

REVIEW

Innate immunity

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ABSTRACT In this review, we discuss how studying the *Drosophila* immune system contributes to a better understanding of the basic principles of innate immunity. We describe the homologies between the insect and the vertebrate immune-regulatory mechanisms and convergent evolutionary traits of the *Drosophila* and the vertebrate immune system.

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KEY WORDS

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Defense and immunity

Metazoans have inborn structures and mechanisms for locomotion, sensing, reproduction, maintaining the homeostasis and defending against foreign invaders. The proper, coordinated functioning of these systems is essential for the evolutionary success and survival of the species. The inborn defense system, which protects the organism from infection by microorganisms and parasites, includes mechanical, humoral and cellular barriers. The innate immune system consists of cells and proteins which are present in the organism from birth and are ready to combat invaders. The basic task of this defense system is to recognize and eliminate anything foreign, *i.e.* to discriminate self from non-self, thereby protecting the organism from invaders (Janeway Jr and Medzhitov 2002). In consequence of its versatility and the available powerful genetic, genomic and immunological tools, the fruit fly *Drosophila melanogaster* has become a model organism in which to study and understand the basic mechanisms of innate immunity and host-pathogen interactions (Sackton et al. 2007; Kounatidis and Ligoxygakis 2012). The results of such studies have revealed that there are substantial similarities between the *Drosophila* immune response and vertebrate innate immunity (Hoffmann et al. 1999), and that most of the genes involved in the regulation of *Drosophila* immunity are similar to those involved in the innate immunity of vertebrates (Engström et al. 1993; Hoffmann et al. 1999).

Innate immunity - adaptive immunity

All multicellular organisms are constantly exposed to microbes, which are generally in balance with the host organism. On the epithelial surfaces of the skin, in the respiratory system and in the gut, they far outnumber cells of the body. Some organisms, such as *Drosophila*, even live in a soup of microorganisms, in fermenting material. In *Drosophila*, these microbes normally serve as a source of food or survive in equilibrium with the organism by becoming part of the normal gut flora (Broderick et al. 2014). Injury or any disturbance of the gut flora permits microbes to pass through the epithelial surfaces, multiply in the body fluids and use the host's components as a source of energy. This leads to pathogenicity and a systemic activation of the immune system (Ferrandon et al. 2007; Fig. 1).

In vertebrates the immune system is composed of two arms, the innate immune system and the adaptive immune system. The innate immune system, which is evolved in all metazoans, is already functional at birth, constantly present and ready to act immediately upon detecting an invader. It comes into action efficiently through preformed effectors, encoded for by the germ line, with a restricted array of specificity to common molecular patterns of invaders (Beutler 2004). The adaptive immunity present in vertebrates is normally silent, however, when an invader is sensed, it adapts to its presence and develops mechanisms to eliminate it. This development after exposure to an invader requires time for the protective action to become functional and needs the contribution of the innate system (Medzhitov et al. 1997). Its specificity is engineered by somatic cell gene rearrangements, which generate an enormous array of receptors which distinguish

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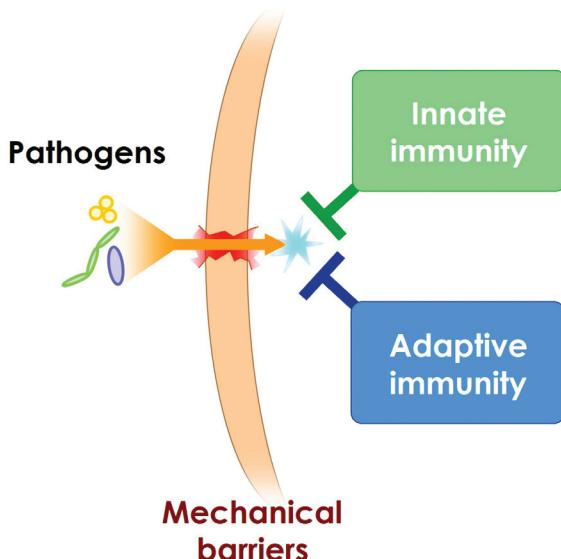


Figure 1. The activation of the immune response against invaders.

minor differences between closely-related structures of the invaders (Cook and Tomlinson 1995; Krangell 2009).

