “immune memory” of insects, a property thought so far to be specific to vertebrates could possibly involve recognition of bacteria by specific membrane proteins expressed by hemocytes such as the immunoglobulin-domain Down-syndrome-cell-adhesion (Dscam) molecules (Dong *et al.*, 2006). Finally, it was very recently shown that *Drosophila* plasmatocytes use macro-autophagy to fight against invading intracellular bacteriae, such as *Listeria monocytogenes*, through intracellular recognition of a bacterial peptidoglycan by a pattern recognition receptor, (PGRP-LE) that is expressed by hemocytes. Autophagy which prevents intracellular growth of *L. Monocytogenes* and promotes host survival following this infection was confirmed in *ex vivo* cultured hemocytes (Yano and Kurata, 2008). Together, the recent reports on new plasmatocyte functions open the way to compare analyses of the signalling pathways involved in immune memory and autophagy as an immune response between insects and mammals.

The second type of *Drosophila* hemocytes, the crystal cells are also generated during both embryonic and larval hematopoiesis but their role in embryos remains unknown (Wood and Jacinto, 2007). They make about 5% of circulating hemocytes in the larval hemolymph and are not found in adult flies (Lanot *et al.*, 2001). Crystal cells owe their name to big crystals of zymogen proPhenolOxydase (proPO1 and ProPO2), a component of the melanisation enzymatic cascade that are stored in their cytoplasm (Ashida, 2004). Crystal cell-release of components of the melanisation cascade plays a key role in formation of the clot which immobilises bacteria, prevents their spreading and promotes their killing since melanin and its biosynthetic by-products are directly toxic to micro-organisms. Clotting is critical in limiting hemolymph loss and creating a physical immune barrier following would healing in insects. The phenoloxydase originating from crystal cells acts during clot maturation to produce a hard clot by cross-linking components of the primary soft clot (Jiravanichpaisal *et al.*, 2006). Melanisation could also be mediated by Pro-PO3 a third proPO isoform active in its zymogen form and expressed by lamellocytes, the third type of hemocytes (Nam *et al.*, 2008).

The *Drosophila* lamellocytes are flat, large, adhesive cells which, unlike plasmatocytes and crystal cells, are produced by larval hematopoiesis (Lanot *et al.*, 2001). They form a multi-layered, overlapping sheath of cells around foreign objects too large to be phagocytised, a process called encapsulation. Lamellocytes are rarely observed in healthy larvae but differentiate in very large number in response to specific challenges such as wasp parasitism. In nature, at least around 50 hymenoptera species parasite *Drosophila* larvae (Carton and David, 1985) among which *Leptopilina boulardi,* a species widely used in laboratories to study the *Drosophila* immune response. *L. boulardi* adult females lay eggs into the hemocel of young *Drosophila* larvae and use the host body as a nutrition source for the developing offspring. The detection of parasitoid eggs, probably by circulating plasmatocytes (Russo *et al.*, 1996), triggers lamellocyte differentiation in the LG and release in the hemolymph. Encapsulation of the egg is followed by its melanisation and death, probably through the effect of cytotoxic free radicals or quinons generated by the melanin synthesis process (Vass and Nappi, 2000). The killed egg stays as a melanotic capsule in the larva and adult as an inert body. If the fly immune response fails, a wasp hatches from the pupa at the expense of the fly. Parasitoid wasps use different infection strategies to avoid the host response (Schlenke *et al.*, 2007). For example, *Asobara tabida* can passively evade the immune response because its eggs attach to the host tissue thanks to their sticky chorion and get embedded in host tissue inaccessible to hemocytes. *L. boulardi* eggs typically attach to host tissues which provide a partial passive protection (Prevost *et al.*, 2005; Rizki *et al.*, 1990). Another way to escape is an active suppression based on specific components introduced into the larval host by the female wasp at the time of oviposition. For example, *L. heterotoma* wasps inject virus like particles (VLP) produced by the so-called long glands. The proteins present in the *L. heterotoma* VLP inhibit the encapsulation by changing the morphology of the lamellocytes, resulting in their diminished adhesive ability and, eventually, apoptosis whereas the venom of *L. Boulardi* virulent strains appears to block the induction and release of lamellocytes from the LG (see references in Schlenke *et al.*, 2007).

