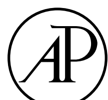
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Blood Cells, Molecules, and Diseases 30 (2003) 223–228

BLOOD CELLS, MOLECULES, & DISEASES

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Transcriptional regulation of hematopoiesis in *Drosophila*

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Submitted 6 January 2003
(Communicated by M. Lichtman, M.D., 13 January 2003)

Abstract

As in mammals, blood cells in *Drosophila* are derived from a common multipotent hematopoietic precursor population. In the embryo, these precursors are derived from the head mesoderm, whereas larval hematopoietic precursors are found in a specialized organ called the lymph gland. This shift in location of hematopoietic differentiation is reminiscent of similar events that occur during mammalian development. Recent analysis has identified several transcriptional regulators in *Drosophila* that influence hematopoietic lineage commitment. Interestingly, many of these factors are similar to factors directing mammalian hematopoietic differentiation. Although *Drosophila* blood cells are much less varied in terms of specific lineages, it would appear that many mechanistic aspects by which hematopoietic cell fate is determined have been conserved between *Drosophila* and mammals. Herein, we describe the *Drosophila* blood cell types, their physical origin, and the transcriptional regulators that govern this process.
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Introduction

Recent investigations have determined that several factors important for hematopoiesis in vertebrates are also functionally conserved in *Drosophila.* In vertebrate hematopoiesis, a common progenitor cell gives rise to all blood cell lineages [1]. Differentiating progenitors then take on either a lymphoid or myeloid cell fate, which then limits lineage commitment to any fate within a particular subgroup. The lymphoid progenitors give rise to B and T lymphocytes, while myeloid progenitors give rise to many cell types including erythrocytes and monocytes [1]. Although distinct, *Drosophila* blood cells most closely resemble cells of the vertebrate myeloid lineage, particularly monocyte/macrophages and granulocytes. This distinction is based upon both morphological and functional similarities, including phagocytic and immune-related activities. As will be described further below, *Drosophila* blood cells are similar to those of vertebrates not only in terms of functionality but also in terms of a genetic hierarchy directing hematopoietic differentiation. This review describes the *Drosophila* blood cell lineages and what is known about the transcriptional regulators that lead to their specification and differentiation.

Hemocyte cell types

In *Drosophila,* blood cells are referred to as hemocytes, of which there are at least three terminally differentiated types: plasmatocytes, crystal cells, and lamellocytes. Plasmatocytes are the predominant cell type at all developmental stages and represent approximately 95% of all hemocytes, with crystal cells making up the majority of the remainder [2]. Lamellocytes are produced in very small numbers, if at all, under normal conditions, but can be induced in large numbers under conditions of immune challenge [3,4]. Unlike plasmatocytes and crystal cells, lamellocytes have not been observed in embryos or adult flies. Other cell types, such as the podocyte, have also been described [5]; however, it is unclear whether these cells represent distinct lineages or rather differentiating forms of one of the three classes described above.

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doi:10.1016/S1079-9796(03)00028-7

Plasmatocytes, which are relatively small, rounded cells, primarily function as professional phagocytes and are often referred to as macrophages [6]. As macrophage-like cells, plasmatocytes engulf and degrade dead cells and debris, as well as invading pathogens such as bacteria. The ability of plasmatocytes to recognize and engulf apoptotic cells is mediated by the cell-surface receptor Croquemort (Crq), which is a member of the CD36 family of scavenger receptors [7,8]. Because phagocytic capacity is a characteristic of mature plasmatocytes [6], it has been suggested that Crq expression may mark the terminal transition between immature plasmatocytes and macrophages [7]. Phagocytosis also allows macrophages to play a critical role in innate immune responses by engulfing and destroying foreign invaders [9]. In addition to this cellular immunity, macrophages also participate in humoral immune responses by synthesizing and secreting antimicrobial peptides [10,11]. Furthermore, macrophages secrete proteins composing the extracellular matrix (ECM)[12], which contributes to the establishment of basement membranes that are important for various developmental processes [13].

Crystal cells are somewhat larger in size than plasmatocytes and derive their name from the paracrystalline inclusions they contain. Crystal cells, which are nonphagocytic, function in the process of melanization, which facilitates innate immune and wound-healing responses [14,15]. The production of melanin begins through activation of a serine protease cascade that converts the enzyme prophenoloxidase to phenoloxidase [14]. Phenoloxidase then catalyzes the oxidation of phenols to quinones that then nonenzymatically polymerize into melanin. Melanin and many of its intermediate compounds and side products (such as hydrogen peroxide) are directly toxic to microorganisms [16]. It is believed that the crystal cell inclusions consist of mass quantities of one or more components of the prophenoloxidase enzymatic cascade, which can be mobilized upon demand. Interestingly, ultrastructural analysis of crystal cells has shown that a membrane does not surround the inclusions [3,5], indicating that these components are not actively secreted. Furthermore, it suggests that lysis of crystal cells may be a prerequisite for proximal exposure of melanization products to their targets.

