Minireview

# Intrinsic and Extrinsic Regulation of Hematopoiesis in *Drosophila*

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***Drosophila melanogaster* lymph gland, the primary site of hematopoiesis, contains myeloid-like progenitor cells that differentiate into functional hemocytes in the circulation of pupae and adults. Fly hemocytes are dynamic and plastic, and they play diverse roles in the innate immune response and wound healing. Various hematopoietic regulators in the lymph gland ensure the developmental and functional balance between progenitors and mature blood cells. In addition, systemic factors, such as nutrient availability and sensory inputs, integrate environmental variabilities to synchronize the blood development in the lymph gland with larval growth, physiology, and immunity. This review examines the intrinsic and extrinsic factors determining the progenitor states during hemocyte development in the lymph gland and provides new insights for further studies that may extend the frontier of our collective knowledge on hematopoiesis and innate immunity.**

**Keywords:** *Drosophila* hematopoiesis, hemocyte differentiation, inter-organ regulation, lymph gland, niche regulation, progenitor cell maintenance

## INTRODUCTION

*Drosophila* hematopoiesis is similar to that of vertebrates (Crozatier and Vincent, 2011; Evans et al., 2003). Vertebrates exhibit two waves of hematopoiesis: the first wave, called primitive hematopoiesis, occurs in the yolk sac of the developing embryo at 7.5 days and 17 days post-coitum for mice and humans, respectively (Baron et al., 2012; Evans et al., 2003; Swain et al., 2014). Similarly, flies undergo the primitive wave as early as 2 h after gastrulation in the procephalic mesoderm of the embryo (Crozatier and Meister, 2007; Tepass et al., 1994). Definitive hematopoiesis, which is the second wave, leads to the generation of hematopoietic stem cells in vertebrates (Durand and Dzierzak, 2005; Dzierzak and Bigas, 2018). Hematopoietic stem cells generated in the second wave of hematopoiesis in the aorta-gonad-mesonephros are essential for the maintenance of the blood system in adult vertebrates (Orkin and Zon, 2002; Swain et al., 2014). Notably, in mice, the transition from primitive to definitive hematopoiesis occurs at about 12.5 days post-coitum in the fetal liver, and the definitive phase completely replaces the primitive wave (Crozatier and Vincent, 2011; Evans et al., 2003; Swain et al., 2014). In invertebrates such as the fruit fly, the lymph gland (LG), the organ where the second wave of blood cell development occurs, begins to form around stage 5 of the embryonic development; however, it becomes more prominent at stage 17, where it is observed as a kidney-like structure flanking either side of the dorsal vessel, an organ that behaves like the heart muscles in pumping hemocytes to maintain their circulation (Banerjee et al., 2019; Krzemien et al., 2010a; Lo et al., 2002; Rugendorff et al., 1994).

*Drosophila melanogaster* is a holometabolous organism and undergoes the following four stages of complete transformation: embryo, larva, pupa, and adult; the LG continues to develop well after the embryonic stage until pupariation under normal conditions (Banerjee et al., 2019; Snodgrass, 1954).

During the initial wave, a group of cells called plasmatocytes, which usually occupy ~95% of the total *Drosophila* blood population, are observed in circulation and perform functions that are analogous to those of vertebrate macrophages (Holz et al., 2003; Honti et al., 2014; Rizki, 1957; Tepass et al., 1994). Crystal cells, named so due to crystalline inclusions within their cytosol, make up the remaining 5% of the fly blood population (Meister and Lagueux, 2003; Rizki, 1957; Shrestha and Gateff, 1982). They participate in immune responses and wound healing and are sensitive to changes in ambient $O_2$ (Cho et al., 2018; Lebestky et al., 2000; Mukherjee et al., 2011; Rizki and Rizki, 1959). Notably, a third group of blood cells, called lamellocytes, exists in the fly blood system, but these are rarely observed under normal conditions (Lanot et al., 2001; Rizki, 1957). Nonetheless, when larvae are challenged by wasps, such as *Leptopilina boulardi*, which are their natural enemy, the LG produces copious amount of lamellocytes and releases them into the circulation to mount an immune defense against the deposited wasp eggs (Carton et al., 2008; Keebaugh and Schlenke, 2014; Lemaitre and Hoffmann, 2007; Russo et al., 1996).