Ontogeny of the larval hematopoietic organ, the lymph gland

The embryonic primordium of the LG is part of the cardiogenic mesoderm which gives also rise to the dorsal vessel, the *Drosophila* heart made of two cell types, the contractile cardiomyoblasts and the associated non-myogenic pericardial cells (Rugendorf *et al.*, 1994) reviewed in (Cripps and Olson, 2002; Zaffran and Frasch, 2002). Homo and hetero-topic cell transplantation experiments mapped the blastoderm LG anlage between 50 and 55%

Egg Length in the thoracic lateral mesoderm, correlating with patterns of expression of some enhancer trap lines (Rugendorf *et al*., 1994). The fate of the transplanted cells further showed that the LG originated from only a few progenitors which are not specified before the second post-blastoderm mitosis (Holz *et al*., 2003). More recently, the pattern of expression of the COE transcription factor Collier/Knot (Col) suggested that the LG originates from discrete clusters of few cells in the dorsal mesoderm of thoracic segments and confirmed that LG progenitors are already specified at the time of germ-band elongation (stage 11), at least partly independent of *srp* (*serpent*) which encodes a GATA factor required for embryonic hematopoiesis ((Crozatier *et al*., 2004); Fig. 1A). The clusters of Col-expressing cells subsequently coalesce to form the primary LG lobes. At the same time, Col expression gets restricted to the 2-3 posterior-most cells of the LG, in segment T3 (Fig. 1A). This late expression of Col prefigures the so-called Posterior Signalling Center (PSC), first identified in LG of 3rd instar larvae ((Lebestky *et al*., 2003), see below). Although Col expression in stage 11 embryos suggested that the LG originates from two thoracic cell clusters, expression of another transcription factor (TF), the C2H2 zinc-finger protein Odd (Odd-skipped), at stage 13 suggests that the LG could be contributed by cells originating from each of the 3 thoracic segments. Unlike Col, however, Odd is also expressed from stage 13 in precursor cells of pericardial cells in abdominal segments and thus constitutes a marker of both lymph gland and pericardial cells, termed also nephrocytes (Mandal *et al.,* 2007; Ward and Coulter, 2000). A detailed appraisal of the transcriptional control of cardiogenic mesoderm formation and LG specification is outside the scope of this review. In brief, the GATA factor Pannier (Pnr) and the Homeobox Tinman (Tin) are required for both development of the dorsal vessel and lymph gland while Srp, another GATA factor is specifically required for specification of the lymph gland, in addition to specification of embryonic hemocytes from the anterior mesoderm (Fig. 1A, (Mandal *et al*., 2004), and review by (Waltzer *et al*., 2010)). *tailup* (*tup*) which encodes a LIM homeodomain transcription factor expressed in all cardioblasts, pericardial cells and LG cells is also required for normal heart and LG formation (Mann *et al*., 2009; Tao *et al*., 2007) as well as *hand*, a direct target of Tin, Tup and GATA factors which encodes a conserved bHLH transcription activator (Han and Olson, 2005; Han *et al*., 2006; Lo *et al*., 2007). *Antennapedia* (*Antp*) is the only homeotic gene expressed in the thoracic cardiac mesoderm. Antp expression is restricted to cardiac cells in the posterior T3 and anterior A1 segments and LG cells in posterior T3, those cells that keep expressing Col and prefigure the PSC (Mandal *et al*., 2004; Perrin *et al*., 2004). Homothorax (Hth) is expressed in a complementary pattern to Antp in the LG, such that at the end of embryogenesis, two populations of cells, Hth and Col/Antp positive, respectively, can be distinguished ((Mandal *et al.,* 2007), Fig. 1B). Antp is required to maintain the PSC-specific *col* transcription from stage 15 (Mandal *et al.,* 2007). This observation was somewhat unexpected since, in absence of homeotic gene function (*Scr-, Antp-, Ubx-, abdA-, AbdB-* embryos) or *Ubx-, abdA-* double mutants the whole cardiac tube develops as anterior aorta with Odd-expressing cells along the entire length of the tube also expressing Col. Conversely, ectopic expression of either Antp, Ubx, or AbdA in the entire mesoderm results in the loss of Col-expressing cells (Mandal *et al*., 2004; Perrin *et al*., 2004). Together, these homeotic gain-of-function and loss-of-function data suggested that pericardial cells and lymph gland progenitor cells are closely related fates, with pericardial cells adopting a “LG identity” in absence of homeotic gene function. Antp could possibly exert a secondary role in specifying PSC cells. This interpretation is supported by Odd and Srp staining of *tup* mutant embryos. While most of the LG and pericardial cells are lost in these embryos, the number of Col-positive cells detected at stage 15 is comparable to wild type, indicating that the posterior part of the LG forms in absence of *tup* and *srp* function (Tao *et al*., 2007). Together, these observations indicate that the specification of PSC cells is controlled by a specific regulatory network. Clonal analyses have suggested that at least a subset of LG cells could originate from bipotential cardioblast-lymph gland progenitors (Mandal *et al*., 2004). A complete view of the lineage relations between the different cell types in the lymph gland, and *Drosophila* heart is, however, still lacking. In summary, LG speci-

