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Hematopoiesis and Hematopoietic Organs in Arthropods

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Abstract

Hemocytes (blood cells) are motile cells moving throughout the extracellular space and exist in all clades of the animal kingdom. Hemocytes play an important role in shaping the extracellular environment and in the immune response. Developmentally, hemocytes are closely related to the epithelial cells lining the vascular system (endothelia) and body cavity (mesothelia). In vertebrates and insects, common progenitors, called hemangioblasts, give rise to the endothelia and blood cells. In the adult animal, many differentiated hemocytes seem to retain the ability to proliferate; however, in most cases investigated closely, the bulk of hemocyte proliferation takes place in specialized hematopoietic organs. Hematopoietic organs provide an environment where undifferentiated blood stem cells are able to self renew, and at the same time generate offspring that differentiate into different blood cell types. Hematopoiesis in vertebrates, taking place in the bone marrow, has been subject to intensive research by immunologists and stem cell biologists. Much less is known about blood cell formation in invertebrate animals. In this review we will survey structural and functional properties of invertebrate hematopoietic organs, with a main focus on insects and other arthropod taxa. We will then discuss similarities, at the molecular and structural level, that are apparent when comparing the development of blood cells in hematopoietic organs of vertebrates and arthropods. Our comparative review is intended to elucidate aspects of the biology of blood stem cells that are more easily missed when focusing on one or a few model species.

Keywords

blood; hematopoiesis; stem cell; invertebrate; arthropod

Introduction

Most animals possess populations of cells that are not fixed in place as part of an organ, but that roam within the body cavities to fulfill functions involved with the immune response, cell degradation and replacement, and the distribution of nutrients and gases. The blood cells, or hemocytes, of the vertebrate body, which comprise many different classes each responsible for one of the functions mentioned above, represent the best known example. All invertebrates which possess a body cavity and, to various degrees, a vascular system also have hemocytes of different types. Even in many animals without a body cavity, including cnidaria, sponges and flatworms, one observes motile cells that primarily function as pools of pluripotent stem cells. Such cells may represent the ancestral type of motile cell from which the diverse, specialized classes of hemocytes of higher animals evolved (van de Vyver, 1981).

Hemocytes typically have a lifespan of days to weeks, and need to be replaced constantly. Furthermore, given that the demand for hemocytes (in particular those involved in the immune response, and the related processes of wound healing and regeneration) varies over time, the rate at which hemocytes are produced must be able to adapt to these variable

conditions. Blood cells are produced in specialized tissues, called hematopoietic organs. Hematopoietic organs generate blood cells in high numbers throughout the life time of the animal, implying that hematopoietic organs must contain, or be supplied with, self renewing stem cells. The study of hematopoiesis thus addresses a central issue of stem cell biology, namely the “decision” of an undifferentiated cell to either continue to proliferate, or to leave this state to initiate differentiation. We will in the following start out by briefly summarizing pertinent facts about blood cell development among the invertebrates and then provide more details illustrating the architecture and proliferative function of hematopoietic organs in the arthropods.

Hematopoietic cells are part of the vasculature or derive from common blood-vascular progenitors (hemangioblasts)

Hematopoietic organs are built as more or less complex, mesenchymal or gland-like structures that are closely associated with the epithelial cells surrounding blood vessels (endothelia) and/or the coelomic cavity (mesothelial or peritoneal cells), which reflects the shared developmental origin of the motile blood cells and the fixed epithelial cells lining the cavities in which blood cells move. In many phyla, cells scattered randomly all over the endothelia/mesothelia, or located in dedicated hematopoietic “niches” within these epithelia, show higher rates of proliferation and bud off hemocytes into the lumen of the coelom or blood vessels (Fig.1A, B; Eckelbarger, 1976; reviewed in Dales and Dixon, 1981). In these cases, progenitors of hemocytes and endothelial/mesothelial cells form one and the same cell type. In other cases, hematopoietic cells form mesenchymal clusters (“nodules”) or gland-like structures (“lymph glands”) that are separate from the vascular linings, but always remain in close contact with them (Fig.1C, D). In *Drosophila*, where the development of an invertebrate hematopoietic organ was studied, it became clear that a common progenitor (hemangioblast) gives rise to both vascular cells and hemocytes (Mandal et al., 2004), which is similar to the situation in vertebrate embryos (Choi et al., 1998; Teixeira et al., 2011). In the *Drosophila* embryo, vascular progenitors undergo a mesenchymal-epithelial transition to form the lining of the blood vessels; blood progenitors assemble into mesenchymal clusters that remain attached to the basal surface of the vascular epithelia. These clusters proliferate rapidly and develop into the lymph glands of the larva. Lymph glands have been observed in many insects. They form metamerically reiterated encapsulated structures that flank the wall of the dorsal blood vessel (Cuenot, 1897; Hoffmann et al., 1979, 1979). Similar structures have also been described for mollusks (e.g., the amebocyte producing organ in gastropods; Jeong et al., 1983; the “white glands” of cephalopods; Cowden and Curtis, 1981) and crustaceans (lymph glands along the vessels and over the foregut; reviewed in Bauchau, 1981). In lower deuterostomes (e.g., ascidians), “blood forming nodules” are grouped around the transverse bars of the pharyngeal basket (Wright, 1981).