The first wave of *Drosophila* hematopoiesis produces hemocytes that are functional at the embryonic and larval stages, and thereafter, the second-wave blood cells largely operate at the pupal and adult stages (Holz et al., 2003; Honti et al., 2014; Lanot et al., 2001; Wood and Jacinto, 2007). A subset of hemocytes originating from the primitive wave is housed and maintained in segmental regions of the larval body wall called hematopoietic pockets. Blood cells in these pockets are released into the circulation on demand and can proliferate or differentiate into the other two cell types (Banerjee et al., 2019; Leitao and Sucena, 2015; Makhijani and Brückner, 2012; Márkus et al., 2009; Petraki et al., 2015). Recent studies have shown that the two groups of hemocytes survive to the adult stage with the ratio of primitive to definitive blood cells being approximately 3:2. Interestingly, no signs of hematopoiesis have been observed in the adult fly (Sanchez Bosch et al., 2019).

The complex system of hematopoiesis in flies is governed by several intrinsic and extrinsic factors that ensure that blood development is coherent with the homeostatic needs and developmental growth of the organism. This review aims to summarize the intrinsic and extrinsic regulators of *Drosophila* definitive hematopoiesis to provide novel insights for advancing the field.

## *DROSOPHILA* LYMPH GLAND

At full maturity under normal conditions, the LG consists of four pairs of lobes on either side of the dorsal vessel: the first is called the primary lobe and lies anteriorly, while the second, third, and fourth are commonly called posterior lobes (Fig. 1) (El Shatoury, 1955; Jung et al., 2005; Krzemien et al., 2010b). The pairs of lobes are separated by pericardial cells,

which are akin to nephrocytes in mammals, and the dorsal vessel (Crossley, 1972; Lo et al., 2002; Mills and King, 1965; Na and Cagan, 2013). The most inward zone and closest to the dorsal vessel in the primary lobe is the medullary zone, which consists of progenitors that give rise to plasmatocytes and crystal cells of the outermost cortical zone (Jung et al., 2005; Krzemien et al., 2010b; Sorrentino et al., 2002). An intermediate zone consisting of cells expressing markers for both the medullary and cortical zones was identified using genetic tracing experiments (Krzemien et al., 2010b; Spratford et al., 2021). With recent advances in single-cell RNA sequencing technology, significant heterogeneity of the progenitor population and endogenous gene expression have been established in the LG (Cho et al., 2020; Girard et al., 2021; Spratford et al., 2021). The complexity of hemocytes and mechanisms controlling multiple developmental paths in the LG hematopoiesis require further investigation. Nearest to the first pair of pericardial cells is a small cluster of cells called the posterior signaling center (PSC), which provides a microenvironment for progenitor maintenance (Krzemień et al., 2007; Minakhina and Steward, 2010). Recently, cardiac tubes have been shown to function as a second niche similar to that observed in vertebrates (Destalminil-Letourneau et al., 2021; Morin-Poulard et al., 2016).

Whereas previous findings on the LG were associated
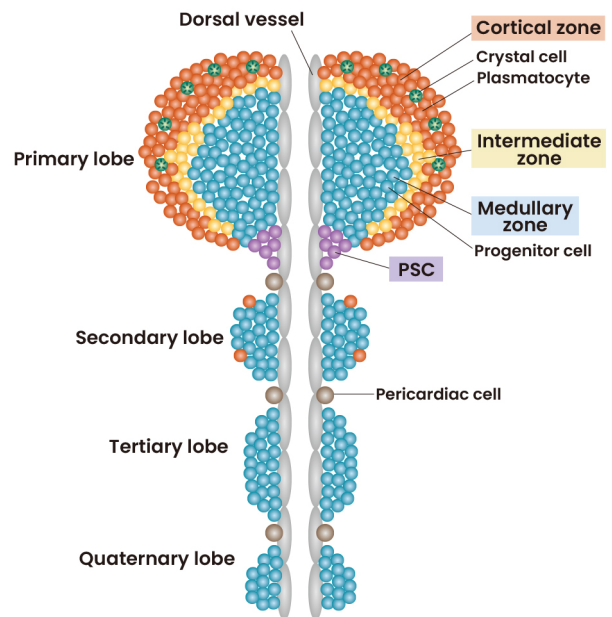


