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# There and back again: The mechanisms of differentiation and transdifferentiation in *Drosophila* blood cells

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ABSTRACT

Transdifferentiation is a conversion of an already differentiated cell type into another cell type without the involvement of stem cells. This transition is well described in the case of vertebrate immune cells, as well as in *Drosophila melanogaster*, which therefore serves as a suitable model to study the process in detail. In the *Drosophila* larva, the latest single-cell sequencing methods enabled the clusterization of the phagocytic blood cells, the plasmatocytes, which are capable of transdifferentiation into encapsulating cells, the lamellocytes. Here we summarize the available data of the past years on the plasmatocyte-lamellocyte transition, and make an attempt to harmonize them with transcriptome-based blood cell clustering to better understand the underlying mechanisms of transdifferentiation in *Drosophila*, and in general.

## 1. *Drosophila* immune cells and hematopoiesis – an overview

The key to survive – both in the case of vertebrates and invertebrates – is a functional and dynamic immune system that can counteract parasites and pathogens. The immunity of *Drosophila melanogaster* consists of cellular and humoral defenses, which are built upon evolutionary conserved mechanisms, such as pattern recognition, immune cascades and the production of antimicrobial peptides. These traits make the fruit fly a perfect candidate for the investigation of innate immunity.

The cellular arm of the immune system employs specialized immune cell types responsible for distinct defense mechanisms. These effector cells originate from progenitors via a process of differentiation controlled by both intrinsic and extrinsic cues. In *D. melanogaster*, the immune cells are collectively called hemocytes, and correspond to the vertebrate myeloid blood cell lineage (Hultmark, 1994; Akira et al., 2006; Kleino and Silverman, 2014). In the larva, three hemocyte types can be distinguished: the plasmatocytes, the crystal cells and the lamellocytes (Rizki and Rizki, 1980; Brehélin, 1982). Plasmatocytes represent the bulk of the hemocyte population in the naive larva. These cells phagocytose microorganisms and cell debris, secrete signal molecules, antimicrobial peptides and extracellular matrix proteins similarly to mammalian macrophages, neutrophils or monocytes (Brehélin, 1982; Yasothornsrikul et al., 1997; Basset et al., 2000; Sears et al., 2003; Ferrandon et al., 2004; Baer et al., 2010; Gold and Brückner, 2015).

In early studies of *Drosophila* blood cells, the plasmatocytes were defined according to their morphological features and phagocytic function (Rizki and Rizki, 1980). Later, specific molecular markers, such as antibodies (NimrodC1) and *in vivo* transgenic reporters (*eater-GAL4*, *pxn-GAL4*) were identified and used for the definition of plasmatocytes (Kurucz et al., 2003, 2007a, 2007b; Márkus et al., 2009; Tokusumi et al., 2009; Stramer et al., 2005). However, these markers did not allow for the detailed characterization of plasmatocyte subsets. The most modern genetic and molecular techniques, such as *in vivo* lineage tracing and single-cell RNA sequencing (scRNA-seq) studies revealed that plasmatocytes represent a diverse group of hemocytes, comprising 10–15 different functional clusters (Cattenoz et al., 2020; Fu et al., 2020; Tattikota et al., 2020). Due to this controversy, referring to plasmatocytes does not always allow the distinction between mature effector phagocytic cells and partially differentiated plasmatocytes with the capacity to transform into other cell types. The harmonization of the latest scRNA-seq data and the development of novel, cluster specific markers would help to correlate plasmatocyte subsets to various functions exerted by this diverse cell type.

The remainder of the larval circulation comprises crystal cells that contain prophenoloxidase (PPO) crystals in their cytoplasm, which, upon wound healing and parasitic attack, are released and activate the

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melanization cascade (Rizki and Rizki, 1959; Nappi et al., 1995). The third cell type, the lamellocytes are activated immune cells; they differentiate only upon immune induction *e.g.* following parasitic wasp infestation (Lanot et al., 2001; Honti et al., 2009; Gold and Brückner, 2015). Together with the crystal cells and the plasmatocytes, they form a tight capsule around the intruders inside the body cavity and separate it from the internal organs (Nappi, 1973; Holz et al., 2003; Honti et al., 2010, 2014; Dudzic et al., 2015). Eventually, the capsule undergoes melanization, which eliminates the parasite. It was also demonstrated that lamellocytes arising upon immune induction do not only differentiate from undifferentiated prohemocytes, but also transdifferentiate from another effector cell type, the plasmatocytes. During this process, morphological, transcriptional and functional changes occur, which were extensively analyzed by monitoring marker expression changes and transcriptomic changes on the single cell level (Honti et al., 2010; Anderl et al., 2016; Cattenoz et al., 2020; Fu et al., 2020; Tattikota et al., 2020).