Invertebrate lymph glands consist of dividing stem cells and hemocyte progenitors, called prohemocytes. In addition we find clusters of cells which continue to divide, but have embarked on a differentiative pathway. As will be discussed further below, both types of cells are usually separated; the undifferentiated hemocyte progenitors typically form a central core which is surrounded by an outer layer, or multiple “nodules”, of hemocytes that are more differentiated (insects: Hoffmann et al., 1979; Shrestha and Gateff, 1982; Lanot et al., 2001; Nardi et al., 2003; Jung et al., 2005; cephalopods: Cowden and Curtis, 1981; ascidians: Ermak, 1976). A prominent “stroma”, similar to the networks of capillaries and reticular cells found in the hematopoietic tissue of vertebrates, is missing in invertebrates. However, “reticular cells” that surround prohemocytes and may act as stem cells have been described in several insect species (Hoffmann, 1970). In addition, individual hemocyte progenitors or groups thereof are surrounded by lamellae of extracellular matrix, formed by proteins that are found ubiquitously in basement membranes and other ECM assemblies of

Drosophila and vertebrates (Lin et al., 2008; Martinek et al., 2008; Grigorian et al., 2011, 2012; Huynh et al., 2011;

We will in the following survey compare the hematopoietic organs found among different arthropods, guided by the question of how the processes of hematopoietic cell renewal and differentiation are laid out in space and time, and are controlled by signaling mechanisms acting on the hematopoietic organs from within and from outside. Before going into these matters it is necessary to briefly mention the types of hemocytes and their functions found in arthropods.

Blood Cell Types in Arthropods

Hemocytes have been studied in a number of insects, crustaceans and chelicerates, with much controversy associated with the number of different hemocyte cell types observed and the terminology used to designate them. Thus, often researchers used different terms for the same type of cell; or, differences in cell structure that lead to different names merely reflected artefacts of fixation, or different stages during the development of the same cell. Attempts to simplify and unify the terminology have been made repeatedly. The more recent literature recognizes the following main cell types (reviewed in Jones, 1970; Rowley and Ratcliffe, 1981; Pelc, 1986; Lavine and Strand 2002; Ribeiro and Brehelin, 2006): prohemocytes, plasmatocytes, granulocytes, oenocytoids, and spherule cells (also called adipohemocytes). (1) Prohemocytes (Fig.2D) are small, rounded cells with a high nucleus:cytoplasm ratio and no specialized cytoplasmic organelles; these cells represent the immature hemocyte progenitors, or stem cells. All other blood cell types are derived from prohemocytes. (2) Plasmatocytes (Fig.2A) have more cytoplasm than prohemocytes, and their membrane forms ruffles and filopodia. Plasmatocytes are phagocytotically active, ingesting cellular debris during normal development, and engulfing microorganisms during the immune response. Plasmatocytes are also able to differentiate into large, flattened cells that arrange themselves around foreign bodies that have gained access to the body cavity, forming a capsule around these invaders (Fig.2J). These enlarged, flattened plasmatocytes were called lamellocytes in *Drosophila* and other insects (Rowley and Ratcliffe, 1981). (3) Granulocytes (Fig.2B) have electron-dense, regularly sized, acidophilic granula. Like plasmatocytes, granulocytes also act as phagocytes. Some insects, including *Drosophila*, lack granulocytes; here, plasmatocytes take on the function of granulocytes. The loss of granulocytes is apparently not evolutionary ancient; other Dipterans, such as *Calliphora*, possess granulocytes (Kaaya and Ratcliffe, 1982). (4) Oenocytoids (Fig.2E) are large, oval cells whose cytoplasm contains agglomerates of crystalline material and/or microtubules. The so called crystal cells of *Drosophila* (Fig.2C) correspond to the oenocytoids defined for other arthropods (Brehelin, 1982). Oenocytoids store prophenol oxidase, an enzyme that plays an important role in the innate immune response (see below). (5) Spherulocytes (Fig. 2F) or adipohemocytes are blood cells with basophilic, variably sized and shaped granules. These cells may represent late stages in the differentiative pathway of phagocytic plasmatocytes. The range of blood cells characterized above is simpler, or modified, in some cases. For example, in *Drosophila*, we do not encounter granulocytes or spherulocytes. Crustaceans and many chelicerates lack oenocytoids; prophenol oxidase is produced by granulocytes (Zhang et al., 2006; Lin and Soderhall, 2011).

Arthropod hemocytes mainly function during the innate immune response and wound healing (Rowley and Ratcliffe, 1981; Nappi et al., 2009; Jiang et al., 2010; Krzemien et al., 2010; Lin and Soderhall, 2011). Invading microorganisms attract plasmatocytes and granulocytes, which release reagents that entrap the invaders (Fig.2G, H). Products secreted by granulocytes also play a role in hemolymph coagulation. Plasmatocytes, differentiating as lamellocytes, form a capsule around the invaders (Fig.2I, J). Oenocytes release prophenol

oxidase, which is converted into activated phenol oxidase by a cascade of enzymes that form part of the hemolymph, and that are activated by foreign molecules (e.g., microbial lipopolysaccharides). Phenoloxidase catalyzes a reaction by which melanin is synthesized (Fig.2H-J), and toxic metabolites (e.g., quinones) are released. Encapsulation and melanization act to neutralize the invading microbes or parasites.