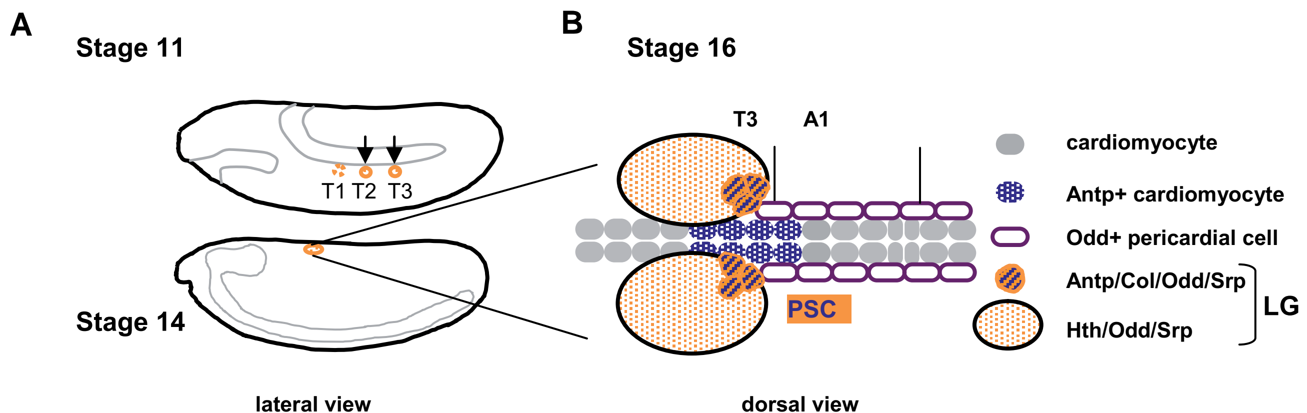

Fig. 1. Ontogeny of the lymph gland in the *Drosophila* embryo. (A) *Diagrammatic representation of Col expression (orange) in the lymph gland anlage. Col expression is observed in two separate clusters of cells (black arrows) in the dorsal-most mesoderm of thoracic segments T2 and T3 at the germ band extension stage (stage 11 embryos). Odd expression identifies a third cluster in segment T1 (broken circle). The clusters of Col + Odd expressing cells coalesce to form the primary lobe of the LG at stage 14. Col then becomes progressively restricted to the posterior-most cells of the LG as shown by the partial overlap with Odd expression.* **(B)** *Schematic drawing of the LG, cardiac and pericardial cells at embryonic stage 16. The expression domains of Antp, Col, Odd and Srp are colour-coded (see text for references).*

fication within the embryonic cardiac mesoderm is controlled by a complex hierarchical network of transcription factors (Fig. 1 and review by (Waltzer *et al.*, 2010)). One key event, more specifically emphasized here, is the restriction of *col* transcription to cells issued from the LG primordium which express Antp and will give rise to the PSC.