Similarly to mammals, hematopoiesis in *Drosophila* starts early in the embryonic development, and takes place in immune compartments in multiple waves. Differentiation of blood cells is controlled by conserved epigenetic and transcription factors (Lebestky et al., 2000; Evans et al., 2003; Holz et al., 2003). The first wave of embryonic hemocytes differentiates from the procephalic mesoderm and gives rise to the embryonic macrophages and crystal cells (Tepass et al., 1994). The macrophages are highly motile cells, which undergo a directed migratory program during embryonic development (Holz et al., 2003; Brückner et al., 2004; Wood et al., 2006; Siekhaus et al., 2010; Parsons and Foley, 2013). They are responsible for the clearance of apoptotic cells, the deposition of ECM-related molecules and the proper development of the nervous system (Nelson et al., 1994; Yasothornsrikul et al., 1997; Zheng et al., 2017). The embryonic crystal cells colonize in two groups close to the anterior part of the midgut, but whether they play a functional role at this developmental state remains unknown (Franc et al., 1996; Lebestky et al., 2000; Kurant et al., 2008; Evans et al., 2010; Nagaosa et al., 2011; Fujita et al., 2012; Koranteng et al., 2020).

The second hematopoietic wave takes place during the larval stages, where hemocytes are located in three immune compartments: the lymph gland, the circulation and the sessile compartment. The lymph gland is situated along the anterior end of the dorsal vessel and consists of paired primary and secondary lobes (Roehrborn, 1961; Shrestha and Gateff, 1982). The primary lobes are organized into three functionally distinct zones. At the posterior base, a dedicated group of cells called Posterior Signaling Center (PSC) is responsible for the maintenance of lymph gland hematopoiesis. The medullary zone (MZ) contains precursor cells, which - according to their position and gene expression pattern - can be separated to core progenitors (the innermost region of the MZ) and distal progenitors (outer MZ region) (Blanco-Obregon et al., 2020). As primary lobe hemocytes complete their differentiation, they populate the cortical zone (CZ), where the mature plasmatocytes and crystal cells are located (Lanot et al., 2001; Sorrentino et al., 2002; Crozatier et al., 2004; Jung et al., 2005; Mandal et al., 2007; Grigorian et al., 2011). At the border of the medullary and cortical zones lies the intermediate progenitor zone, comprising progenitors, immature plasmatocytes and crystal cells (Krzemien et al., 2010).

The sessile compartment is the segmentally repeating pattern of hemocytes adhered to the epidermis in a dorsal patch and lateral islets on both sides of the larva in innervated epidermal-muscular pockets (Makhijani et al., 2011). Under normal conditions, the lymph gland and the sessile islets remain intact until the end of the larval stage, while in response to infection or injury, these release hemocytes into the circulation, thereby contributing to the lamellocyte pool (Márkus et al., 2009; Owusu-Ansah and Banerjee, 2009; Honti et al., 2010, 2014; Dragojlovic-Munther and Martinez-Agosto, 2012).

In this review, we summarize the current knowledge on the blood cell differentiation in *Drosophila*, shedding light on novel differentiation routes of hemocytes based on lineage tracing and single cell transcriptional changes with a perspective on the vertebrate hematopoiesis.

## 2. The regulatory mechanisms of the lymph gland niche

The lymph gland is the most well studied model of *Drosophila* hematopoiesis, which yielded extensive details on how hemocyte differentiation is regulated on the transcriptional level (reviewed in Fosset, 2013; Banerjee et al., 2019). However, these studies also highlighted non-cell autonomous mechanisms consisting of sensing and interpreting external signals and hemocyte-hemocyte interactions, which serve as an additional layer of regulation.

The posterior-most region of the primary lymph gland lobes is occupied by a set of 30–40 specialized cells together known as the posterior signaling center (PSC), which provides molecular cues to the MZ progenitors (Mandal et al., 2007; Krzemien et al., 2007). To maintain its function of keeping the equilibrium between progenitor maintenance and differentiation, the number of PSC cells is tightly regulated (Mandal et al., 2007; Tokusumi et al., 2012; Pennetier et al., 2012; Lam et al., 2014; Hao and Jin, 2017). Furthermore, the integrity of the PSC depends on its interaction with the cardiac tube, which is mediated through Slit-Robo signaling (Morin-Poulard et al., 2016) (Fig. 1A).

Microscopic analysis showed that the PSC cells extend long filopodia (cytonemes) into the MZ, which could be potential avenues of transferring short range signals (Mandal et al., 2007). In fact, in the ovarian stem cell niche of *Drosophila*, similar filopodia emanated by the cap cells provide Hedgehog signals to escort cells (Rojas-Ríos et al., 2012). Furthermore, in the wing imaginal disc epithelium, cells from the anterior compartment extend basal filopodia towards the posterior half, which are also implicated in receiving Hedgehog signals and establishing a morphogen gradient in the tissue (Bischoff et al., 2013; Chen et al., 2017). While functional evidence for cytoneme-dependent signal transfer in the PSC remains elusive, it has been established that this group of cells provides additional molecular cues to the progenitor cells besides Hedgehog, such as Serrate and Decapentaplegic (Lebestky et al., 2003; Dey et al., 2016; Blanco-Obregon et al., 2020). Importantly, the signaling center also regulates progenitor maintenance by producing the PDGF- and VEGF-Receptor Related (Pvr) ligand Pvf1 to CZ hemocytes, which in turn secrete the Adenosine-dependent growth factor Adgf-A that modulates the levels of adenosine in the MZ (Mondal et al., 2011) (Fig. 1A).