Hematopoietic organs in arthropods

Even though typically associated with the vascular system, the location and internal structure of arthropod hematopoietic organs is extraordinarily variable. Furthermore, for a long time, compact “lymphoid tissues” were thought to represent sites of phagocytosis, mainly because they typically contain mature plasmatocytes or granulocytes that are phagocytotically active (Nutting, 1951; Jones, 1970; Hinks and Arnold, 1977). The multiplication of hemocytes was thought to occur by division of circulating cells (Tauber, 1936; Feir and O’Connor, 1965; Feir and McClain, 1968; Hoffmann et al., 1979). Clear experimental evidence for the hematopoietic role of the compact organs came when by ablating these structures, the number of circulating hemocytes was drastically reduced (Nittono et al., 1964). Following these experiments, numerous investigations were undertaken to characterize the formation and lineage diversification of hemocytes in the hematopoietic centers in detail.

In insects, hematopoietic organs are closely associated with the dorsal vessel and are called lymph glands. This location may reflect the fact that embryonically, as shown in *Drosophila*, prohemocytes and cardioblasts (the cells forming the dorsal vessel) share a common origin. In some cases, as in *Drosophila* or *Gryllus* (Hoffmann, 1970; Roehrborn, 1961; Jung et al., 2005; Mandal et al., 2004), the lymph glands are located anteriorly in the abdomen (Fig.3A); in other cases, they are seen in the entire abdomen (*Locusta*; Hoffmann, 1970; Fig.3B), or alongside the posterior part of the dorsal vessel (*Calliphora*; Zachary and Hoffmann, 1973; Hoffmann et al., 1979; Fig.3C). In lepidopterans, which were studied quite extensively in regard to hematopoiesis, hematopoietic organs are located more laterally near the imaginal discs (Hinks and Arnold, 1977). Typically, they form prominent, paired organs in the mesothoracic and metathoracic segments (Hinks and Arnold, 1977; Nardi et al., 2003), but smaller organs also occur along the segmentally reiterated tracheal spiracles.

In other arthropod groups, the hematopoietic tissue is also connected to the vasculature. In crustaceans, it consists of nodules or sheets located in the head, connected to the ophthalmic artery, or draped over the foregut (Bauchau, 1981; Lin and Soderhall, 2011; Fig.3D). In spiders, the thickened wall of some blood vessels/heart produces prohemocytes which differentiate into the different types of blood cells (Seitz, 1972).

Functional architecture of arthropod hematopoietic organs

The compact hematopoietic organs found in insects and other arthropods are subdivided into distinct zones occupied by either immature hemocyte precursors (prohemocytes) or differentiating/mature hemocytes. In addition to hemocytes and hemocyte precursors, defined by their round shape and characteristic cytoplasmic/nuclear features, other mesenchymal cells, called fibroblast, fibroblast-like cell, or reticular cell, are present in numerous species. In *Drosophila*, the lymph gland appears in the late embryo as a bilobed structure, located on either side of the anterior part of the dorsal blood vessel. Each lobe contains approximately 20 prohemocytes (Fig.4A). During the larval stage, prohemocytes proliferate rapidly. By the third instar, the lymph gland is composed of three or more paired lobes, together possessing many thousands of cells. The anterior (primary) lobe is the largest one and exhibits three distinct zones (Fig.4B,D). In the center of the lymph gland is the medullary zone which contains densely packed, undifferentiated prohemocytes (Jung et al.,

2005). Individual prohemocytes are surrounded by lamellae of extracellular material (“basal laminae” Grigorian et al., 2011; Fig D-E). Surrounding the medullary zone is the cortical zone, which contains more loosely packed, differentiating hemocytes (Fig 4B,D, F-G). These cells express differentiation markers. Two markers (“early differentiation markers”), peroxidase and hemolysin (Jung et al., 2005), are expressed as soon as cells enter the cortical zone. “Late” differentiation markers are the protein P1 for plasmatocytes, and Runx1 for crystal cells (Jung et al., 2005). Hemocytes expressing early differentiation markers are still mitotically very active (see below); those with late differentiation markers become postmitotic. The third zone of the *Drosophila* lymph gland is a cluster of prohemocytes located at the posterior base of the lymph gland, called the posterior signaling center (PSC; Lebestky et al., 2003; Mandal et al., 2007; Krzemien et al., 2007). The PSC, recognizable, by specific molecular markers, such as the homeobox gene Antennapedia, plays an important role in controlling the rate at which prohemocytes located in the adjacent medullary zone are allowed to differentiate. In normal development, differentiation of hemocytes starts at the beginning of the third instar; by the end of the larval period, approximately half of the cells of the lymph gland are differentiated. During the first hours of metamorphosis, the remainder of the prohemocytes differentiates in one rapid burst, and all cells are released from the lymph gland (Fig.4C). Loss of the PSC, or mutations in genes encoding signals (including Hedgehog) produced by the PSC, result in the precocious differentiation of all hemocytes prior to the onset of metamorphosis (Mandal et al., 2007).