**Fig. 1. The *Drosophila* lymph gland.** The lymph gland is made up of four pairs of lobes. The anterior primary lobe is divided into the medullary zone (MZ) made of progenitors, the cortical zone (CZ) consisting of mature blood cells, and the intermediate zone (IZ) which is a mixture of cells from the MZ and CZ. While the dorsal vessel separates the opposite lobe pairs, the pericardial cell splits adjacent posterior lobes. The primary lobe consists of the posterior signaling center (PSC), MZ, IZ, and CZ, while the other lobes comprise the posterior lobes.

with the primary lobe, recent studies have elucidated the composition and role of the other pairs of lobes. Posterior LG lobes are made up of progenitors that differentiate under specific stress conditions such as wasp infestation (Banerjee et al., 2019; Krzemień et al., 2007; Lan et al., 2020; Lanot et al., 2001; Letourneau et al., 2016). Rodrigues et al. (2021) revealed heterogeneity of posterior progenitors and characterized useful genetic tools to further study the population. In addition, Kanwal et al. (2021) highlighted the significance of Ultrabithorax (Ubx) and collier in posterior progenitor maintenance. These studies provide grounds for understanding the posterior lobe hematopoiesis; future studies will expand our knowledge regarding the posterior lobes and their function and proportion in the pupa and adult fly blood.

## INTRINSIC REGULATION OF HEMATOPOIESIS IN THE LYMPH GLAND

Like vertebrate hematopoietic stem cells, LG progenitor cells

require a niche microenvironment, called the PSC, that secretes various signaling factors for their maintenance (Mandal et al., 2007). At the first instar stage of larval development, Decapentaplegic (Dpp) signal from the PSC activates Notch in early progenitor cells. In the absence of Dpp, Notch-positive progenitor cells disappear, and the size of the LG decreases (Dey et al., 2016; 2019) (Fig. 2A). Serrate (Ser) is another Notch-activating regulator known to be expressed in the PSC. PSC-dependent Notch-Serrate signaling in progenitor cells is necessary to prevent their differentiation (Blanco-Obregon et al., 2020; Lebestky et al., 2003). In addition, the size of the PSC is reduced upon the expression of the dominant-negative form of Ser (Krzemień et al., 2007). These findings reveal the cell-autonomous and non-cell-autonomous functions of Notch in the PSC as well as the essential role of Notch in progenitor maintenance.

Progenitor cells express the Hh receptor patched (ptc), whose activation inhibits the cleavage of Cubitus interruptus (Ci) to attenuate their differentiation (Mandal et al., 2007;
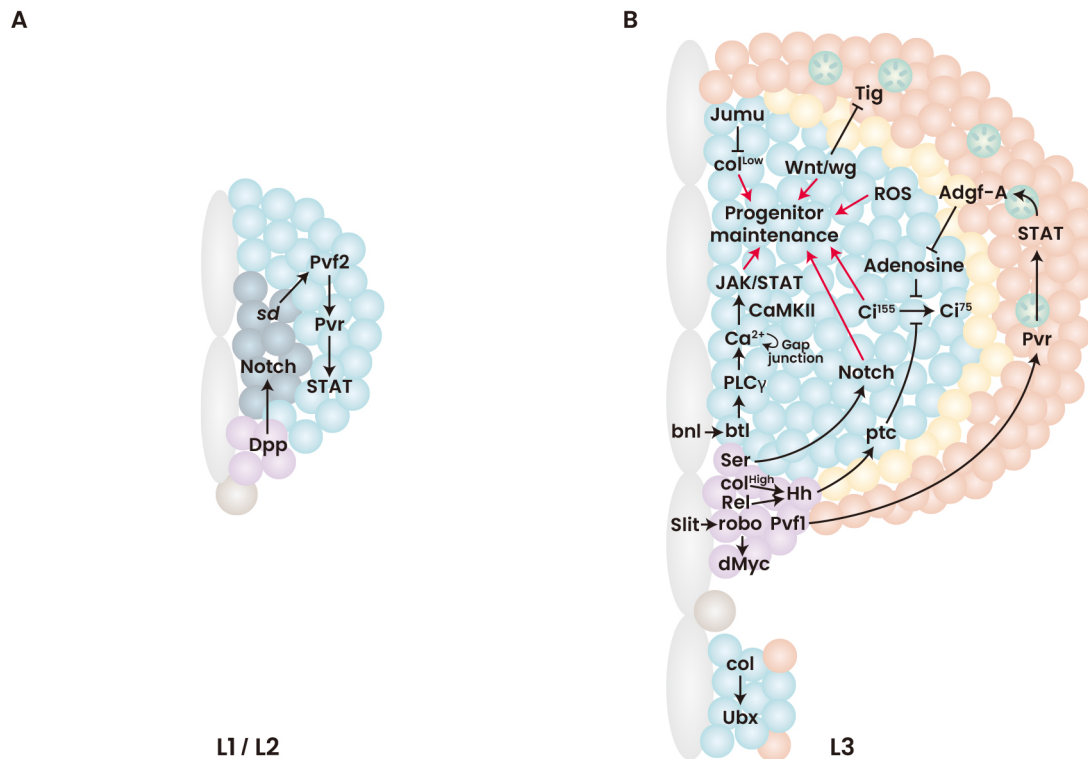