The MZ progenitor cells are small in size compared to differentiated effector hemocytes either in the lymph gland or the circulation, tightly packed and strongly express E-cadherin (Jung et al., 2005; Gao et al., 2013). These features suggest active cell-cell contact via adherens junctions, which in turn can maintain a three-dimensional structure where cells proliferating in the inner layers can “push” cells toward the CZ, which can then initiate the plasmatocyte or crystal cell differentiation program. Further evidence also shows that septate junction components and polarity determinants, such as Scribble and Discs large 1 regulate the cell fate choice of hemocytes in the MZ through a Yorkie-dependent mechanism (Khadilkar and Tanentzapf, 2019). In addition to cell-cell contacts through Cadherin-mediated homophilic interactions, the specific extracellular matrix (ECM) composition in the MZ also influences progenitor behavior. The presence of the heparan-sulfate proteoglycan Trol has been demonstrated to be instrumental for hematopoiesis and the maintenance of undifferentiated state in these cells, which suggests that specific receptors on the progenitor cell surface interact with the ECM and through a currently unknown signaling mechanism, repress differentiation (Grigorian et al., 2013). Similarly, the ECM protein Tiggrin suppresses precocious differentiation of effector cells, while further increasing its levels in the cortical zone stalls the hemocytes in a partially differentiated state (Zhang and Cadigan, 2017). Interestingly, the modulation of immune cell differentiation by niche ECM composition, structure and stiffness has also been observed in mammalian hematopoietic stem cell (HSC) niches (Domingues et al., 2017).

Furthermore, some MZ cells contain high levels of Reactive Oxygen Species (ROS), which, if increased further locally, cause the differentiation of MZ cells and loss of progenitors, while if inhibited, result in the absence of differentiated effector hemocytes in the lymph gland through

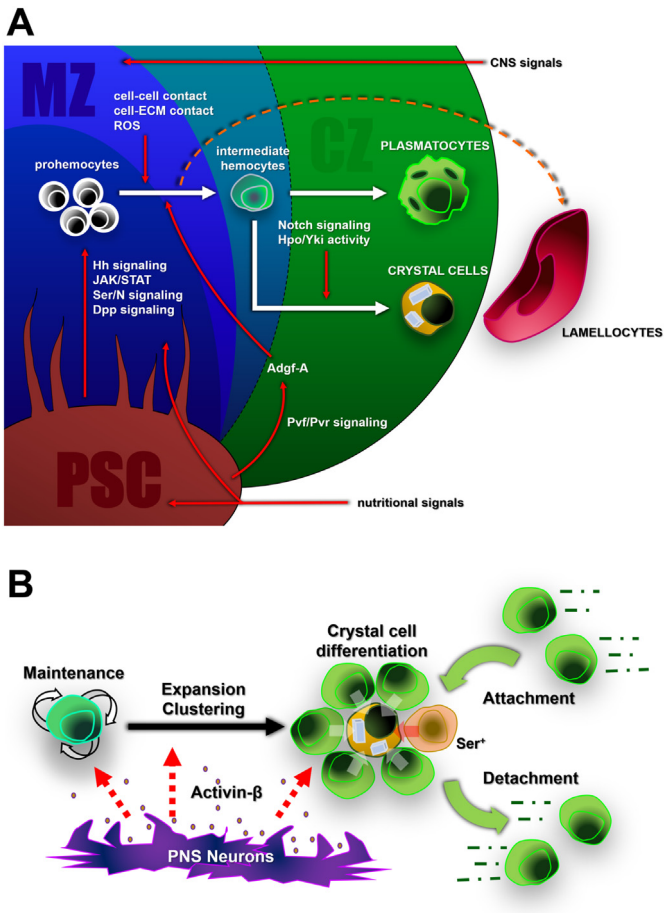


**Fig. 1.** Regulatory mechanisms of hematopoiesis in the lymph gland and in the sessile compartment. A) The compact structure of the lymph gland enables tight regulation of hemocyte differentiation. The posterior signaling center (PSC), the medullary zone (MZ) and the cortical zone (CZ) of the organ are interconnected via several regulatory pathways mediated by Hh, Notch, JAK/STAT and Pvf/Pvr. Importantly, the MZ of the lymph gland contains prohemocytes, which can differentiate either into plasmatocytes or crystal cells in the CZ. The direction of this differentiation is regulated in intermediate hemocytes by Notch signaling. In case of immune induction, lamellocyte differentiation is initiated. The PSC is indicated in red, the MZ in blue (core progenitors: dark blue, distal progenitors: light blue), the intermediate zone in teal, and the cortical zone in green. Red arrows indicate signaling pathways, white arrows show lineage relationships, the orange dashed arrow indicates differentiation following parasitization. B) The sessile compartment is in dynamic steady-state with the circulation; hemocytes are constantly exchanged between the two compartments. By secreting Activin-β, peripheral neurons regulate the expansion of the sessile islets and the homing of hemocytes from the circulation into the sessile hematopoietic pockets. The sessile islets contain no dedicated precursor cells, therefore here plasmatocytes transdifferentiate into other cell types, such as crystal cells. This transdifferentiation is directed by signals arriving from neurons and surrounding plasmatocytes.

a combination of Jun N-terminal kinase (JNK) pathway activation and Serpent (Srp) dependent gene expression (Owusu-Ansah and Banerjee, 2009; Gao et al., 2014). Curiously, this mechanism also reduces E-cadherin levels in the progenitors, which implies that the impairment of the previously described cell-to-cell contacts may also contribute to failed hemocyte differentiation observed in these cases.