The generated specificities are fixed in memory cells and, upon repeated exposure, they ensure a fast, specific response. The efficient immune response develops through the cooperation of the two arms of the immune response and any malfunction in the innate immune system has severe consequences, and in many cases lethality, which underlines the importance of the innate immune system. Although the cellular and the humoral elements have different functions, they interact with each other in the course of an efficient immune response (Fig. 2).

Epithelial surfaces provide physical barriers

The mechanical barriers are the epithelium of the skin, the respiratory system and the digestive tract. The tight junctions between epithelial cells block the entry of the microorganisms into the body, but these cells also have anatomical and biological constituents, such as cilia to sweep away and mucus to trap microorganisms and prevent their entry into the tissues. The mucus also contains peptides with a broad spectrum of antimicrobial activity, e.g., the defensins kill Gram-positive and Gram-negative bacteria, fungi and parasites. The respiratory tract and the digestive system accommodate a natural (commensal, symbiotic) flora of fungi and bacteria which are required for normal development and metabolism. This flora forms an important part of the first-line defense as it prevents invasion by other, pathogenic microorganisms by competing for sources of energy. Invasion by pathogenic bacteria elicits

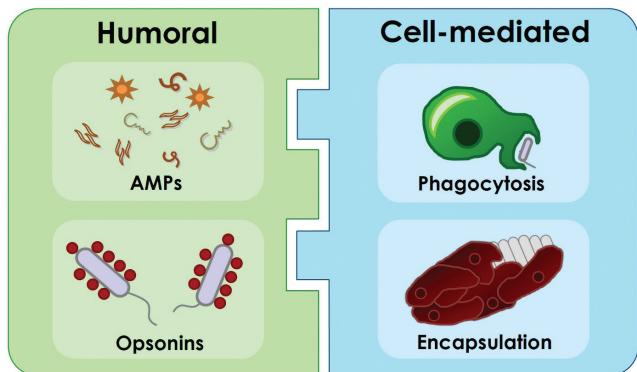


Figure 2. The conserved defense mechanisms of the innate immune system.

both local and systemic immune responses to fight off infection (Nehme et al. 2007).

Sensing and signaling in innate immunity

When barriers are damaged, microorganisms or parasites can enter the tissues. The non-self recognition of the innate immune system is based on a limited number of germline-encoded pattern recognition receptors (PRRs), which detect evolutionarily conserved structures on pathogens, termed pathogen-associated molecular patterns (PAMPs), which are not found in the host (Janeway Jr and Medzhitov 2002). PRRs are also involved in sensing endogenous 'danger' signals by recognizing danger-associated molecular patterns (DAMPs). PRRs are expressed by professional immunocytes that engulf and destroy pathogens or act as humoral factors in the extracellular compartment. Ligand engagement of PRRs induces receptor oligomerization, which subsequently triggers intracellular signaling pathways, and induces effector mechanisms, the activation of gene expression and the synthesis of proinflammatory cytokines, chemokines, antimicrobial peptides and cell adhesion molecules. These responses also initiate the development of adaptive immunity (Barral and Brenner 2007).

The cellular components of innate immunity express the Toll-like pattern recognition receptors (TLRs) at their cellular or endosomal membranes (Medzhitov et al. 1997). TLRs are glycoproteins characterized by an extracellular or ligand-binding domain containing leucine-rich repeat (LRR) motifs and a cytoplasmic signaling Toll/interleukin-1 (IL-1) receptor homology (TIR) domain. The TLRs derived their name from their *Drosophila* homolog Toll receptor, which is involved in embryogenesis and the antimicrobial response in the fruit fly (Lemaitre et al. 2004). In humans, 10 TLRs have been

identified that recognize a variety of PAMPs from bacteria, fungi, parasites and viruses, including lipid-based bacterial cell wall components such as lipopolysaccharide (LPS) and lipopeptides, microbial protein components such as flagellin, and nucleic acids such as single-stranded or double-stranded RNA and CpG DNA (Akira et al. 2003).