In addition to plasmatocytes and crystal cells, *Drosophila* produces a third blood cell type called a lamellocyte, which functions during cellular immune responses by encapsulating and neutralizing objects too large to be engulfed by macrophages [17]. Accordingly, lamellocytes are large, flat, adherent cells that form melanized barriers around foreign objects in conjunction with crystal cells and possibly plasmatocytes. An example of this can be observed upon egg-capsule deposition by the wasp *Leptopilina boulardi,* which parasitizes *Drosophila* larvae [3,4,17]. Interestingly, very few lamellocytes are normally observed in larvae; however, upon parasitization, the differentiation of lamellocytes is rapidly induced [3,4]. Subsequently, lamellocytes adhere to and surround the egg capsule, which begins to melanize, thereby walling it off inside the larvae. Interestingly, this response can be recapitulated with pieces of human hair [3], indicating that the lamellocyte-inducing signal is general and probably linked to object size. How signals directing the differentiation of lamellocytes are communicated from the site of infestation to precursor cells is not known.

Origins of *Drosophila* hemocytes

In the embryo, both plasmatocytes and crystal cells are derived from a precursor population located in the procephalic mesoderm, first observable in around stage 5 [6,18]. The hematopoietic precursors (prohemocytes) express the GATA transcription factor Serpent (Srp), which is required for hematopoiesis in *Drosophila* [18,19]. As these cells begin to differentiate, plasmatocyte precursors initiate expression of the transcription factor Gcm (*glial cells missing*) [20] and begin to migrate throughout the embryo. Crystal cell precursors (CCPs) cluster near the proventriculus and begin to express the transcription factor Lozenge (Lz), which is essential for their differentiation into crystal cells [19].

Embryonic hematopoiesis gives rise to a limited number of hemocytes that do not persist into late larval stages [3,19]. Consequently, the vast majority of hemocytes observed in circulation are derived from a secondary wave of hematopoiesis that occurs during larval stages. As described below, this hematopoietic wave occurs in a spatially distinct site compared to the embryo. Interestingly, this reinitiation and relocation of hematopoiesis in *Drosophila* is similar to what occurs during mammalian development [21]. It is estimated that embryonic hematopoiesis gives rise to approximately 800 macrophages (V. Hartenstein, personal communication); however, the number of all embryonic hemocytes found in first instar larvae is estimated to be less that 200 per animal, whereas greater than 5000 hemocytes are observable by the onset of pupariation [3]. In contrast to the embryo, larval blood cell development occurs in a specialized organ called the lymph gland (LG), which is formed from the dorsal mesoderm during embryogenesis [19]. Although variable, the LG generally consists of 4–6 pairs of cell clusters or "lobes" that flank the dorsal vessel near the anterior abdominal segments. The LG lobes decrease in size from anterior to posterior and consist of hemocytes surrounded by a layer of extracellular matrix that is attached to the dorsal vessel, which functions as a primitive heart/aorta to move hemolymph throughout the larvae and adult. The large, anterior-most LG lobe is the primary site of larval hematopoiesis. A recent ultrastructural analysis has revealed that prohemocytes, in both primary (anterior-most) and secondary LG lobes, appear as cells 4–6 μm in diameter with a relatively high nucleo-cytoplasmic ratio that also contain lipid droplets and many free ribosomes. By these criteria, the vast majority of cells observed in the anterior

lobes during the second larval instar are prohemocytes. In contrast, by the third larval instar the anterior lobes contain only a minor population of prohemocytes, with the majority of cells differentiating into plasmatocytes and crystal cells. A fourth cell type was also observed that exhibited extensive rough endoplasmic reticulum and Golgi vesicles and was accordingly referred to as a secretory cell [3]. Secretory cells are particularly abundant near the periphery of the LG lobes in mid-third-instar larvae; however, what function these cells might have or to what lineage they might belong has yet to be determined.