The CZ hemocytes represent effector cells in the lymph gland, and are mostly composed of plasmatocytes and - to lesser extent - crystal cells at various stages of differentiation. Throughout larval development, this population of cells gradually becomes the dominant zone in the primary lobes due to the constant differentiation of MZ cells. Even though the bulk of CZ cells represents differentiated effector cells, they still partake in controlling the MZ progenitor activity by regulating the expression of Adgf-A, which decreases adenosine levels in the MZ and maintains the cells in a quiescent state (Mondal et al., 2011, 2014) (Fig. 1A).

It is apparent that the intricate control of lymph gland hematopoiesis relies on a multitude of mechanisms, which can account for several physiological and pathological cues, and is executed via a combination of cell-cell contact dependent signaling, distribution of soluble factors and cell-ECM contacts.

With the recent single cell sequencing of naive and immune induced lymph glands, several new subclasses of lymph gland hemocytes were uncovered (Cho et al., 2020). According to this study, the lymph gland not only comprises PSC cells, prohemocytes and the three canonical effector cell types (plasmatocytes, crystal cells and lamellocytes), but also GST-rich cells with active ROS-response gene expression program and adipohemocytes with enhanced lipid metabolic capacity. Furthermore, the prohemocytes were separated into 6 sub-clusters, which according to their gene expression patterns, represent hemocytes in various stages of differentiation towards mature plasmatocyte, crystal cell or lamellocyte fate. Importantly, the single-cell resolution and correlation of marker expression patterns provided positional information on these cells, revealing a gradient-like distribution of hemocytes from undifferentiated progenitors to fully differentiated effector cells in the primary lobes (Cho et al., 2020). Comparing naive and parasitized lymph glands also showed a significant change in cellular composition, hallmarked by the appearance of lamellocytes which primarily differentiated from the iPH-4 sub-cluster of prohemocytes and the decline of the iPH-1 and -2 sub-clusters, representing the least-differentiated groups of hemocytes in the MZ (Cho et al., 2020).

These results refine our classical model of lymph gland hematopoiesis, and provide additional insight into how the lymph gland alters its hemocyte differentiation program upon immune challenge.

## 3. The sessile hematopoietic compartment and the circulating hemocytes

While there is an abundance of data available on hematopoietic regulation in the lymph gland, much less is known about how immune cell differentiation is orchestrated on the periphery. This is partly due to the lymph gland being a discrete, centralized hematopoietic organ, while the sessile compartment was considered a repetitive accumulation of cells along the body wall, which is able to exchange cells with the circulation. Recently it has been established that the organizing principle of the sessile compartment very strongly depends on peripheral sensory neurons (Makhijani et al., 2011) (Fig. 1B). These neurons are usually present near the center of sessile clusters, and secrete Activin-β to attract the hemocytes and locally stimulate their proliferation. Absence of these cells or loss of Activin-β activity causes disruption of the sessile pattern and elevated circulating hemocyte numbers (Makhijani et al., 2011, 2017). In line with the notion that Activin-β serves as a homing signal, when hemocytes are dislocated from the sessile compartment via mechanical agitation, the banded pattern of hemocytes is restored after a short recovery period (Makhijani et al., 2011).

Notably however, the organization of the sessile compartment is not only controlled by local neuronal signals. Activation of Toll signaling in the larval adipose tissue (fat body) was found to drastically disrupt the sessile hemocyte pattern, and the secreted protein Edin, also produced by the fat body, was shown to induce the detachment of sessile hemocytes following parasitoid wasp infestation (Schmid et al., 2014; Vanha-Aho et al., 2015). In addition, the adipokine Nimrod B5 is secreted upon nutrient shortage from the adipose tissue, and aside from its metabolic function, it also curbs hemocyte proliferation and attachment in the sessile compartment (Ramond et al., 2020). Since a subset of hemocytes can also impact organismal growth and metabolism by activating the JAK/STAT pathway in the fat body, the crosstalk of the immune and the metabolic system seems to be an essential component of larval survival in

environments with various nutrient availability and parasitic challenges (Shin et al., 2020).

The mechanistic underpinnings of hemocyte association to the sessile compartment are only partially explored. It has been suggested, and later experimentally proven that there is a dynamic exchange of cells between the circulation and the sessile compartment, which may also contribute to the expansion of the latter (Makhijani et al., 2011) (Fig. 1B). In recent years, the Nimrod phagocytosis receptor family member Eater emerged as a crucial factor required on the surface of the hemocytes to enable sessility (Bretscher et al., 2015).