Components of internalized or intracellular pathogens and their derivatives are detected by cytosolic PRRs. The nucleotide binding oligomerization domain (NOD) receptors NOD1 and NOD2 receptors sense bacterial molecules derived from the synthesis and degradation of peptidoglycan. The NOD-like receptors are characterized by a tripartite-domain organization with a conserved NOD and leucine-rich repeats (LRRs) (Kanneganti et al. 2007). The RIG-I-like receptors, which are involved in the recognition of viruses, constitute a family of three cytoplasmic RNA helicases that are critical for host antiviral responses through the detection of exogenous dsDNA, leading to the induction of interferons and/or the processing of pro-inflammatory cytokines (Yoneyama et al. 2004). C-type lectin receptors (CLRs), including the dectin-1 and mannose binding lectin (MBL), bind to carbohydrates in a calcium-dependent manner through their carbohydrate-recognition domains. CLRs are involved in fungal recognition and in the modulation of the innate immune response (Geijtenbeek and Gringhuis 2009). Cytosolic dsDNA sensor molecules bind dsDNA to prevent cytokine induction.

The sensing of microbes in the extracellular compartment is prompted by humoral factors such as lysozyme, lactoferrin, complement proteins and antimicrobial peptides. Lysozyme destroys the cell wall of Gram-negative and Gram-positive bacteria by enzymatic mechanisms, while lactoferrin forms biofilms on certain bacteria. Complement proteins are proteolytic enzymes activated in the presence of microbes (Dunkelberger and Song 2010). The classical pathway of complement action is activated by antibodies combined with microbes to which the host has previously been exposed. The alternative pathway is triggered directly through the contact of certain complement components C3, factor B, factor D and properdin to microbial surfaces. The lectin pathway is initiated by the interactions of microbial polysaccharides with the soluble mannose-binding lectins. The activation of the complement cascades generates complement component C3b, which binds to microbes and opsonizes them for phagocytosis. The generation of the inflammatory and chemotactic C5a and the activation of C5 initiates the membrane attack complex to lyse Gram-negative bacteria, and also to inactivate viruses.

Humoral defense mechanisms

Injuries and the cell wall components of microbes activate humoral responses like proteolytic cascades, which kill and

eliminate the invaders and facilitate the healing of the lesion; these processes involve the complement system in vertebrates (Dunkelberger and Song 2010), the melanization reaction in insects and the blood clotting system in all metazoans with a circulatory system (Cerenius et al. 2010; Theopold et al. 2014).

In insects, melanin and proteins produced after activation of proteolytic cascades are important to prevent loss of body fluids through lesions, or, for directly killing certain pathogens by their toxic properties (Cerenius and Söderhäll 2004). Melanin is the final, toxic product of a proteolytic cascade. When active forms of the proteins are present in the body fluid of insects, melanin is deposited on the eggs of parasites and tumours (Ashida and Brey 1995). Pathogens are insulated by blood clotting too. Besides preventing blood loss, the main purpose of the coagulation is to localize the infectious agents on the site of injury. The blood clot is formed by the contribution of several proteins, some of them functioning in cleavage of precursor proteins (Karlsson et al. 2004).