During larval development, the embryonic, primary LG lobes grow in size while secondary, posterior lobes form (Johnson *et al.*, 2007). When fully developed in third instar larvae, primary lobes contain each about 3000 cells (compared to ~30 at the end of the embryogenesis) and represent the main *Drosophila* larval hematopoietic site (Lanot *et al.*, 2001). Before the LG disperses and releases hemocytes into circulation in response to a peak of ecdysone at metamorphosis (Sorrentino *et al.*, 2002), all plasmatocytes and crystal cells, either circulating or sessile, originate from embryonic hematopoiesis. A mixture of embryonic and lymph gland hemocytes is thus found in pupae and the adult fly (Holz *et al.*, 2003). Until now, no hematopoietic site has been described in the adult fruit fly (Holz *et al.*, 2003). However, it might be important to consider that, when the LG disperses, it contains a mixture of differentiated and non differentiated hemocytes, including those present in the variable numbers of posterior lobes whose function has not been thoroughly investigated. Lymph gland cells give rise to both circulating and sessile hemocytes which stay attached to the integument after metamorphosis (Holz *et al.*, 2003). Whether some of them are immature progenitors remains a possibility to be explored.

The control of larval hematopoiesis: the key role of the Posterior Signalling Center

Perhaps related to very different time scales, only few parallels have been established between embryonic and larval *Drosophila* hematopoiesis. The transcription factor, Lozenge (Lz) plays a key role in crystal cell specification, in both embryos and larvae (Lebestky *et al.*, 2000). Similarly, U-shaped which acts in repressing the crystal cell fate in embryos by binding to a specific isoform of Srp is also involved in repressing the lamellocyte fate in the LG (Ferjoux *et al.*, 2007; Sorrentino *et al.*, 2007) and accompanying review by (Waltzer *et al.*, 2010). Gcm and Gcm2 which are strictly required to endow embryonic hemocytes with a plasmatocyte fate are not expressed in the lymph gland ((Bataille *et al.*, 2005) review by (Waltzer *et al.*, 2010). At the opposite, Odd is only expressed during larval hematopoiesis (Ward and Coulter, 2000). A more conflicting issue is Notch signalling which has been shown to be essential for crystal cell differentiation in larvae(Duvic *et al.*, 2002; Lebestky *et al.*, 2000) while its role during embryonic hematopoiesis remains controversial (Bataille *et al.*, 2005).

A key step in the description of larval hematopoiesis was the detailed examination of a fully developed 3rd instar LG by optical microscopy. It allowed to distinguish two zones, in addition to the PSC: a cortical zone (CZ) with a granular appearance surrounding a smooth and compact medullary zone (MZ) (Jung *et al.*, 2005). The CZ contains differentiated plasmatocytes and crystal cells while the MZ contains undifferentiated pro-hemocytes (Fig. 2). Powerful cell tracing experiments further showed that CZ cells originate from the MZ. The mode and control of the MZ>CZ transition are currently the subject of intense investigation (see below). Apart from the lack of expression of differentiation markers, the MZ can be visualised by the expression of several genes and reporter expression driven by *domeless*-gal4, a transgene inserted in the *domeless* (*dome*) upstream region ((Bourbon *et al.*, 2002; Jung *et al.*, 2005); Table 1). *dome* encodes the *Drosophila* receptor of the evolutionarily conserved JAK/STAT (Janus Kinase/Signal Transducer and Activators of Transcription) pathway (review by (Agaisse and Perrimon, 2004)). The JAK/STAT signalling pathway was discovered from studying the role of interferon in the control of immune responses in vertebrates. (for review (Heinrich *et al.*, 2003; Kristensen *et al.*, 2005). Initial evidence that JAK/STAT signalling could be involved in *Drosophila* cellular immunity came from analysis of dominant-gain of function mutations of the JAK kinase hopscotch (hop), such as the thermosensitive mutation hop^{Tum-l}. When kept at restrictive temperature, hop^{Tum-l} larvae display numerous circulating lamellocytes and melanotic pseudotumors in absence of wasp infestation

TABLE 1

GENES EXPRESSED IN THE ANTERIOR LOBE OF THE LYMPH GLAND, EITHER IN THE ENTIRE LOBE, THE POSTERIOR SIGNALLING CENTER, THE MEDULLARY ZONE OR THE CORTICAL ZONE