**Fig. 2. Intrinsic regulation of progenitor maintenance in the lymph gland.** (A) Early progenitors of the L1 (1st instar)/L2 (2nd instar) lymph gland are sustained by Decapentaplegic (Dpp), Notch (N), or Pvf2 signaling prior to emergence of the CZ. Secreted Pvf2 from a group of cells near the dorsal vessel that are *scalloped* (*sd*)-positive activates Pvr in the MZ which, in turn, triggers STAT signaling to ensure formation of proper lymph gland size. Also, Dpp from the PSC progresses N signaling for progenitor maintenance. (B) In the late L3 (3rd instar) larvae, bnl and slit secreted from the cardiac tube (dorsal vessel) activate btl and robo, respectively. In the PSC, robo actuates dMyc while btl triggers JAK/STAT signaling via PLCγ, Ca²⁺ and CamKII in the MZ. Gap junctions contribute to the overall calcium levels in the MZ. In contrast to (A), N signaling in the MZ is propagated following interactions with Serrate (Ser) from the PSC while Relish (Rel) and collier (col) serve as inputs for Hedgehog signaling (Hh). Pvr in the CZ is activated by traveling Pvf1 from the PSC. Active Pvr through STAT and Adgf-A regulates adenosine levels for MZ maintenance. Wnt/Wg signaling supports the MZ and inhibits Tiggrin (Tig) in the CZ while moderate levels of reactive oxygen species (ROS) as well as suppression of col by Jumu in the progenitors preserve the MZ. In posterior lobes, col maintains the posterior progenitors via Ultrabithorax (Ubx).

Sharma et al., 2019) (Fig. 2B). Interestingly, Hh signal is transferred by filopodia from the PSC to progenitor cells and directly binds to the ptc receptor. In addition, in the PSC, Relish (Rel) is required for proper cytoskeletal structure, and thus, loss of Rel traps the Hh signal, which impacts progenitor maintenance (Mandal et al., 2007; Ramesh et al., 2021). PDGF- and VEGF-related factor 1 (Pvf1) is secreted from the PSC and travels farther to indirectly promote Ci stabilization through Pvr, a Pvf receptor, in the cortical zone. Pvr in the cortical zone activates adenosine deaminase-related growth factor A (Adgf-A) via STAT92E, and consequently, Adgf-A lowers adenosine in progenitor cells (Mondal et al., 2011). A moderate concentration of adenosine in the LG regulates Hh-dependent Ci activation, and both Adgf-A and Hh signals ensure proper levels of Ci for progenitor cell maintenance. Like Pvf1, Pvf2 from *scalloped* (*sd*)-positive progenitor cells close to the dorsal vessel activate hemocyte proliferation and regulate the size of the LG (Ferguson and Martinez-Agosto, 2017).

In addition to the PSC, the cardiac tube also functions as a niche (Fig. 2B). The cardiac tube secretes slit (Jones et al., 2007), a ligand, to the PSC, which is recognized by round-about (robo) and activates CDC42 and dMyc for proper PSC development (Morin-Poulard et al., 2016). The FGF ligand in *D. melanogaster*, namely branchless (bnl), is also secreted from the cardiac tube. The bnl activates its receptor breathless (btl) in progenitor cells and maintains its calcium levels by activating PLCγ. Therefore, blocking bnl-btl interaction causes progenitor cell differentiation (Destalminil-Letourneau et al., 2021).