In vertebrates, lactoferrin and transferrin limit the growth of bacteria by sequestering free iron, and thereby remove this essential substrate (required for bacterial growth) (Actor et al. 2009). The interferons limit virus replication, the enzyme lysozyme breaks down bacterial cell walls, and the interleukins induce inflammatory proteins, and some have antimicrobial activity too. There are three major families of antimicrobial peptides (AMPs) in vertebrates: the defensins (Lehrer and Ganz 2002), the cathelicidins and the histatins (Ganz and Lehrer 1998). The defensins are an ancient class of antimicrobial peptides that are produced by insects, plants and vertebrates. There are several families of defensins: over 13 defensins are known in plants, over 15 in *Drosophila*, and at least 20 in humans, their genes forming a single gene cluster on chromosome 8. These peptides are characteristic of a disulfide-bound stabilized amphipathic region and are able to disrupt bacterial and fungal cell membranes and also envelopes of some viruses. It is supposed that they are incorporated into the cell membrane and form pores, which make the cell membranes leaky. Cathelicidins have been identified in humans and mice (Kościuczuk et al. 2012). They are produced constitutively by neutrophil granulocytes, macrophages and epithelial cells in the gastrointestinal system and in the lung after infection. They are activated by proteolytic cleavage. The histatins are secreted into the saliva and kill pathogenic fungi such as *Candida albicans* in the oral cavity (Yan and Bennick 1995).

In insects, large families of antimicrobial peptides have been identified (Lemaitre et al. 1997; Bulet et al. 1999). They are produced by the fat body (the equivalent of the vertebrate liver) and by blood cells, called hemocytes and are secreted into the body fluid, the hemolymph. Their production is induced in response to infection by microbes or some parasites. On the basis of their sensitive target, they can be

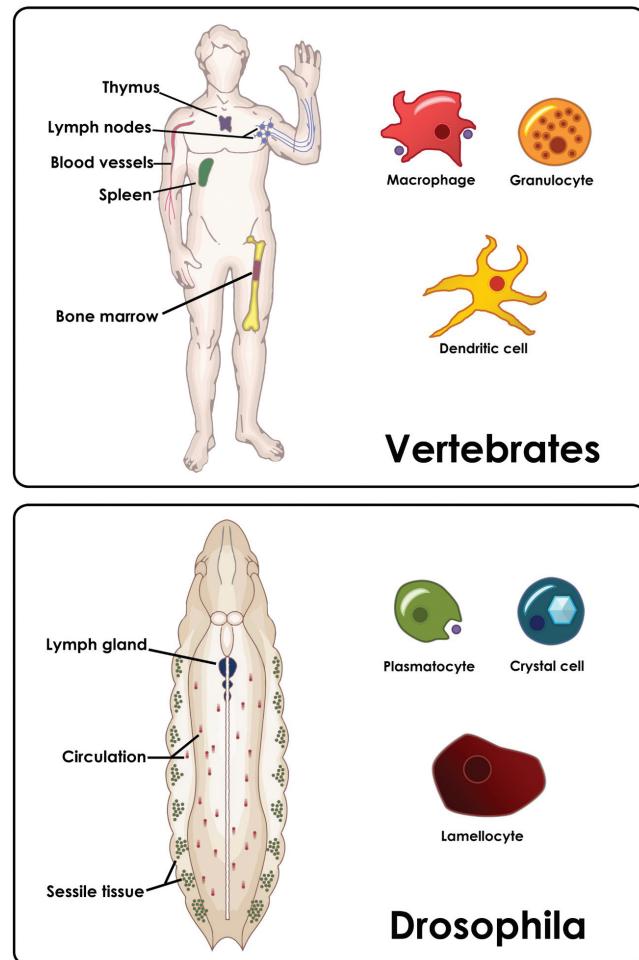


Figure 3. Hematopoiesis in insects and vertebrates. The embryonic and post-embryonic hematopoietic tissues and organs are indicated.