Markers	Expression in the LG	References
Serpent (Srp)	whole	Letbestky *et al.* 2000
Hemese (He)	whole	Kurucz *et al.* 2003
PDGF- and VEGF-receptor related (Pvr)	whole	Jung *et al.* 2005
Odd-skipped (Odd)	whole	Jung *et al.* 2005
Viking (Vkg-GFP)	whole	Jung *et al.* 2005
U-shaped (Ush)	whole	Sorrentino 2007; Gao 2009
dSPARC	whole	Irving *et al.* 2005
cg9372	whole	Irving *et al.* 2005
imaginal disc growth factor (idfg1)	whole	Irving *et al.* 2005
Serrate (Ser)	PSC+scattered cells in LG	Lebestky *et al.* 2003
Collier (Col)	PSC	Crozatier *et al.* 2004
Antennapedia (Antp)	PSC	Mandal *et al.* 2007
Hedgehog (Hh)	PSC	Mandal *et al.* 2007
DE-cadherin (DE-cad)	MZ	Jung *et al.* 2005
domeless (dome) (P-gal4)	MZ	Jung *et al.* 2005
thioester-containing protein (tep4)	MZ	Irving *et al.* 2005
Patched (Ptc)	MZ	Mandal *et al.* 2007
Cubitus interruptus (Ci)	MZ	Mandal *et al.* 2007
Latran (Lat)	MZ	Makki *et al.*, 2010
Cut (Ct)	CZ	Jung *et al.* 2005
Nimrod1 (NimC1)	CZ (plasmatocytes)	Kurucz *et al.* 2007
Crocquemort (Crq)	CZ (plasmatocytes)	Irving *et al.* 2005
Peroxidasine (Pxn)	CZ (plasmatocytes)	Jung *et al.* 2005; Irving *et al.* 2005
Lozenge (Lz)	CZ (crystal cells)	Lebestky *et al.* 2000
Prophenoloxidase (proPO)	CZ (crystal cells)	Duvic *et al.* 2002
diphenol oxidase A3 (doxA3)	CZ (crystal cells)	Crozatier *et al.* 2004
PS4 integrin α chain (αPS4)	(lamellocytes)	Krzemien *et al.* 2007
misshapen (msn) (P-lacZ)	(lamellocytes)	Braun *et al.* 1997
L1	(lamellocytes)	Kurucz *et al.* 2003
puckered (puc) (P-lacZ)	(lamellocytes)	Irving *et al.* 2005
Filamin-240/Cherio (Cher)	(lamellocytes)	Rus *et al.* 2006
Wingless	MZ, PSC, CC precursors	Sinenko *et al.*, 2009
Frizzled 2 (DFz2)	MZ, PSC	Sinenko *et al.*, 2009

Expression in specific hemocytes is indicated in brackets.

Gene names in italic letters indicate that the expression is detected by *in situ* hybridisation, whereas P-element enhancer trap insertions are indicated by P-gal4 or P-lacZ. Abbreviations: LG, lymph gland; Whole, entire lobe; PSC, posterior signalling center; MZ, medullary zone; CZ, cortical zone.