Detailed descriptions of hematopoiesis exist for the lepidopterans *Manduca sexta* (tobacco hornworm) and *Bombyx mori* (silkworm). In both, similar to *Drosophila*, compact lymph glands are only found during the larval stage, but not in the adult. In *Manduca*, the lymph gland forms two bilobed masses draped across the wing imaginal discs (Nardi et al., 2003). The inner surfaces of the lymph gland face towards the wing discs; close to the inner surfaces one finds a zone of densely packed highly coherent cells, which express the adhesion protein neuroglian (Nardi et al., 2003; see Fig.1D). Meanwhile, the outer zone of the HPO, which faces the hemocoel, is composed mainly of differentiating cells which have lost their coherence and which are dispersing into the hemocoel. These cells decrease the expression of neuroglian. A similar pattern is seen in the *Drosophila* lymph gland, where adhesion proteins such as DE-cadherin are highly expressed in the medullary zone, but not in the cortical zone.

In *Bombyx mori* (silkworm) the lymph gland is located in the anterior thorax, close to the wing disc. Within the lymph gland one observes loose and compact islets as well as free hemocytes (Han et al., 1998; Fig.5A, B). Compact islets, which appear at early stages and persist throughout larval development, are comprised of proliferating prohemocytes and plasmatocytes (Fig.5). Loose islets contain differentiated hemocytes, and appear in late (5th instar) larvae. Compact islets are attached to tracheae and the wing disc, while the loose islets peripherally surround the compact islets. This arrangement, strongly reminiscent of the spatial relationship between central medulla and peripheral cortical zone in *Drosophila*, suggests that the loose islets developed from the compact islets (Han et al., 1998). Both types of islets are surrounded by a basal lamina. However, the basal lamina associated with the loose islets is thinner and shows disruptions where maturing hemocytes were dispersed into the hemolymph (Han et al., 1998). In a recent study, Joo et al. (2004) created a 3-D rendering of the 5th instar larval *B. mori* hematopoietic organ, and described the cell composition of both compact and loose islets (Fig.5C). Compact islets are comprised of prohemocytes, plasmatocytes and reticular cells (Fig.5D). Loose islets contained mostly differentiated hemocytes. It has been speculated that reticular cells, similar to the PSC of the *Drosophila* lymph gland, play a role in controlling hemocyte proliferation and differentiation (Joo et al., 2004; Hoffman et al., 1979). Reticular cells surround prohemocytes, and gap junctions join reticular cells with adjacent cells (Joo et al., 2004).

In the hemimetabolans *Gryllus bimaculatus* (Mediterranean field cricket) and *Locusta migratoria* (grasshopper) lymph glands exist not only during development, but also in the adult. In *Gryllus*, the lymph gland takes the form of a bilobed structure that flanks either side of the dorsal vessel (Hoffmann, 1979; see Fig.3A). Interestingly, in contrast to what is seen in *Drosophila*, it is the outer, cortical zone that contains undifferentiated prohemocytes, next to reticular cells (Fig.5E). It is stated that prohemocytes arise as daughter cells to rapidly dividing reticular cells. As the prohemocytes differentiate, they form islets composed of similar hemocyte types. Once fully differentiated, hemocytes accumulate in the central zone (“medullary zone”), which is in direct contact with the dorsal vessel (Fig.5E). The mature cells are then able to move into the blood vessel (Hoffmann et al., 1979).

The lymph gland in *Locusta migratoria* (migratory locust) also contains reticular cells which give rise to undifferentiated prohemocytes (Fig.5F). As seen in *Gryllus bimaculatus*, prohemocytes keep proliferating, while differentiating and forming isogenic hemocyte islets. The lymph gland of *Locusta* stretches out along the dorsal vessel on the dorsal surface of the dorsal diaphragm, a muscular sheet that suspends the dorsal vessel from the body wall (Hoffmann et al., 1979). It appears that release of hemocytes in *Locusta* occurs in a way that is more similar to what is seen in *Drosophila*. That is, the mature cells are released directly into the circulating hemolymph of the pericardial sinus. The ventral domain of the lymph gland, which is in contact with the dorsal diaphragm, is comprised of prohemocytes and reticular cells that may be dividing (Fig.5F). The dorsal region contains differentiated cells, which are located in isogenic cell islets. These cells are able to release themselves from the organ, by breaking free from a network of basement lamellae. This release is very reminiscent of what is seen in *Drosophila* during pupal stages, when the hemocytes penetrate the basal lamina surrounding the lymph gland (Grigorian et al., 2011).

A separation of discrete domains containing undifferentiated versus differentiated hemocytes has also been described for apterygote insects, and for crustaceans. In *Thermobia domestica* (Thysanura; Firebrat) the hematopoietic organ is located close to the dorsal vessel, flanking the dorsal diaphragm and the pericardial cells. The organ is organized into two layers: the upper layer, located dorsally of the diaphragm, consists of reticular cells (also called hematocytoblasts), and the lower layer (ventral of the diaphragm) contains islets of differentiating granulocytes and plasmatocytes (Francois, 1975). In the crustacean *Pacifastacus*, the hematopoietic organ forms a layer, several cells thick, and penetrated by blood vessels (Fig.3D). It stretches over the dorsal side of the foregut and is connected anteriorly to the brain. Actively proliferating cells are concentrated in the anterior proliferation zone (Noonin et al., 2012); more differentiated cells form clusters in the main, posterior part of the hematopoietic organ from where they are released continuously into the hemolymph.