grouped as (a) defensins (Gram-positive bacteria and fungi), (b) cecropins, drosocin, attacins, MPAC (matured pro-domain of attacin C) and diptericin (Gram-negative bacteria) or (c) drosomycin and metchnikowin (fungi). These proteins are generally produced as inactive proproteins, which need to be cleaved to the final amphipathic structure that is integrated into the microbial membrane, leading to disruption of the microbe (Lemaitre and Hoffmann 2007). The *Drosophila* model has revealed that immune genes of insects contain an upstream sequence that shares homology with the binding site of the mammalian nuclear-factor kappa B (NF- κ B) (Sun and Faye 1992), and AMP synthesis is controlled by the NF- κ B protein (Dushay et al. 1996), indicating an ancient origin of certain modules of the innate immune response, conserved from insect to man (Engström et al. 1993). The AMPs are membrane-active, but the mechanism through which antimicrobial peptides kill microbes is still under investigation.

Cell-mediated defense mechanisms

The immune cells involved in the innate immunity of vertebrates are the monocytes, macrophages, dendritic cells, neutrophil granulocytes, mast cells, basophil cells, eosinophils and natural killer cells. After being recruited to the site of infection, monocytes differentiate into tissue macrophages. After taking up and ingesting microorganisms, they present antigen for lymphocytes for induction of the antigen-specific (adaptive) immune response. Dendritic cells are phagocytic cells residing in the tissues (more specifically the lymph nodes and the skin). After ingesting the antigen, they present fragments in the context of major histocompatibility complex (MHC) antigens to the lymphocytes. The antigen-presenting cells, the macrophages and the dendritic cells, serve as an important link between innate and adaptive immunity. The neutrophils recruited from the circulation to the site of the infection differentiate into polymorphonuclear cells, phagocytose and kill microorganisms through the participation of reactive oxygen species. The mast cells and basophils release histamine and active agent-containing granules. The eosinophils take up bacteria by phagocytosis, but are additionally involved in the destruction of parasites. Natural killer cells are able to recognize and kill virus-infected cells or tumor cells. Thus, they provide early protection against viral infections. These cells develop from stem cells in the bone marrow and upon maturation enter the circulation or occupy specific niches in the tissues, e.g., mast cells in the epithelium and basophils in the circulation (Beutler 2004).

In insects, several classes of immune cells have been described (Gupta 1986). The best-studied organism is *D. melanogaster*, where three main classes of effector blood cells (hemocytes) have been identified on the basis of morphological (Rizki and Rizki 1980) and immunological criteria (Kurucz et al. 2003, 2007a, 2007b; Honti et al. 2010, 2014) and with the aid of genetic markers (Lebestky et al. 2000; Kurucz et al. 2003; Zettervall et al. 2004; Honti et al. 2009; Tokusumi et al. 2009). The use of combinations of immunological and genetic markers has allowed a thorough definition of functional cell types and lineages (Evans et al. 2003; Honti et al. 2010; Fig. 3). The phagocytic cells, which resemble the phagocytic cells of vertebrates, are called plasmatocytes; they are spherical cells capable of ingesting and killing microorganisms (Stuart and Ezekowitz 2008). Besides their phagocytic function, they produce antimicrobial peptides and extracellular matrix proteins and may play a role in the elimination of certain tumors. They are also involved in the defense against parasites at an early stage of the isolation and killing of parasite eggs, the encapsulation reaction. Plasmatocytes have been assumed to be terminally differentiated cells. It was recently shown that they are capable of transforming

into another cell type, the non-phagocytic lamellocyte (Honti et al. 2010). The crystal cells are also spherical cells, of the same size as plasmacytocytes. They contain enzymes, in the form of crystals, which are required for the melanization reaction (Gajewski et al. 2007). The third cell type is the lamellocyte (Rizki and Rizki 1992). These are large, flat cells, which are involved in the encapsulation reaction by enveloping the parasite egg and melanizing it (Carton and Nappi 1997). Plasmacytocytes and crystal cells are ready to act when an invader is sensed or after an injury, while lamellocytes develop after parasitic infection or sterile wounding, and just before pupation.