Even though the loss of *eater* completely abolishes the sessile compartment and the circulating hemocyte number significantly increases as a result, crystal cell differentiation is not noticeably affected (Bretscher et al., 2015; Melcarne et al., 2019). This raises the question: is the sessile compartment needed for hematopoiesis on the periphery or is it merely an auxiliary reservoir for immune cells? According to Makhijani and coworkers (2017), hemocyte proliferation in the sessile compartment is enhanced when compared to the circulation, and while this increase can be elicited by the stimulatory effect of peripheral sensory neurons, it is possible that the attachment and proximity of the hemocytes also facilitate their expansion. In support of this argument, it was shown that hemocyte clustering in the sessile pockets controls local crystal cell differentiation, possibly by reaching a critical number of cells within the cluster which produce sufficient level of the Serrate ligand to trigger Notch activity in a centrally positioned hemocyte (Leitão and Sucena, 2015) (Fig. 1B). However, Bretscher et al. (2015) reported that disruption of the sessile compartment caused by *eater* loss did not result in decreased circulating crystal cell numbers, which suggests that other mechanisms may substitute for the clustering-induced differentiation.

Observations by Márkus et al. (2009) suggested that a dedicated function of the sessile compartment could be a reservoir of cells that can be mobilized to contribute to anti-parasite response. Immune induction with the parasitoid wasp *Leptopilina boulardi* was found to disrupt sessile compartment structure and to decrease cell numbers while circulating hemocyte counts increased. Similar observations were made by Vanha-Aho et al. (2015), who established that the disintegration of the sessile compartment post-infection is dependent on the presence of Edin, secreted by the adipose tissue. Notably however, a more recent study suggested that the sessile compartment is only marginally affected by parasitic attack, and circulating cell numbers increase independently of sessile compartment integrity (Anderl et al., 2016). These conflicting findings possibly stem from methodological differences as well as the different degrees of parasitization, and would require comprehensive investigation to determine whether and when sessile hemocytes are mobilized.

With the recent single-cell sequencing studies (Cattenoz et al., 2020; Tattikota et al., 2020; Cho et al., 2020), it has also become clear that similarly to LG hemocytes, the blood cells in the circulation and in the sessile compartment are not composed exclusively of terminally differentiated effector cells. These studies identified hemocytes that run the gamut of differentiation states from proliferative cells, plasmatocyte and crystal cell precursors to mature effector hemocytes. Interestingly, the subgroup of hemocytes with high lipid metabolic activity was found not only in the lymph gland, but in the circulation as well (Cho et al., 2020; Cattenoz et al., 2020).

Taken together, the endogenous transcriptional program and the non-cell autonomous regulation of the hemocyte developmental direction ensure dynamic adjustment at various stages of cellular differentiation to tailor the composition of the immune cell pool to specific immune responses.

## 4. Transdifferentiation of plasmatocytes

Transdifferentiation is a special differentiation process, in which a mature cell type transforms into another mature cell type. The unique feature of the process, as compared to other differentiation processes is that it does not require the involvement of stem cells for the formation of a fully functional cell (Slack and Tosh, 2001; Reid and Tursun, 2018). In case of *Drosophila* hemocytes, it was first speculated by Rizki (1957) that lamellocytes may differentiate from plasmatocytes through a special cell type, the podocyte. Although at that time the phenomenon was not termed as transdifferentiation, the plasmatocyte-lamellocyte differentiation route was believed to be separate from the crystal cell lineage. According to this model, both plasmatocytes and crystal cells differentiate from a common progenitor. Later it was shown that lamellocytes differentiate at the expense of crystal cells in the lymph gland, which further underlined the theory that the crystal cell lineage - although it requires the same precursors - is separate from the plasmatocyte-lamellocyte fate (Krzemien et al., 2010).

The idea of potential pluripotency of plasmatocytes was however forgotten for decades. The notion that plasmatocytes are plastic cells that transdifferentiate into lamellocytes upon induction of the larvae with the parasitic wasp was corroborated more than 50 years later with genetic lineage tracing experiments. Since then, a growing body of evidence shows that plasmatocytes are plastic cells, which are capable of differentiation into lamellocytes both after infestation by a parasitic wasp and in case of tumors (Honti et al., 2010; Avet-Rochex et al., 2010; Stofanko et al., 2010).

Overexpression of certain transcription factors in plasmatocytes also induces lamellocyte fate. One of these is Charlatan, an interacting factor of the CoREST complex, which may be involved in the transdifferentiation of plasmatocytes both in the lymph gland and in the circulation (Stofanko et al., 2010). Surprisingly, plasmatocyte specific overexpression of Serpent, a transcription factor crucial for the embryonal specification of the hemocyte lineage, also results in the differentiation of lamellocytes (Kroeger et al., 2012), which suggests that factors such as Serpent and U-shaped may have a more general role and they can be utilized in a second wave of hematopoiesis. However, the regulatory role of these genes was not validated with loss-of-function studies, and therefore their function in transdifferentiation was not formally proven. Also, the expression of the *eater-Gal4* driver used in the experiment is not restricted to terminally differentiated plasmatocytes, therefore from these data, it is not clear which plasmatocyte cluster is capable of producing lamellocytes.