As in embryonic prohemocytes, all LG prohemocytes express Srp. Lineage differentiation is first observed during the second larval instar when a small number of prohemocytes in the anterior lobes begin to express Lz, eventually reaching 50 to 100 cells per lobe during the third larval instar [19]. These Lz^{+} cells, which define crystal cell precursors, are uniformly distributed throughout the anterior lobes, while very few of the secondary-lobe cells express Lz. At some unknown stage of differentiation, hemocytes become competent to leave the LG and enter circulation, presumably by crossing the surrounding ECM layer [3]. When this can first occur is unknown, although it is clear that exiting the LG is not required for differentiation since both mature plasmatocytes and crystal cells can be observed in the anterior lobes of wandering third instar larvae.

It has been suggested that hemocytes found in adult flies are of larval hematopoietic origin. The larval lymph gland does not perdure into the adult nor give rise to an adult-specific hematopoietic organ, but rather it expels its cellular content and degenerates during metamorphosis [3]. Furthermore, no adult hematopoietic tissue has, so far, been found and adult hemocytes have not been observed to be actively dividing. Macrophages are clearly present in adults (observable by injection and subsequent phagocytosis of India ink), although they are not uniformly distributed throughout the adult but rather tend to accumulate in various locations such as the legs, halteres, and near the dorsal vessel [3,22]. Crystal cells are also present and are readily made visible by the *Black cells* mutation, which causes premature melanization within the cells [23]. As mentioned above, lamellocytes have not been observed in adults, even after immune challenge [3].

Transcriptional regulation during hematopoiesis

The transcription factor Serpent (Srp) is expressed in all hemocyte precursors and is required for hematopoietic development [18,19]. Serpent is a member (one of five in *Drosophila*) of the highly conserved GATA family of zinc-finger transcription factors that recognize the consensus DNA binding site WGATAR [24]. All vertebrate GATA factors contain two zinc finger motifs of the Cys-X2-Cys-X17-Cys-X2-Cys type separated by 29 amino acids; the amino-proximal zinc finger is often referred to as the "N-finger" while the carboxy-proximal zinc finger is the "C-finger." The C-finger principally mediates DNA binding while the N-finger can modulate the selection of particular DNA binding sites. Additionally, both zinc fingers can interact with other transcription factors to influence gene expression. In mice, an erythroid-specific requirement for the N-finger of GATA-1 has been described and mutation of the N-finger of the *Drosophila* GATA factor Pannier/dGATAa leads to defects in sensory bristle formation [25,26]. Interestingly, many invertebrates appear to encode GATA family homologs that only contain C-finger motifs. Until recently, Srp was described as just such a factor.

An analysis of *srp* transcripts has revealed that alternative splicing can give rise to Srp isoforms containing both an N-finger and a C-finger separated by a conserved 29-amino-acid spacing (SrpNC) [27]. Furthermore, as in vertebrates, SrpNC was shown to enhance DNA binding compared to SrpC (C-finger only) as well as to mediate specific interaction with the *Drosophila* Friend-of-GATA homolog U-shaped (Ush) [28]. Both isoforms are expressed in the same pattern and both are capable of supporting hematopoiesis independently; however, misexpression of either isoform in CCPs leads to a reduction in crystal cell number [27]. This reduction was independent of Ush function, which had previously been shown to reduce crystal cell numbers when expressed in CCPs [29]. Additionally, the expression of the gene *prophenoloxidase* (a mature crystal cell marker) was lost in the remaining crystal cells, suggesting that, in addition to negatively influencing crystal cell numbers, *srp* expression negatively influences commitment to the crystal cell lineage. This interpretation is consistent with the previous observation that cells taking on a crystal cell fate (i.e., that begin to express *lozenge*) downregulate *srp* expression (see below) [19].

The transcription factor Lozenge, a member of the "Runt domain" family of transcription factors and known regulator of cell fate specification in the eye [30,31], is required for crystal cell development [19,32]. In *Drosophila,* Runt-domain transcription factors form heterodimeric complexes with the partner proteins Brother (Bro) or Big Brother (Bgb) [33]. The Runt domain is a conserved 128-amino-acid region that binds DNA and helps mediate partner protein interaction. The partner proteins themselves have no intrinsic DNA binding capacity but rather enhance the DNA binding capacity of Runt domain proteins. Interestingly, the Lozenge Runt domain exhibits 71% identity with that encoded by the mammalian AML-1 gene [19], the most frequent target of chromosomal translocations leading to acute myeloid leukemia [34]. In mammals, AML-1 forms a complex with CBFβ, a protein homologous to Bro and Bgb [35]. Importantly, mutations in both AML-1 and CBFβ have been shown to lead to a loss of definitive hematopoiesis [36].