The hemocytes are organized in three compartments: the circulation, the lymph gland, and the sessile hematopoietic tissue (Fig. 3). The hemocytes circulate freely in the open circulatory system. Over 90% of the circulating cells are plasmacytocytes, crystal cells account for only a minor fraction in this compartment. Lamellocytes appear in a low number before pupa formation, but arise rapidly after infection by parasitic wasps, in which case their number constitutes more than 30% of the circulating hemocyte pool (Honti et al. 2010). The lymph gland (comprising paired primary and accessory lobes) serves as an organ for precursors and differentiating effector cells. The differentiation of the hemocytes takes place in zones, defined by transcription factors and immunological markers (Mandal et al. 2007; Kurucz et al. 2007b). A specific zone, involving a cluster of a few cells, defined by a transcription factor, Collier, is postulated to be the master regulator of hemocyte development in this organ (Crozatier et al. 2004). After immune induction by parasitic wasps, the structure of the organ is disrupted and the effector hemocytes are released and enter the circulation. If the development of the larva is unhindered, the plasmacytocytes and the crystal cells from this organ contribute to the hemocyte population in the adult. A set of hemocytes, named the sessile hematopoietic tissue, attaches to the body wall in a striped pattern, and comprises plasmacytocytes and crystal cells. The function of this tissue was recently revealed (Márkus et al. 2009) to serve as a source of lamellocytes in response to immune stimulation. It was also observed that plasmacytocytes constantly attach to and detach from the sessile compartment, thereby establishing a dynamic steady state with the circulating hemocyte pool (Makhijani et al. 2011).

Microbial contacts stimulate a phagocytic response by the innate immune cells, the professional phagocytes such as the macrophages, neutrophils and dendritic cells. In the course of phagocytosis, particles bind to cell-surface receptors directly or via opsonins, activating complex signaling networks, which rapidly lead to a cytoskeletal reorganization followed by internalization and killing of the microbes (Aderem and Underhill 1999). Phagocytosis is a complex process (Fig. 4); phagocytes express a broad spectrum of receptors, and the recognition and internalization of particles recognized

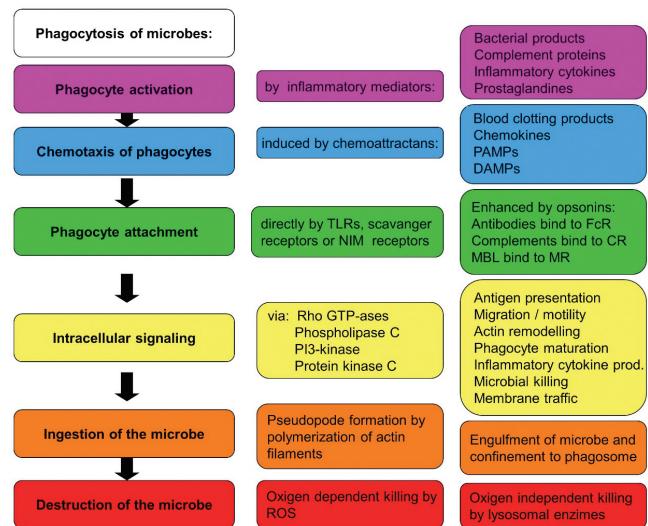


Figure 4. The underlying mechanisms of phagocytosis. The six stages are indicated by different colors.

as non-self are usually mediated simultaneously by multiple receptors. Phagocytosis receptors induce different signaling pathways that interact cooperatively. Signals induce inflammatory responses that affect the efficiency of particle internalization, clustering neighboring phagocytes, and produce the molecules required for efficient antigen presentation to the adaptive immune system. Phagocytes express a broad spectrum of receptors; many of them transduce signals into the cytoplasm that trigger phagocytosis, while others participate in the binding of microbes or increase the efficiency of internalization.