The lamellocyte fate is often regarded as a suppressed cell fate. This hypothesis is also underlined by the finding that the mutation of certain repressors that act at the chromatin level, such as the Polycomb-group genes *multiple sex combs*, *polyhomeotic proximal* and *Enhancer of Polycomb* results in lamellocyte differentiation (Remillieux-Leschelle et al., 2002; Owusu-Ansah and Banerjee, 2009; Theopold, 2009; Kurihara et al., 2020). In accordance with this, a recent study found that fruit fly populations which are continuously exposed to a parasitic wasp develop a more efficient immune response than populations that do not face parasitism. This increased resistance, which manifests in an increase of hemocyte number and a higher rate of melanotic capsule formation, is the result of constitutive upregulation of immune-induced genes. Interestingly, immature lamellocytes are present in resistant larvae, while mature lamellocytes remain inducible by the parasitoid wasp. This result suggests that the last step of lamellocyte differentiation is the change of cell morphology (Leitão et al., 2020, preprint). The high number of parasitic wasp species suggests that most insect populations are parasitized in nature (Forbes et al., 2018). Therefore, the hemocyte composition of larvae in lab stocks used for the past decades without parasitism may be very different from that of natural populations.

During the transdifferentiation process, plasmatocytes change their morphology, function and gene expression profile. Since, unlike plasmatocytes, lamellocytes do not phagocytose bacteria, it is reasonable that plasmatocytes switch off genes encoding phagocytosis receptors, such as NimC1 and Eater during transdifferentiation (Honti et al., 2010; Stofanko et al., 2010). This is also underlined by the finding that intermediate cells expressing lamellocyte markers are still able to phagocytose bacteria at early time points following immune induction (Honti et al., 2010).

Similar observations were made in *D. ananassae*, where multinucleated giant cells perform the encapsulation task instead of lamellocytes. In this species, plasmatocytes do not only differentiate, but also fuse with each other during the formation of the encapsulating cells (Márkus et al., 2015).

Moreover, plasmatocytes were also found to be differentiating into crystal cells in the sessile compartment under naive conditions, which is in line with the finding that - unlike the lymph gland - sessile islets do not contain prohemocytes, therefore proliferative plasmatocytes act as precursors for all other cell types outside the lymph gland (Leitão and Sucena, 2015; Cattenoz et al., 2020). These findings suggest that the plasmatocyte can be regarded as a pluripotent cell type and can serve as a progenitor to other effector blood cell types in the circulation and in the sessile compartment, where the equivalents of LG precursor cells are not present. The transdifferentiation into crystal cells was also underscored with a lineage tracing study by Corcoran et al. (2020, preprint). It was shown that precursors of the crystal cells in the sessile compartment are phagocytosis competent, active plasmatocytes. The transdifferentiation requires a signal conveyed by sensory cone neurons at the caudal hematopoietic pockets at the posterior end of the larva. However, the connection between signaling by sensory neurons and the Notch pathway required for crystal cell differentiation is still unclear.

The transition of regular plasmatocytes to lamellocytes is still debated and contradicted by certain findings. According to a study by Anderl et al. (2016), lamellocytes do not belong to a homogenous subset of encapsulating cells, but can be categorized into two different types (type I and type II) according to their marker expression pattern and their site of differentiation. While type I lamellocyte corresponds to the previously described large flat encapsulating lamellocytes, type II lamellocytes are smaller cells simultaneously expressing the lamellocyte specific *msn-Cherry* and the plasmatocyte specific *eater-GFP* markers. The origin of the two lamellocyte types was found to be different; type II lamellocytes differentiate from plasmatocytes, while type I lamellocytes originate from dedicated precursors. In the latter differentiation route, precursors first differentiate into lamelloblasts, which are similar to plasmatocytes, but express the plasmatocyte marker, *eater-GFP*, at a low level (Anderl et al., 2016). This finding however contradicts the previous results from lineage tracing experiments, in which *eater* positive, phagocytic plasmatocytes were shown to differentiate into large flat (type I) lamellocytes (Honti et al., 2010). Besides the type II lamellocyte, the study reveals the existence of activated plasmatocytes, which are similar to type II lamellocyte cells in size and in marker expression. Surprisingly, activated plasmatocytes are not capable of differentiating into lamellocytes (Anderl et al., 2016).

The latest methods that enabled the analysis of hemocyte transcriptome on a single-cell level allowed for the redefinition of plasmatocyte types and for the studies of differentiation routes in detail (Cattenoz et al., 2020; Tattikota et al., 2020; Fu et al., 2020). These investigations suggest that plasmatocytes are more diverse than they were believed before. The clustering of plasmatocytes suggests that certain plasmatocyte subsets may take specific parts in the defense; *e.g.* plasmatocytes expressing genes important for phagocytosis were distinguished from cells whose main function may be the production of antimicrobial peptides. A notable, newly emerged class of plasmatocytes expresses metabolic storage proteins most associated with the adipose tissue. Furthermore, a plasmatocyte cluster expressing genes that are characteristic to proliferating cells were identified in the sessile compartment. These cells may serve as pluripotent precursors in this compartment. Notably, the study by Fu et al. (2020) also identified reservoir plasmatocytes at the root of the differentiation-trajectory tree, suggesting that these cells are the most plastic plasmatocytes, possibly giving rise to three other plasmatocyte clusters. According to the model by Tattikota et al. (2020), this cluster of oligopotent cells serves as a progenitor for both activated plasmatocytes, which are characterized by high expression of genes encoding antimicrobial peptides and lamellocytes. This hypothesis was also underlined by the finding that breaking the cell cycle arrest by silencing *polo* in the self-renewing plasmatocyte subset results in the differentiation of lamellocytes. Since the transdifferentiation takes places following immune stimulation, it is noteworthy that, according to their transcriptome, a difference was found in the plasmatocyte composition between larvae after mechanical wounding and those parasitized by a wasp. The model suggests that through different intermediate cells, the same subset of plasmatocytes which transdifferentiates into lamellocytes, can also differentiate into crystal cells.