*Drosophila* early B cell factor, collier (col), is expressed in progenitor cells and the PSC, although its targets are segregated (Fig. 2B). The level of col expression in the PSC is higher (col$^{High}$) than that in the progenitors (col$^{Low}$), but col expression in both regions supports progenitor maintenance. While progenitor-specific overexpression of *col* represses blood cell differentiation, expression of *col* RNAi in the same cells leads to robust and precocious differentiation of plasmatocytes. Loss of *col* in the PSC reduces Hh expression as well as filopodia extension, whereas overexpression of *col* in the PSC does not alter the size of PSC or the number of crystal cells (Benmimoun et al., 2015; Oyallon et al., 2016; Pennetier et al., 2012). These studies imply that col exhibits bifurcated signaling pathways in the PSC and progenitor cells despite its identical function. As an upstream of col, Jumeau (Jumu), a member of the forkhead transcription factor family, cell-autonomously maintains a low level of col cells and indirectly suppresses the number of PSC, collectively maintaining progenitor homeostasis (Hao and Jin, 2017). In addition to col, the Wnt/Wg signal is expressed in the progenitors. Secreted Wg from progenitor cells is recognized by the Wnt receptor, frizzled 2 (fz2), and it maintains the level of *Drosophila* E-Cadherin, shotgun (shg) (Sinenko et al., 2009). Overexpression of Wg or constitutive activation of Wnt signaling pathway by the expression of armadillo (arm), a *Drosophila* homolog of beta-catenin, inhibits progenitor cell differentiation. Consistently, upregulation of Wnt suppresses Tiggrin (Tig), a cortical zone marker, and keeps the medullary zone intact (Zhang et al., 2014). These results indicate significant

cell-autonomous roles of col and Wnt signaling in progenitor cell maintenance.

In the medullary zone, free radical oxygen species (ROS) function as signaling molecules to support progenitor cells (Fig. 2B) (Owusu-Ansah and Banerjee, 2009). High ROS upregulates FOXO and induces the JNK pathway, which triggers the precocious differentiation of progenitor cells. Likewise, scavenging ROS induce the same phenotype, indicating that moderate ROS levels are required for progenitor maintenance. In contrast to progenitor cells, PSC cells do not exhibit the presence of ROS under normal conditions. However, under wasp infestation, an increase in the level of ROS in the PSC stimulates the secretion of spitz (spi) and activates the EGFR signaling pathway, causing lamellocyte differentiation (Sinenko et al., 2011).

JAK/STAT signaling is involved in the intrinsic regulation of progenitor cell maintenance (Fig. 2B) (Jung et al., 2005; Makki et al., 2010). Through single-cell RNA sequencing, the expression of *domeless*, a JAK/STAT receptor, in the medullary zone has been confirmed, and Rodrigues et al. (2021) have shown that progenitor cells in the posterior lobes also express *domeless-Gal4* (Girard et al., 2021; Rodrigues et al., 2021). Additionally, the expression of JAK/STAT is highlighted in the earliest progenitor cells in the first to second instars (Cho et al., 2020), indicating that JAK/STAT is a universal pathway for progenitor cell fate. The level of calcium, shared through gap junctions, induces phosphorylation of calcium/calmodulin-dependent protein kinase II (CamKII), and inhibits the trafficking of domeless receptors to prevent progenitor cell differentiation (Ho et al., 2021). In response to wasp infestation, JAK/STAT pathway is attenuated in the primary lobe through an antagonistic effect of Latran (lat), a short type I cytokine-related receptor (Makki et al., 2010). However, JAK/STAT pathway in posterior lobes is enhanced against wasp parasitism, implying functional compartmentalization of JAK/STAT in the progenitors (Rodrigues et al., 2021).

## EXTRINSIC REGULATION OF HEMATOPOIESIS IN THE LYMPH GLAND

Developmental homeostasis of progenitor and differentiated hemocyte populations in the LG is regulated by systemic factors from other organs (Fig. 3). *Drosophila* insulin-like peptide 2 (dIlp2) is secreted from insulin producing cells (IPCs) in the brain and promotes growth and metabolism similar to mammalian insulin (Boulan et al., 2015; Brogiolo et al., 2001; Graham and Pick, 2017). Malnutrition reduces the release of dIlp2 from the IPCs into the hemolymph and subsequently decreases Wnt signaling in progenitor cells. Consequently, starved larvae show a smaller population of progenitor cells and an increase in differentiated hemocytes (Shim et al., 2012). Insulin signaling in the PSC supports the number of PSC cells and promotes progenitor maintenance (Benmimoun et al., 2012; Tokusumi et al., 2012).