(Hanratty and Dearolf, 1993; Harrison *et al.*, 1995; Luo *et al.*, 1995; O'Shea *et al.*, 2002; Sorrentino *et al.*, 2007). Conversely, loss of hop function leads to a complete absence of lamellocyte differentiation upon wasp infestation (Sorrentino *et al.*, 2004). This led to the conclusion that up-regulation of JAK/STAT signalling was instrumental in triggering lamellocyte differentiation. The situation is certainly more complex, however, since JAK/STAT signalling has recently been shown to play an opposite role in hemocyte homeostasis, that is, preserving the pro-hemocyte character of MZ cells (Krzemien *et al.,* 2007). Maintenance of JAK/STAT signalling in the MZ depends upon activity of the PSC. The PSC was initially described as a small cluster of posterior LG cells expressing the Notch (N) ligand Serrate (Ser) in third instar larvae. Based in part on the roles of N signalling in crystal cell formation and Ser as the *Drosophila* N ligand in this process (Duvic *et al.*, 2002; Lebestky *et al.*, 2003), it was proposed that the posterior cluster of Ser positive LG cells could act as a signalling centre instructing neighbouring cells to become crystal cells (Lebestky *et al.*, 2003). This conclusion was contradicted, however, by the observation that crystal cells differentiate in col^1 mutant LG (col^1 is a null allele) which lacks the PSC, suggesting that Ser expression in scattered cells in the LG, and not the PSC, was required for crystal cell differentiation (Crozatier *et al.*, 2004). Nevertheless, the notion of a posterior signalling centre was fundamental, since the PSC turned out to control other essential aspects of larval hematopoiesis. The first observation was that PSC-depleted larvae are unable to mount a cellular immune response in response to parasitoid wasp egg-laying (Crozatier *et al.*, 2004). The authors also noticed an increased differentiation of hemocytes that occurs in LG lacking a PSC in absence of wasp infestation, suggesting other roles of the PSC in normal physiological conditions. Two independent reports have now shown that the PSC acts as a specific micro-environment, for hematopoietic progenitors (Fig. 2). In the first report, Krzemien *et al.,* (2007) reinvestigated the col mutant phenotype using MZ and CZ specific markers and showed that, in absence of PSC, there is a global, premature differentiation of all hematopoietic progenitors. This premature differentiation is linked to the loss of JAK/STAT signalling activity in MZ cells, indicating that remote control of JAK/STAT activity by the PSC is a key to the control of hemocyte homeostasis in the *Drosophila* LG. Upon wasp infestation, JAK/STAT signalling is switched off in the entire MZ, allowing massive differentiation of lamellocytes at the expense of the pool of prohemocytes (Makki *et al.,* 2010). Since in absence of PSC, no lamellocytes differentiate after wasp infestation, it indicated that the maintenance of a pool of multipotent progenitors in the MZ is a prerequisite for *Drosophila* larvae to be able to mount a dedicated cellular response against parasites (Crozatier *et al.*, 2004; Krzemien *et al.,* 2007). The Friend of GATA protein U-shaped has now to been shown to act downstream of JAK/STAT signalling to preserve pro-hemocyte potency (Gao *et al.*, 2009). In the second report, (Mandal *et al.,* 2007) used Antp mutants which lack Col expression in the PSC to assess the role of the PSC in 3rd instar larvae. They first showed that PSC cells specifically express Hedgehog (Hh) downstream of Antp, while MZ cells express downstream components of the Hh pathway, the receptor Patched (Ptc) and transcription factor Cubitus interruptus (Ci) (Table 1). As observed with col^1 mutants, a massive differentiation of hemocytes was observed in hh^{ts} mutants or when expression of a dominant-negative form of Ci, the transcriptional effector of the pathway was targeted to the MZ. It was therefore proposed that Hh produced by PSC cells could act at distance in the lymph gland, as it was described in other *Drosophila* tissues (Chuang and Kornberg, 2000; Mandal *et al.,* 2007) to maintain a pool of prohemocytes. Brought together, the observations of Krzemien and Mandal and co-authors suggest that PSC cells could non cell-autonomously control the level of JAK/STAT signalling in the MZ, via the secretion of Hh (Fig. 2). Other possible hypotheses should be considered, however, such as the existence of relay cells which would transform short-range signalling from the PSC into long-range signalling in the entire MZ, similar to the activation of Dpp expression in cells close to the Hh source in the wing disk (review by Lawrence and Struhl, 1996). Very recent evidence from UAS-Gal4 targeted loss and gain of function of components of the