According to the same study, lamellocytes differentiate from a subset of plasmatocytes whose gene expression pattern is reminiscent of that of hemocytes located in the PSC of the lymph gland. These intermediate states are also characterized by a lower Atilla expression, while high Atilla levels were observed in another cell population appearing after immune induction. Based on these results, two clusters of lamellocytes were distinguished; an intermediate and a terminally differentiated cluster, both of which express genes involved in cytoskeleton rearrangement, melanotic capsule formation and integrin mediated cell adhesion (Tattikota et al., 2020). These two clusters of lamellocytes are not equivalents of the type I lamellocyte and type II lamellocyte cells described in Anderl et al. (2016), which may mean that in this study, the type II lamellocyte was recognized as a specific plasmatocyte cluster according to their transcriptome profile.

The scRNA-seq studies also uncovered two novel hemocyte types which show only partial similarity to the currently established effector cells. One of these is the primocyte. Primocytes are very rare in the circulation and express pan-hemocyte markers such as *srp* and *asrij* (Fu et al., 2020). According to their gene expression pattern, these cells may represent a special precursor population of hemocytes which was found so far exclusively in the lymph gland. Interestingly, primocytes also express *Antennapedia* and *knot/collier*, characteristic markers of both PSC cells and TepIV positive MZ cells of the lymph gland. The other newly found hemocyte type was termed thanacyte (Fu et al., 2020). Thanacytes are activated cells that express many stimulus response genes and are involved in the defense against bacteria. According to their transcriptome, thanacytes can be clustered as plasmatocytes with high *Tep4* expression. Interestingly, thanacytes do not express *nimC1*, one of the most characteristic markers of plasmatocytes.

A similar scRNA-seq study was performed to investigate the details of hemocyte ontogeny in the lymph gland. In this compartment, real self-renewing HSCs were described previously (Dey et al., 2016). The transcriptome data underlined the finding that the progenitors are in contact with the cells of the PSC. The ontogenic map based on the transcriptome data shows that progenitors differentiate through several prohemocyte clusters into plasmatocytes. Then the different intermediate plasmatocyte clusters serve as a basis for the differentiation of transcriptomically distinct terminally differentiated plasmatocyte clusters, as well as adipohemocytes, crystal cells and lamellocytes. The lymph gland is a compact organ, and hemocytes are arranged in the primary lobe according to their differentiational stage, which enabled the correlation of the spatial pattern of hemocyte subsets with the transcriptome-based clustering (Cho et al., 2020).

While the various approaches used to establish these datasets resulted in a plethora of new cell subtypes that only partially overlap among the studies, there are certain commonalities which can be recognized among them (Fig. 2.). The currently available scRNA-seq data suggest that the term “plasmatocyte” refers to a broad spectrum of immune cells, some of which seem to specialize to execute distinct gene expression programs and possibly, distinct functions. The results however also agree on the fact that the majority of plasmatocytes are in a transitionary state, derived from mitotic cells, but not expressing the characteristic markers for the specialized clusters. Since all scRNA-seq experiments were performed from late third instar larvae, one can speculate that these cell populations represent the “responsive/plastic” subsets of hemocytes that can conclude their differentiation when the presence of the specific plasmatocyte subclasses is required, or transdifferentiate into