Genetic control of stem cells, progenitors and hemocyte lineages in arthropods

The well studied hematopoietic pathway in the bone marrow of vertebrates includes a number of steps. (1) Pluripotent hematopoietic stem cells (HSCs) self renew in response to signals that originate systemically or locally from their microenvironment, the “niche” (2) when HSCs no longer receive niche signals, they become blood progenitors, cells which are committed to a specific blood lineage, and which proliferate rapidly; (3) signals called cytokines control the number and proliferatory activity of progenitors, thereby adjusting the type of blood cells produced at any given moment to the special needs existing at that moment. Does the hematopoietic system in arthropods function in a similar manner?

Stem cells and stem cell renewal—The prohemocytes found in the compact zones or “islets” of arthropod lymph glands exhibit a number of attributes of hematopoietic stem

cells. They appear to be pluripotent, giving rise to most, if not all, blood cell lineages. They are maintained for extended periods of time: in crustaceans and some insects, throughout the lifetime of the animal; in other cases, such as *Drosophila*, until the onset of metamorphosis. It is currently not clear what the relationship is between prohemocytes and the type, found in a few insect species, of presumed blood stem cell called “reticular cells”, or “hematocytoblast” (Hoffmann, 1970; Hoffmann et al., 1979; Francois, 1975). Reticular cells are said to proliferate and give rise to prohemocytes. That is possible in cases where both cell types exist side by side, as for example in *Gryllus* (Hoffmann, 1970). On the other hand, in *Thysanura*, undifferentiated (dividing) cells appear to comprise only reticular cells (Francois, 1975), and no prohemocytes are described. If true, this means that reticular cells differentiate directly into plasmatocytes, granulocytes and the other types of hemocytes found in that species. In *Drosophila* (and other insects) reticular cells do not seem to exist, which makes it difficult to study the relationship between this cell type and prohemocytes using genetic techniques.

Detailed studies of prohemocyte renewal in *Drosophila* revealed numerous fundamental similarities to how this process is regulated in vertebrates. Prohemocytes react to local and systemic factors which, similar to vertebrate “niche signals”, promote their self renewal and inhibit differentiation. Some of these factors even play similar roles in vertebrates and arthropods. In *Drosophila*, the posterior signaling center acts via Hedgehog and Jak/Stat to block differentiation of prohemocytes (Fig.6). Ablation of either the *Hedgehog* signal or the PSC leads to mass-differentiation of all the cells within the lymph gland, leading to a loss of the medullary zone. Conversely, increasing the size of the PSC leads to a larger medullary zone and a smaller cortical zone (Mandal et al. 2007). A study looking at *Stat* loss of function mutants showed a loss of the medullary zone and an increase in differentiation (Krzemien et al. 2007).

In addition to signals acting on the prohemocytes from the PSC, there are factors expressed within the medullary zone, notably Wingless, which maintain prohemocytes in an undifferentiated state (Sinenko et al. 2009). Wingless, which is a key player in the maintenance of vertebrate stem cells (Haegebarth and Clevers, 2009), also plays a role in dictating PSC cell number, as an overexpression of *Wg* in the PSC leads to an increase in PSC cell number. Wingless signaling responds to systemic signals, such as insulin-like growth factor, which adjust hematopoiesis to the nutritional state of the larva (Shim et al., 2012).

Finally, signals derived from differentiated hemocytes act back on to the prohemocytes. This has been shown for the *Drosophila* lymph gland, where Adenosine deaminase growth factor A (Adgf-A), produced by differentiating cells in the cortical zone, antagonizes prohemocyte differentiation (and prolongs proliferation) in the medullary zone (Mondal et al., 2012). Signals secreted by differentiated hemocytes also act on the hematopoietic tissue in crustaceans; here, the peptide astakine 1 promotes differentiation and prevents apoptosis of prohemocytes. Astakine 1 is found in many arthropods, but is lacking in some insect taxa including *Drosophila*. Vertebrate homologs of Ast1 are PROK1/2 peptides; among other functions, these signals promote differentiation of the monocyte lineage (Lin and Soderhall, 2011).

The extracellular matrix that surrounds the densely packed prohemocytes may play an important role in mediating the effect of signals on these cells. This matrix, which forms structurally distinct basement lamellae around individual prohemocytes (Fig.4F, G), acts as a barrier to differentiation and release. In crayfish, astakine 1 acts by decreasing the activity of trans-glutaminase, an enzyme that crosslinks collagen and stabilizes the ECM. Reduction in transglutaminase in the peripheral domain of the hematopoietic organ weakens the ECM

and promotes differentiation (Lin et al., 2008). A similar effect of the ECM has been described in a recent study in *Drosophila* (Grigorian et al., 2012). The homolog of the ECM protein Perlecan, Trol (Datta and Kankel, 1992), is highly expressed in basement lamellae in the fly lymph gland. Mutations in the *trol* gene alter the structure of the ECM; instead of basal lamellae, the ECM forms a spongy mass. Correlated with this ECM phenotype, hemocyte differentiation occurs precociously (Grigorian et al., 2012).