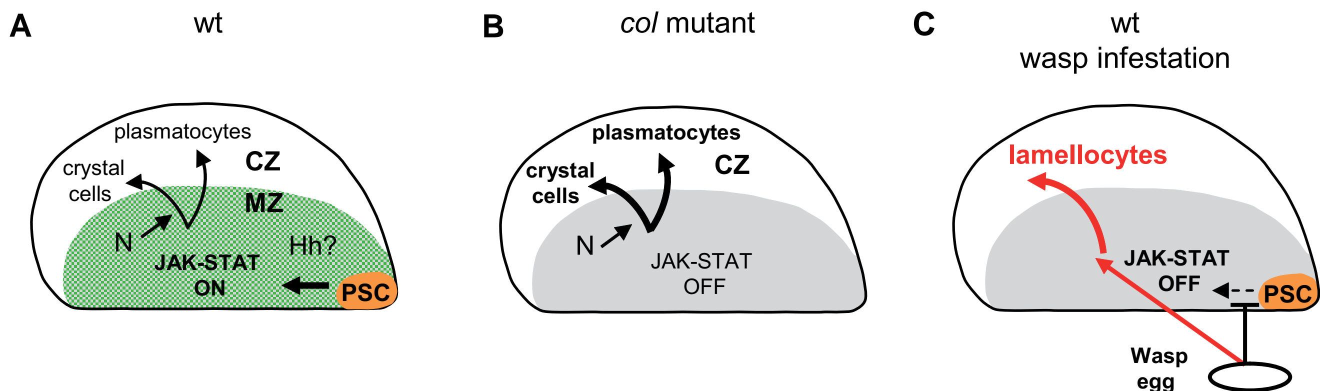

Fig. 2. A model for larval hematopoiesis in the *Drosophila* lymph gland in normal conditions and upon wasp parasitization. **(A)** *Schematic drawing of a LG primary lobe in wt mid-3rd instar larvae, showing the PSC (orange), the medullary zone (MZ) which contains pro-hemocytes (shaded green) and the cortical zone (CZ) which contains differentiating hemocytes (white). During normal development, the PSC acts non-cell autonomously, possibly via Hh signalling, to maintain JAK/STAT signalling in the MZ, thereby maintaining a pool of pro-hemocytes.* **(B)** *In* col *mutant larvae that lack a PSC, there is loss of the MZ and premature differentiation of hemocytes.* **(C)** *Wasp parasitization antagonises the signalling from the PSC to the MZ, leading to down regulation of JAK/STAT signalling. Pro-hemocytes are instructed to differentiate into lamellocytes but the nature of the instructive signal remains unknown. (Krzemien* et al., *2007).*

Wingless (Wg) signalling pathway suggest a dual role of Wg signalling during larval hematopoieisis: maintenance of prohemocytes and maintenance of the PSC (Sinenko *et al.*, 2009). In this context, it is worth emphasizing that PSC cells extend numerous long filopodia, raising the possibility that they could communicate with a specific subset of MZ cells via direct cell-cell contacts (Krzemien *et al.,* 2007). Finally, Owusu-Hansa and Banerjee (Owusu-Ansah and Banerjee, 2009) observed that the pro-hemocyte population in the LG shows significantly increased ROS levels in the last larval stage but not at earlier developmental stages. By monitoring the timing of hemocyte differentiation upon either further increased or reduced level of ROS in the MZ, these authors could conclude that high ROS level in progenitors are an intrinsic factor sensitising these progenitors to differentiation. High ROS levels activate JNK signalling in hematopoietic progenitors, resulting in FoxO activation and down regulation of Polycomb activity which combinatorially can induce the differentiation of all three type of *Drosophila* hemocytes. Signalling via ROS needs now to be connected to other signalling pathways that operate in the hematopoietic progenitors, such as JAK/STAT signalling, in order to get an integrated view of the control of hematopoiesis in different environmental conditions (Vincent and Crozatier, 2010).

The key role of the PSC in maintenance of hematopoietic progenitors and regulation of their differentiation is very reminiscent of the vertebrate hematopoietic stem cell (HSC) niche, a term coined 30 years ago to describe the structural and regulatory micro-environment sustaining long-term renewal of HSC located in the bone marrow. Although the concept of stem cell niche has long been proposed, the cellular and molecular basis for HSC niche activity has remained a complicated issue (see Kiel and Morrison, 2008). One difficulty in drawing detailed parallels between the PSC and vertebrate HSC is that a functional test for an HSC character, that is the ability to give rise to all hematopoietic lineages and restore long–term hematopoiesis in lethally irradiated recipients (Domen and Weissman, 1999; Matsuzaki *et al.*, 2004), is not available in *Drosophila*. Other types of *Drosophila* niches have been well characterized, especially the ovarian and testicular niches which control Germ-line Stem Cell (GSC) maintenance. Communication from the niche to GSCs and reciprocal involves multiple signalling pathways, including the TGFβ, JAK/STAT, Hh and Wnt pathways in the ovary and TGFβ and JAK/STAT in the testis, pointing to the complexity and versatility of stem cells/niche interactions in *Drosophila*. (Fuller and Spradling, 2007). In parallel to GSCs, other types of, tissue-specific, *Drosophila* stem cells have recently been characterized, including neural and intestinal stem cells, bringing to light a diversity of cellular and molecular mechanisms involved in stem cell maintenance (Kohlmaier and Edgar, 2008).