Loss-of-function mutations in *lozenge* (*lz*) have been shown to lead to a loss of the crystal cell lineage. During hematopoiesis, a subset of Srp^{+} prohemocytes begins to

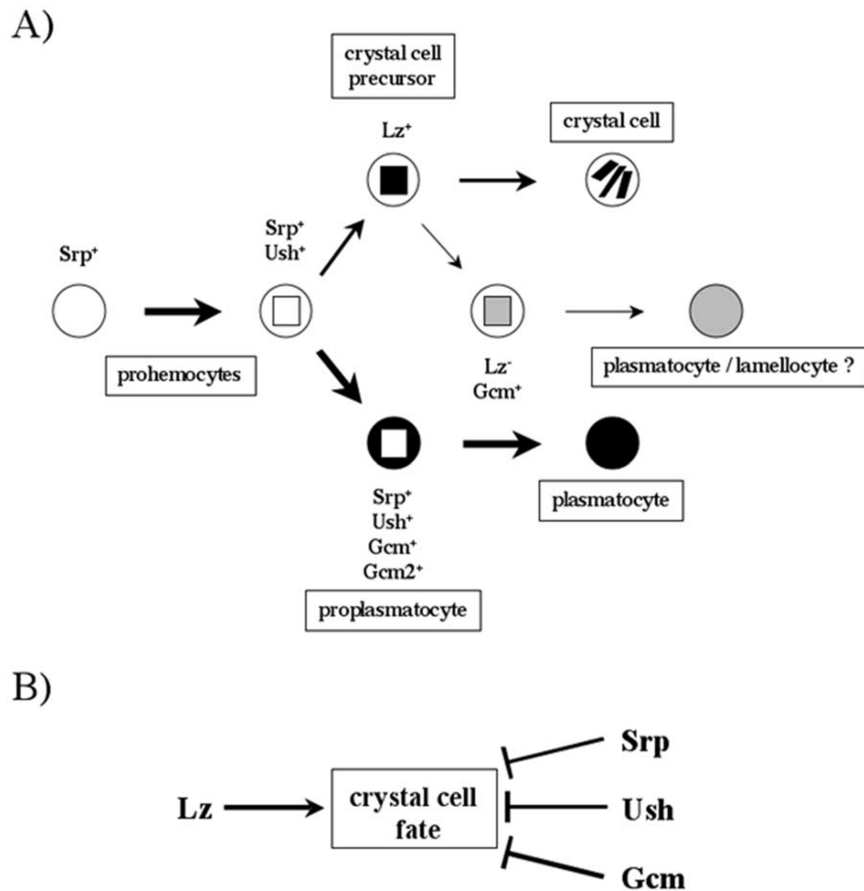

Fig. 1. Transcriptional regulation of hematopoiesis in *Drosophila.* (A) Prohemocytes express the GATA factor Serpent (Srp), the earliest known marker of hematopoietic fate. This is followed by expression of the FOG homolog U-shaped (Ush). A small subset of cells begins to express Lozenge (Lz), a Runt-domain transcription factor similar to the mammalian hematopoietic factor AML-1, while downregulating Srp and Ush. These cells are referred to as crystal cell precursors, which eventually become mature crystal cells. The vast majority of prohemocytes maintain Srp and Ush expression and begin to express the transcription factors Gcm and Gcm2. These cells compose the proplasmatocyte pool, which eventually become plasmatocytes. A small fraction of cells with plasmatocyte-like characteristics is derived from Lz-positive precursors, which turn off Lz expression and turn on Gcm expression. It is not known whether these cells are equivalent to plasmatocytes derived from the normal pathway or whether they represent a novel plasmatocyte-like lineage or possibly a lineage that gives rise to lamellocytes. (B) A schematic of factors influencing crystal cell fate. Induction of Lz expression is required for the establishment of the crystal cell lineage, whereas Srp, Ush, and Gcm repress crystal cell fate determination.

express Lz while simultaneously downregulating Srp. An allele of *srp* (*srp*^{neo45}) that specifically abolishes Srp expression in embryonic prohemocytes also blocks Lz expression, indicating that Srp is required to initiate Lz expression [19]. The majority of Lz$^+$ cells continue to differentiate and eventually become Lz$^+$ crystal cells, while a small population of Lz$^+$ cells turns off *lz* expression and appears to take on a macrophage-like cell fate. What influences these cells to downregulate *lz* is not known.