Sensory input from olfactory neurons triggers γ-aminobutyric acid (GABA) secretion from the brain into the hemolymph. Circulating GABA, in turn, regulates calcium signaling in the progenitors to systemically control hematopoiesis in the LG (Fig. 3) (Shim et al., 2013). From an immune perspec-
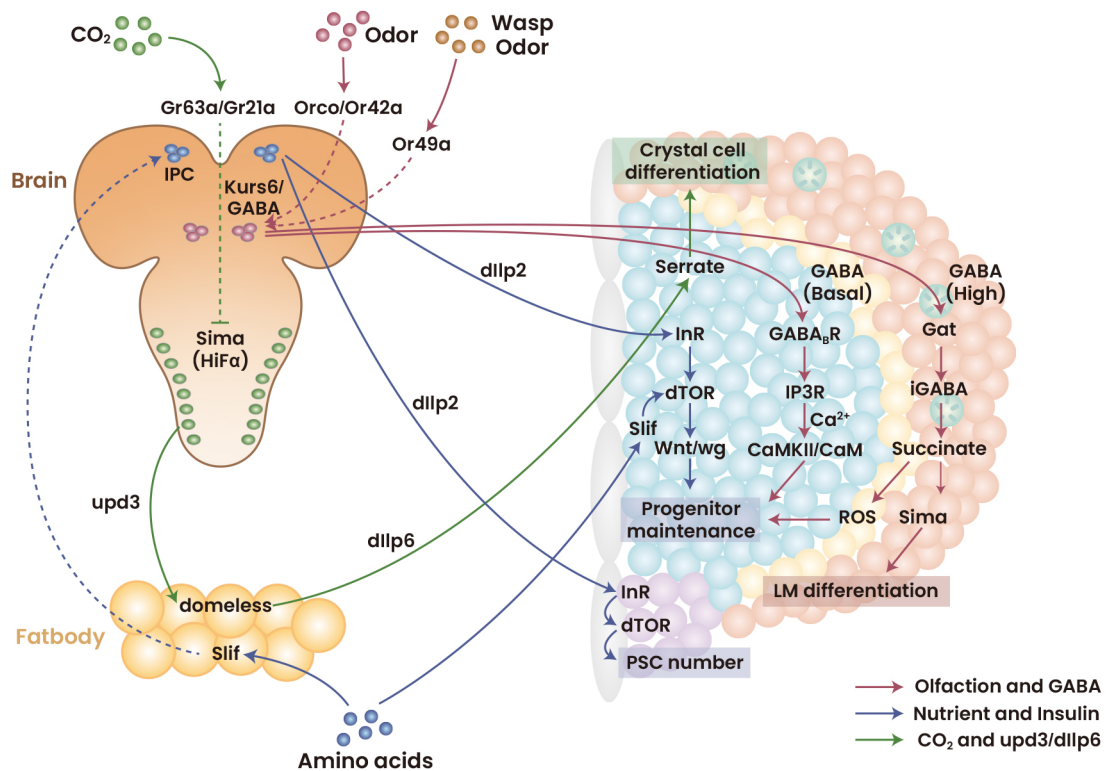
**Fig. 3. Inter-organ communication for the regulation of progenitors in the lymph gland.** Odor is detected by Orco/Or42a which triggers release of GABA from the Kurs6 neuron in the brain. Basal levels of GABA detected by GABA$_B$ receptor (GABA$_B$R) in the lymph gland support progenitor maintenance through IP3 receptor (IP3R), cytosolic calcium, and CamKII/CaM. Under wasp infestation, combination of Or49a and Or42a produces high levels of GABA secretion from the brain. High GABA is transported and internalized by GABA transporter (Gat) after which it is converted through the GABA shunt pathway to generate succinate. Succinate stabilizes sima and enhances lamellocyte (LM) differentiation. A portion of succinate is utilized by the TCA cycle to create ROS for progenitor maintenance. Nutrient including amino acids is sensed by the fat body which in turn increases *Drosophila* insulin-like peptide 2 (dIlp2) release from the brain insulin producing cells (IPCs). dIlp2 progresses insulin receptor (InR) signaling in the MZ which eventually sustains progenitors through dTOR and Wnt/wg signaling. Alternatively, Slimfast (Slif) directly uptakes amino acids and merges into dTOR pathway. Also, in the PSC, dIlp2 activates InR and dTOR to control PSC number. Ambient CO$_2$ is recognized by Gr63a/Gr21a inhibiting sima (Hif-α) in the ventral nerve cord (VNC). sima stabilization induces release of unpaired 3 (upd3) which is detected by domeless in the fat body. Fat body secrets dIlp6 which increases Serrate in the IZ and causes crystal cell differentiation. Red arrows, olfaction and GABA axis; Blue arrows, nutrient and insulin axis; Green arrows, CO$_2$ and upd3/dIlp6 axis.