col plays a key role in the PSC but does not seem to be expressed in other known *Drosophila* niches. Col is a member of the family of COE transcription factors which includes mammalian EBF (Early B cell Factor) (Daburon *et al.*, 2008; Dubois and Vincent, 2001). *ebf(1)* was shown to be essential for B-lymphocyte specification and differentiation (Lin and Grosschedl, 1995; Pongubala *et al.*, 2008) but possible links between *col* and *ebf* functions in the immune system have remained scant. Mouse *ebf2* expression in immature osteoblasts which constitute a major component of the HSC niche in the vertebrate bone marrow (Kieslinger *et al.*, 2005; Wilson and Trumpp, 2006) raises, however, the intriguing possibility of functional parallels between the roles of Col and EBF2 in *Drosophila* and vertebrate hematopoiesis, respectively. It further suggests an ancestral role of COE proteins in controlling cellular aspects of metazoan innate immunity.

Hemocyte types and numbers in different insects; plasticty in the cellular immune responses

Drosophila melanogaster has become a major invertebrate model to study developmental and evolutionary aspects of innate immunity, owing to the power of genetic manipulations. But how does the knowledge acquired on fruit flies compare with what has been learnt from studies performed in other insects? Insect hemocytes have traditionally been classified according to morphological, histochemical and functional criteria and only more recently on additional, molecular criteria. Description of hemocytes in diverse insect orders including *Lepidoterae*, *Dipterae*, *Orthopterae*, *Blattariae*, *Coleopterae*, *Hymenopterae*, *Hemipterae* and *Collembola* considered four types of hemocytes: granular cells, plasmatocytes, spherule cells and oenocytoids (Lavine and Strand, 2002; Ribeiro and Brehelin, 2006. Only the spherule cells have not been described in *D. melanogaster. Drosophila* plasmatocytes can be assimilated to granular hemocytes, the professional phagocytes and crystal cells to oenocytoids, the cells that carry phenoloxidase pro-enzymes and play a prominent role in melanization. The lamellocytes are a more complex issue. Ultra-structural and functional characteristics suggest that *D. melanogaster* lamellocytes are equivalent to circulating "plasmatocytes" of other insect orders, described as the cells involved in encapsulation of foreign bodies too large to be engulfed by phagocytosis. However, it is necessary to recall that lamellocyte fate is a cryptic fate in *D. melanogaster*. Another case of cryptic hemocyte fate has been described in the tobacco hornwporm Manducta sexta, that is only revealed following infection (Dean, 2004) and shares morphological properties with *Drosophila* lamellocytes, suggesting that they may be functionally equivalent cells. It is also interesting to recall that the ability to differenciate lamellocytes in response to an immune challenge is not shared by all *Drosophila* species. *Drosophila subobscura* larvae, for example, are unable to differentiate lamellocytes and encapsulate parasitic eggs or other types of foreign bodies (Eslin and Doury, 2006). Parasitoid wasps are a common threat to many insects, but it appears that different parasites of the same host use different infection strategies, including immune-suppressive strategies (Schlenke *et al.*, 2007). The molecular co-evolution of these strategies and the dedicated cellular immune responses displayed by different insect larvae is an entirely new field of investigation.

Concluding remarks

Over the years, our understanding of hematopoiesis in the fruit fly *Drosophila melanogaster* has made considerable progress. Many unsolved questions subsist, however, among which the question of whether hematopoietic cells with *bona fide* stem-cell character do exist in the *Drosophila* lymph gland and survive metamorphosis. Further characterisation of cell lineages in the

lymph gland and the role of the PSC as a hematopoietic niche will certainly provide new insights into the cellular and molecular mechanisms by which niches control the balance between stem cell self-renewal and differentiation. The role of the filopodia extending from PSC cells and the extracellular matrix surrounding the LG and the PSC, in the control of LG growth, and hemocyte differentiation and dispersal at metamorphosis remain to be explored. Finally, studies of hematopoiesis in other insect models, in both normal and immune-challenged conditions will give a broader evolutionary perspective to studies so far mainly performed in *Drosophila*.

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