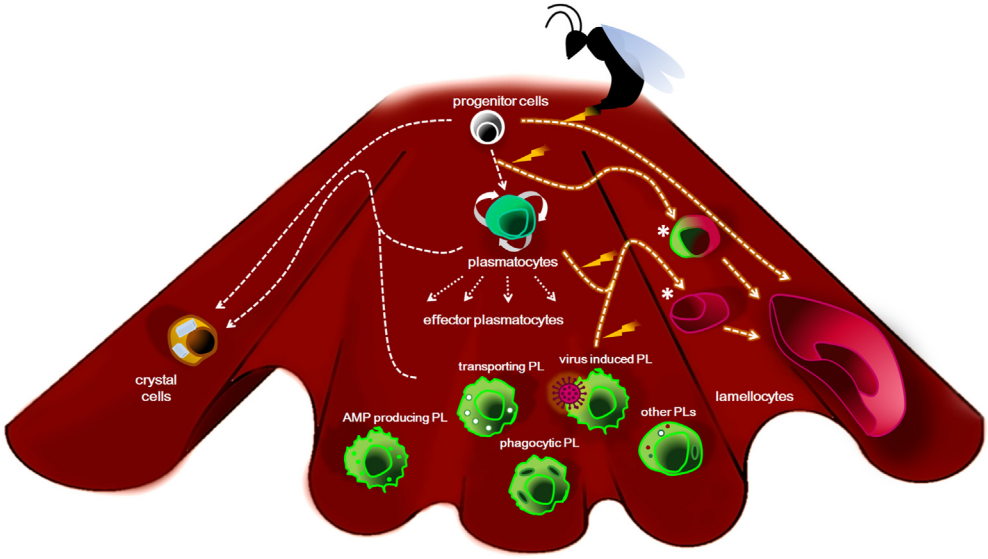


**Fig. 2.** The red carpet of *Drosophila* hemocyte fate-map. The circulation of the larva contains three main hemocyte classes: plasmatocytes, crystal cells and - in case of immune induction (bolt) - lamellocytes. Effector hemocytes (cells with dedicated immune or homeostatic function) can differentiate from progenitors - either directly, or in case of lamellocytes, through multipotent intermediates (*) following immune induction. In the circulation and in the sessile compartment, effector hemocytes differentiate from self-maintaining, proliferating plasmatocytes. The transdifferentiation from plasmatocytes to lamellocytes can occur at different developmental stages from the plasmatocytes. The cells can change their fate before they reach the early self-maintaining plasmatocyte stage (red-green intermediate cell), they can form from the self-maintaining stage or even from the late mature effector stage (red intermediate cell). The latest transcriptome studies enabled the clustering of plasmatocytes based on their gene expression patterns and revealed that the plasmatocytes are not just a single group of cells with phagocytic function; there are 10–15 subgroups with different effector functions. Virus induced plasmatocytes, transporting plasmatocytes, AMP producing plasmatocytes and phagocytic plasmatocytes may represent the main functional subgroups of effector plasmatocytes, which were not distinguished before. Therefore, we think, the plasticity of plasmatocytes - suggested a decade ago - may be an exclusive feature of a self-maintaining cluster of this cell population.

lamellocytes upon immune challenge. Additionally, data from transgenic expression or knockdown of genes that cause lamellocyte differentiation never resulted in the transformation of the entire plasmatocyte pool, which suggests that the specialized plasmatocyte types might be incapable of undergoing transdifferentiation. This notion is further strengthened by the findings of Leitão et al. (2020, preprint), who established a *Drosophila* line by infecting it with *L. boulardi* over several generations. In the circulation of these larvae even under non-infested conditions, cell numbers were increased and plasmatocyte-lamellocyte intermediates were enriched, suggesting the possibility of a dynamic adaptation of the hemocyte pool to the environment (Leitão et al., 2020; preprint).

Hematopoietic transdifferentiation is not a unique feature of *Drosophila* immune cells. In mammals, the most studied plastic blood cell type is the Th17 cell, a $CD4^{+}$ helper cell type of the adaptive immune system. Th17 cells, as well as Th1, Th2, Th9, Th22, follicular Th cells, peripheral Tregs and Tr1 regulatory cells differentiate from naive Th0 cells, as a response to various instructive signals such as ILs, TNFα and TGFβ. However, Th17 cells themselves can differentiate into all the other cell types originated from Th0. Those Th17 cells which switch off their IL-17A expression are regarded as unstable, and when they start expressing cytokines characteristic to other lineages, they become plastic, and start the transdifferentiation (Liu et al., 2017; Agalioti et al., 2018). Although there are similarities between the plasticity of Th17 cells and *Drosophila* plasmatocytes, the extent of these similarities may differ. It is not clear whether Th17 cells appear to be plastic due to the expression of the certain cytokine genes, or these cells undergo a complete epigenetic lineage reprogramming, which is assumed in case of the plasmatocyte to lamellocyte transdifferentiation in *Drosophila*. Moreover, it was shown that B cell fate is reprogrammable to differentiate macrophages and granulocyte-macrophage precursors. Macrophages form rapidly due to the enforced expression of C/EBPα and C/EBPβ in differentiated B cells (Xie et al., 2004; Di Tullio et al., 2011; Rapino et al., 2013; Cirovic et al., 2017). The dynamics of B cell into macrophage transition was analyzed with logical modeling (Collombet et al., 2017). Moreover, it was shown that manipulation of certain transcription factors and epigenetic regulators induces the transdifferentiation of T cells into NK cells and erythroblasts into megakaryocytes (Li et al., 2010; Siripin et al., 2015). Together, these data argue that the phenomenon of immune cell transdifferentiation is a mechanism adapted by phylogenetically distant organisms to optimize available resources to environmental challenges.

## 5. Summary

Transdifferentiation of immune cells provides additional adaptability to animals with which they can overcome immune challenges. The fact that effector hemocyte types can differentiate from other effector cells on demand suggests a peculiar adaptation of the immune system to maximize the available resources, as either maintaining all hemocyte types as differentiated effector cells or to have dedicated precursors for each cell type may be energetically more costly.

Recent evidence from scRNA-seq studies indicates that only certain hemocyte subtypes which were previously categorized as “plasmatocytes”, synonymous with a phagocytic, terminally differentiated effector hemocyte class can differentiate into lamellocytes and crystal cells. This suggests that some of the plasmatocyte clusters may actually be “latent-progenitor hemocytes” with potential to complete their differentiation into mature plasmatocytes, or switch their fates to other effector cell types depending on external inputs or the hematopoietic

microenvironment.

The scRNA-sequencing and clustering of hemocytes opened new avenues to understand hemocyte function and plasticity, and will no doubt prove an invaluable resource for discovering new functional hemocyte subsets that develop to tackle the variety of insults that *Drosophila* faces during different stages of development.

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