tive, GABA secreted from the brain is used as a metabolite in progenitor cells through succinate conversion to enhance lamellocyte differentiation (Madhwal et al., 2020). The level of food odors through olfactory receptor 42a (Or42a) sets up the basal GABA concentration enough to activate the GABA$_B$ receptor, while combinatorial stimulation of Or42a and wasp-odor-sensing Or49a boosts GABA secretion to trigger GABA internalization. The GABA shunt pathway converts imported GABA in immune precursors into succinate to stabilize sima, *Drosophila* Hif-α, which is required for lamellocyte differentiation. In addition, succinate derived from extrinsic GABA can be utilized in the tricarboxylic acid (TCA) cycle to generate moderate levels of ROS in the LG to promote progenitor maintenance (Goyal et al., 2022). These findings highlight the context-dependent role of GABA in hematopoiesis and immunity.

Atmospheric gaseous molecules control blood cell development in the LG (Fig. 3). Gr63a/Gr21a in the terminal organ of larvae senses ambient CO$_2$ levels and normally triggers behavioral responses (Jones et al., 2007; Kwon et al., 2007). However, during the development, low levels of CO$_2$ cause sima accumulation in the ventral nerve cord (VNC) and up-regulates unpaired 3 (upd3) secretion from the brain. Subsequently, upd3 targets the fat body, an organ akin to the liver, and induces the secretion of *Drosophila* insulin-like peptide 6 (dIlp6) (Arrese and Soulages, 2010). dIlp6 augments the expression of Serrate in the intermediate zone and leads to excessive crystal cell differentiation (Cho et al., 2018). Likewise, hypoxia stabilizes sima to non-canonically activate Notch receptor and promote crystal cell survival (Mukherjee et al.,

2011). Together, these studies suggest a close relationship between the level of ambient gases and hemocyte differentiation.

## PERSPECTIVE

In a nutshell, LG progenitor maintenance is regulated by various intrinsic signaling factors, such as Dpp, Notch, Hh, col, Wnt, ROS, and JAK/STAT, largely mirroring the mechanisms underlying myeloid differentiation in vertebrates (Chavakis et al., 2019; Dzierzak and Bigas, 2018; Kim et al., 2021; Yamashita et al., 2020; Zhu and Emerson, 2002). Notably, the heterogeneity of the LG progenitor population and existence of intermediate progenitor cells have been frequently proposed (Cho et al., 2020; Ferguson and Martinez-Agosto, 2014; Krzemien et al., 2010b; Spratford et al., 2021). These studies suggest that signaling factors may not work in the same way for all progenitor subtypes, and hence, more complex signaling mechanisms may be active. Further studies may elucidate such unknown cascades in diverse progenitor populations.

Extrinsic signaling factors reflect environmental condition —examples include nutritional content, odor diversity, and atmospheric gaseous composition. However, previous studies involving external factors have heavily focused on behavioral and physiological contexts (van Breugel et al., 2018; Vermehren-Schmaedick et al., 2010; Wang et al., 2013). This may be due to the difficulty in observing direct interactions between these factors and *in vivo* hematopoietic systems. Since *Drosophila* LG is a sentinel for stress responses and provides intuitive phenotypes for observation, it serves as a potent model to further characterize the effects of extrinsic factors on hematopoiesis and immunity.

## AUTHOR CONTRIBUTIONS
F.K., B.C., and J.S. wrote the manuscript. F.K. and B.C. drew figures. J.S. supervised the manuscript.

## CONFLICT OF INTEREST
The authors have no potential conflicts of interest to disclose.

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