Cells entering the plasmatocyte lineage from the Srp$^+$ prohemocyte pool begin to express the transcription factor Gcm (*glial cells missing*) [20], which is also the primary regulator of glial cell differentiation in the nervous system [37,38]. Gcm expression is first detectable in proplasmatocytes at the end of the blastoderm stage and begins to disappear after embryonic stage 11 [20]. Gcm expression is never observed in CCPs [19]. Consistent with its role in establishing plasmatocyte cell fate, early misexpression of *gcm* leads to an increase in the number of cells expressing the macrophage maker Peroxidasin [20]. Furthermore, ectopic expression of Gcm in the CCPs, in both the embryo and the larvae, leads to the adoption of plasmatocyte characteristics including the eventual expression of Crq [19], indicating that Gcm can override Lz-mediated crystal cell fate determination. The reverse is not true, however, because expression of Lz in all prohemocytes did not inhibit the establishment of the plasmatocyte lineage or increase the number of crystal cells [19].

Although Gcm is clearly important in regulating the plasmatocyte lineage, embryos mutant for *gcm* show only a reduction in the number of plasmatocytes (approximately 30%) [20], indicating that factors other than Gcm also play a role. One such factor is the *gcm* homolog *gcm2* [39,40]. Like *gcm, gcm2* is expressed in early prohemocytes until stage 11, although at much lower levels. Deletion of *gcm2* resulted in a reduction in plasmatocyte number by approx-

imately 25% [40]. Deletion of both *gcm* and *gcm2* simultaneously did not result in a complete loss of plasmatocytes, but only a reduction to about 40%. The remaining plasmatocytes had aberrant morphologies but still expressed Peroxidasin, although none were observed to express Crq. Consistent with a Gcm-independent mechanism of crystal cell specification, the *gcm*/*gcm2* double deletion did not reduce the number or position of crystal cells. The presence of plasmatocyte-like cells in *gcm*/*gcm2* double mutants suggests that other factors initially function to specify this lineage. Accordingly, it is proposed that Gcm and Gcm2 have overlapping roles in the early expansion of the prespecified plasmatocyte lineage as well as in the transition from a proplasmatocyte stage into mature macrophages [40]. The possibility also exists that plasmatocytes are the "default" hematopoietic cell lineage that only requires factors like Gcm to modulate its characteristics. This possibility is supported by the fact that plasmatocytes maintain the expression of Srp and Ush observed in prohemocytes, while crystal cells must downregulate these genes in addition to upregulating Lz.

The *Drosophila* FOG protein U-shaped can directly interact with the N-terminal zinc finger of the hematopoietic GATA factor Srp [27]. Expression of Ush in *Drosophila* embryonic hemocyte precursors, which is dependent upon Srp expression, is first observable during stage 8. Ush expression is subsequently maintained in plasmatocytes but is downregulated in CCPs and is eventually lost in mature crystal cells. Loss-of-function mutations in *ush* lead to an expansion of the crystal cell population by approximately 30%, while misexpression of Ush in CCPs results in a loss of mature crystal cells [29]. Together, this indicates that Ush normally functions to repress crystal cell fate. Such a function is consistent with FOG-mediated lineage repression observed in vertebrates [41,42]. The inhibitory activity of Ush may be mediated by its conserved PXDL motif. This motif has been shown to mediate interaction between murine FOG-2 and the transcription repressor C-terminal binding protein (CtBF) in vitro. In support of a conserved mechanism of Ush function, expression of FOG-2 in CCPs of transgenic flies also resulted in a loss of mature crystal cells, while mutation of the PXDL motif abrogated this loss [29].

In summary, the *Drosophila* blood cell types and the known transcriptional regulators (see Fig. 1) governing hematopoiesis in *Drosophila* have many similar components in the vertebrate system. Several facets of *Drosophila* hematopoiesis remain unknown, including what regulates the activity of the known transcriptional regulators described above. Although not touched upon here, the JAK/STAT and Toll/Cactus (NF-κB) signal transduction pathways have previously been implicated in *Drosophila* hematopoiesis [43–45]. This is particularly interesting in light of the fact that hyperactivation of these pathways results in hyperproliferation phenotypes that appear similar to leukemic conditions observed in mammals [46]. In addition to proliferation control, signal transduction pathways must influence the decisions of precursor cells to take on particular fates. Along this line, it has been observed that Notch, functioning in conjunction with the ligand Serrate, regulates the crystal cell lineage by modulating Lz expression in CCPs [47].

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