Hemocyte progenitors and the formation of distinct hemocyte lineages—The prohemocytes which populate specialized domains within the hematopoietic organs described in the previous sections start out as pluripotent, rapidly dividing cells, producing most, if not all, of the blood cell types found in the arthropod hemolymph. During a later phase of hematopoiesis, as shown in *Drosophila*, niche signals set in and put a brake on prohemocyte proliferation and differentiation until the end of the larval period, when all prohemocytes appear to differentiate. During this late larval phase, those prohemocytes that are not subject to the niche signal (i.e., cells in the cortical zone) continue to proliferate and at the same time start to express differentiation markers. How are the different cell types, such as crystal cells, plasmatocytes and lamellocytes found in *Drosophila*, specified? What kind of signals are involved, and when do these signals act?

An interesting aspect of arthropod hematopoiesis lies in the observation that, unlike vertebrate blood cells, many types of arthropod hemocytes are interconvertible. Thus, lamellocytes and granulocytes differentiate from circulating plasmatocytes (Markus et al., 2009; Stofanko et al., 2010); plasmatocytes, in other insects, are also capable of differentiating into oenocytoids (Nakahara et al., 2010); spherule cells may be late stages in the life cycle of plasmatocytes and/or granulocytes. That being said, it is also clear that several different types of hemocytes are determined during blood development while still residing within the lymph gland. In *Drosophila*, the specification of crystal cells is triggered by the expression of the transcription factor Lozenge/Runx1, homolog of the vertebrate blood HSC determinant AML1 (Lebestky et al., 2000), in a subset of hemocytes. Loss of this factor results in the absence of crystal cells. Another finding hinting at a fate restriction that sets in before hemocytes differentiate is that certain hemocyte types form only at certain locations, and certain time points, in the developing animal. The origin of hematopoietic organs has been described, using specific markers, in two insects, *Drosophila* (Tepass et al., 1994; Rugendorff et al., 1993; Mandal, 2004) and *Manduca* (Nardi et al., 2003). In these insects, haematogenic centers are set up at two locations in the embryo. The first one is the head mesoderm, which gives rise to a pool of prohemocytes that transform into the hemocytes acting as phagocytes and immune cells in the embryo and larva. The second haematogenic center is the cardiogenic mesoderm of the trunk region, from which the lymph glands discussed in this paper originate. Whereas both haematogenic centers produce plasmatocytes and crystal cells, lamellocytes, specialized differentiative end forms of plasmatocytes, only arise in the cardiogenic mesoderm of the trunk, that is, the lymph gland (Nardi et al., 2003). It is likely that transcription factors expressed in a specific spatial context (that is not met in the head mesoderm of the embryo) play a role in the plasmatocyte-lamellocyte transition. One of these factors is Collier, expressed in the PSC of the larval lymph gland, and required for the formation of lamellocytes (Crozatier et al., 2004). Lack of this factor prevents any formation of lamellocytes upon parasitic infection.

Even though hemocyte-specific determinants clearly do exist and are switched on in prohemocytes prior to the time point when these cells differentiate, there seems to be variation across arthropod species as to whether there will be an onset of rapid proliferation in prohemocytes once they become committed to a specific cell type and start expressing markers for this cell type. In other words, it is not clear whether (as described for vertebrates) there exists a step in which a “stem cell-like, pluripotent prohemocyte becomes

committed and then enters a phase of rapid proliferation (“progenitor”) which produces a single type of differentiated hemocyte, thereby forming clones or “colonies” (Fig.7A). In *Gryllus* and *Locusta* the prohemocytes divide as they start to differentiate and are said to give rise to isogenic clusters of hemocytes of the same type (Hoffmann et al., 1979). However, the evidence documenting this notion is scarce; more detailed studies need to be undertaken to ascertain that the “islets” (clones) of cells derived from a single, rapidly dividing progenitor are really all expressing the same fate (keeping in mind that even certain types of differentiated hemocytes can convert into each other). Islets shown photographically in the literature mostly contain more than one cell type (e.g., Han et al., 1998, their Figs.9-12).

In the hematopoietic organ of crayfish, non-committed prohemocytes (“type 1 and type 2 precursors”) give rise to two different blood cell types, granular cells and semi-granular cells (Fig.7B). Prior to final differentiation of these two cell types, intermediate stages (“type 3, 4, and 5 precursors”) of prohemocytes appear that already express specific attributes of granular cells and semi-granular cells. Proliferation occurs predominantly among the non-committed prohemocytes, and slows down once differentiation sets in (Lin and Soderhall, 2011; Fig.7B).

The same may be true for *Drosophila* hemocytes. As these cells enter the cortical zone and express early differentiation markers, they maintain their proliferative activity. Notch-dependent signaling between these “early hemocytes” (called “intermediate progenitors” in Krzemien et al., 2010a) is important to determine the fate of crystal cells (Lebestky et al., 2003; Krzemien et al., 2010). Subsequently, once cells of the cortical zone express terminal differentiation markers, such as Runx1 (crystal cells) or P1 (plasmatocytes), they exit the cell cycle and become postmitotic (Krzemien et al., 2010a). This would mean that clones or colonies of cells derived from one committed hemocyte “progenitor” (akin to the blood progenitor in the vertebrate bone marrow) may not exist in *Drosophila*. However, even if that were confirmed, more information is needed to generalize this notion to other arthropod species. As discussed earlier, insects like *Bombyx* or *Locusta* have lymph glands containing islands of isogenic cells that may well present clones. Clearly, more research is needed to establish how the processes of proliferation, cell commitment and differentiation relate to each other in the hematopoietic systems of different arthropod groups.

Recruitment of hemocyte lineages—It is clear from a multitude of experimental studies that the rate of hematopoiesis in arthropods is adjustable and responds to demand. For example, wounding and infection leads to a rapid acceleration of hemocyte formation in all systems studied. Signaling pathways involved in this response start to become clear. In the fruit fly, as in vertebrates, the Jak/Stat pathway forms a pivotal link between infection and hematopoietic response (Boutros et al., 2002; Agaisse and Perrimon, 2004). Mutations in genes encoding members of this pathway can modify hemocyte proliferation and differentiation. In line with the observations made in the previous section, the signaling pathways controlling hematopoiesis in the fly mostly appear to act (1) on proliferation of uncommitted prohemocytes, resulting in changes in the production of all blood cell types, or (2) on the differentiation of specific cell types, such as lamellocytes, from other types. Unlike the vertebrate, where it is known that specific cytokines, which can act through the Jak/Stat pathway, target cell type-specific progenitors and expand the clones produced by these progenitors, analogous signals in *Drosophila* are not yet as clear. For example, activation of the Jak/Stat pathway leads to a strong increase in plasmatocytes which then give rise to lamellocytes (reviewed in Agaisse and Perrimon, 2004); on the other hand, loss of the ligand (Unpaired; Upd) also appears to lead to precocious differentiation of prohemocytes in the lymph gland (Minakhina et al., 2011; Krzemien et al., 2007). This

suggests that Jak/Stat may play multiple roles in hemocyte proliferation and differentiation, and that it is affected by other factors that help vary its role (Makki et al., 2010).

Conclusion

Research discussed in this review has revealed many similarities between the types of blood cells, their function, and their development among different arthropod groups. Blood cells are produced in hematopoietic organs that are developmentally and structurally associated with the open vascular system. Immature prohemocytes and differentiating hemocytes are spatially segregated within these organs. One hallmark of undifferentiated prohemocytes is their aggregation into compact “islets” or “zones” (e.g., medullary zone in *Drosophila*) characterized by a high level of cell-cell adhesion. As hemocytes differentiate, their packing density decreases and the formerly “compact islets” transition into “loose islets”/zones. The release of differentiated hemocytes during normal development may be continuous, or is coupled to developmental steps (as, for example, molting or onset of metamorphosis in many insects). Increased demand, caused by immune challenges, is able to override this pattern and result in enhanced proliferation and accelerated differentiation of hemocytes. Molecular pathways controlling the balance between immature, stem cell-like prohemocytes and differentiating hemocytes in the genetic model system *Drosophila* are increasingly revealing homologies to the corresponding pathways in vertebrates. Continued research on this model will lead to a more complete understanding of the network of signals and transcription factors that specify blood cells during normal development and pathological conditions. At the same time, in view of the fact that several aspects of the *Drosophila* blood system (such as its small number of different blood cell types) are most likely derived features, one should welcome and promote genetic-molecular approaches in other arthropods.

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Figure 1.

Origin of hematopoietic tissues. A: In many coelomate invertebrates (such as polychaete annelids represented here as schematic cross section) hemocytes are produced in hematopoietic centers within the somatopleura, splanchnopleura, and blood vessel wall B: Histological section of somatopleura of the polychaete *Nicolea zostericola*, showing hematopoietic center from where groups of hemocytes bud off into the coelom (Scale bar: 18 μ m; from Eckelbarger, 1976, with permission). A: Schematic cross section of insect showing hematopoietic lymph gland (lg) closely associated with the dorsal blood vessel. D: Histological section of the compact hematopoietic organ (lymph gland) of lepidopteran *Manduca sexta* (Scale bar: 50 μ m; from Nardi et al., 2003, with permission).

Figure 2.

A-F: Types of arthropod hemocytes (summarized in E), including (A) plasmatocytes (pl) (from Rowley and Ratcliffe, 1981, with permission), (B) granulocytes (gr) (from Rowley and Ratcliffe, 1981, with permission), (C) oenocytoids/crystal cells (oe/cc) (from Rizki and Rizki, 1984, with permission), (D) prohemocytes (pr) (from Hoffmann et al., 1979, with permission), and (F) spherulocytes/adipohemocytes (sp/ad) (from Rowley and Ratcliffe, 1981, with permission). G-J: Schematic depiction of innate immune reaction carried out by different classes of hemocytes in response to invading microorganisms (ba bacteria; from Rowley and Ratcliffe, 1981, with permission). For detail, see text. Scale bar (A-D, F): 2 μ m.

Figure 3.

Hematopoietic organs (hpo) in different arthropods. (A) *Gryllus bimaculatus*. The drawing shows abdominal segments of a cricket with two paired lymph glands (dark shading) in the body cavity of the 2nd and 3rd abdominal segments (from Hoffmann, 1979, with permission). (B) *Locusta migratoria*. Shown is the abdomen of the locust; the hematopoietic organ is formed by a sheet of cells (stippling) flanking the dorsal vessel (dv; black) (from Hoffmann, 1970, with permission). (C) In the blowfly *Calliphora erythrocephala*, the hematopoietic organ flanks the posterior tip of the dorsal vessel (dv) (from Hoffmann, 1979, with permission). (D) Head of the lobster *Orconectes limosus*, (crustacea); the hematopoietic organ forms a multilayered sheet covering the foregut (fg) (from Böhm and Gersch, 1983, with permission). Other abbreviations: am alary muscle (forms the diaphragm); br brain; tr trachea.

Figure 4.

Cellular architecture of the *Drosophila* lymph gland. A-C: Confocal sections of lymph gland, dorsal view, anterior to the top. A: Late embryo; B: Third instar larva; C: Eight hours after puparium formation (from Grigorian et al., 2011, with permission). Basement membranes around lymph gland cells are labeled by reporter for ECM protein Trol/Perlecan (green). Prohemocytes of embryonic lymph gland, as well as pericardial cells, are labeled by antibody against Odd skipped (Odd, red); differentiating hemocytes of late larval and pupal lymph gland are labeled by antibody against Peroxidase (Pxn). Nuclei are labeled with Sytox (blue). D-G: Electron micrographs of section of late larval lymph gland (From Grigorian et al., 2011, with permission). E: Low magnification of section of primary lymph gland lobe. Medial border of lymph gland (left) is marked by the wall of the dorsal vessel (dv). Cells in the medial two thirds of the lymph gland form the medullary zone (mz). These cells are densely packed and show the ultrastructural characteristics of prohemocytes (pr; shown at higher magnification in E). In the periphery of the larval lymph gland (cortical

zone, cz; demarcated from medullary zone by dotted line) cells are more loosely packed and show features of differentiated plasmotocytes (pl; see higher magnification in F, G).

Abbreviations: bl basal lamina; cz cortical zone; dv dorsal vessel; lgpl primary lobes of lymph gland; lgsl secondary lobes of lymph gland; mz medullary zone; pc pericardial cells; pl plasmotocytes; pr prohemocytes; psc posterior signaling center. Bars: 10 μ m (A-C); 5 μ m (D); 1 μ m (E, F); 0.5 μ m (G)

Figure 5.

Cellular architecture of lymph gland in lepidopteran *Bombyx mori* (A-F) and the orthopterans *Gryllus bimaculatus* (G) and *Locusta migratoria* (H). A, B: Low power electron micrographs of compact islet (ci; A) and loose islet (li; B) of the *Bombyx* lymph gland (from Joo et al., 2004, with permission). Scale bars = 5 μ m. C: Digital 3D model showing number and pattern of compact islets (shades of green) in *Bombyx* lymph gland. D: Digital 3D model of compact islet, depicting distribution of reticular cells (rc), prohemocytes (pr), and plasmatocytes (pl; C and D from Joo et al., 2004, with permission). E, F: Line drawings of sections of lymph glands in *Gryllus* and *Locusta* (from Hoffmann, 1970, with permission). In both drawings medial is to the left. In *Gryllus*, compact zone with prohemocytes (pr) is located underneath the capsule surrounding the lymph gland (right of drawing); loose zone (“medullary zone”) containing differentiated granulocytes (gr) seen at the left. Reticular cells (rc) surround hemocytes and secrete fibers of extracellular matrix (ef). In *Locusta* (F), compact islets (ci) with prohemocytes, and loose islets (li) with differentiated hemocytes are scattered throughout the lymph gland.

Figure 6. Schematic drawing of the *Drosophila* larval lymph gland (primary lobe), showing zonation and known signaling interactions involved in controlling the balance between undifferentiated prohemocytes (medullary zone) and differentiating hemocytes (cortical zone). For detail see text.

Figure 7.

Hematopoietic stem cells, progenitors and blood cell lineages. **A:** Mammalian bone marrow. When leaving the niche, daughter cells of the hematopoietic stem cells become committed as specific blood progenitors; these undergo rapid divisions and produce clones of specific blood cell types ("Type A, Type B"). **B:** Hematopoietic organ of crustacean (based on Lin and Soderhall, 2011). Prohemocytes go through a series of morphologically distinguishable phases (types). Initially, type 1 and 2 prohemocytes are not yet committed to a specific blood cell fate. Proliferation occurs mostly during this phase, and is promoted by the signal astakin 1 (ast1). Subsequently, as the split between two lineages occurs, proliferation slows down; prohemocytes type 3 and type 4 give rise to granular cells, type 5 to semi-granular cells. **C:** *Drosophila* lymph gland. The medullary zone contains undifferentiated, uncommitted prohemocytes. As these cells leave the niche environment, they become "intermediate progenitors": they simultaneously express early differentiation markers, and undergo several rapid rounds of division. Signaling events during this phase differentiate between two cell types, plasmatocytes and crystal cells. Once these express their final ("late") differentiation markers, they become postmitotic.