In vertebrates, the expression of Fc-receptors, complement receptor 3 and the mannose receptor in a non-phagocytic cell demonstrated that they are involved in the internalization of specific target particles. Macrophages and neutrophils express different combinations of Fc receptors for the recognition of IgG-opsonized particles containing immunoreceptor tyrosine-based activation motifs in their intracellular domains that recruit kinases and activate phosphorylation cascades, or containing immunoreceptor tyrosine-based motifs that recruit phosphatases that inhibit signaling (Ravetch and Bolland 2001). Activating receptors with high affinity (Fc γ RI) and low affinity (Fc γ RIIA and Fc γ RIIIA) bind to the IgG-opsonized particles and trigger their engulfment through actin polymerization. Complement receptors bind complement proteins in the serum, opsonize microbes through antibody-dependent or antibody-independent mechanisms. Complement receptor 1 (CR1) is expressed on erythrocytes, B cells, monocytes, neutrophils, eosinophils and dendritic cells, Complement receptor 3 is found on monocytes, macrophages, neutrophils, granulocytes, dendritic cells and NK cells, and complement receptor 4 binds to microbial opsonins such as

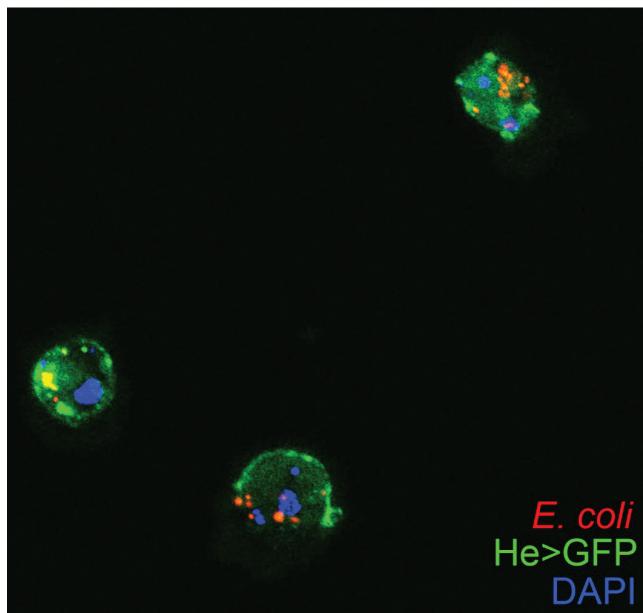


Figure 5. Phagocytosis of TRITC labelled *E. coli* (red) by GFP expressing *Drosophila* blood cells (green). Blue staining indicates the nuclei.

certain complement components (Klickstein et al. 1997) and MBL (Ghiran et al. 2000).

The scavenger receptors are structurally unrelated multiligand receptors that bind polyanionic ligands expressed by different pathogens and modified self-cells (Dunne et al. 1994) and it is likely that coreceptors generate the internalization signals (Peiser et al. 2000).

SR-A is a transmembrane homo-trimer expressed on most macrophages; it binds whole bacteria and also the microbial cell wall components, lipoteichoic acid and LPS. The class B SRs, CD36 and its *Drosophila* homolog known as Croquemort (Franc et al. 1996), are multifunctional receptors in mammals and flies. The CD36 receptors not only act as phagocytic receptors, but also regulate TLR4 and TLR2 signaling (Wright et al. 1990). Croquemort is a receptor for apoptotic cells and also binds *S. aureus*; this led to the identification of mammalian CD36 as a phagocytic receptor for *S. aureus*. Another class B SR, Peste, was identified in *Drosophila* to be involved in the recognition of mycobacteria, suggesting that mammalian class B SRs may also participate in the recognition of mycobacteria (Philips et al. 2005).

Mammalian phagocytes express a wide variety of surface lectins that recognize self and foreign carbohydrates. The mannose receptor expressed on subpopulations of macrophages and dendritic cells binds α -mannan (Ezekowitz et al. 1990), and dectin-1, originally defined as a dendritic cell-specific receptor, binds β -glucan (Brown and Gordon 2001) in the yeast cell wall.

A new family of PRRs that use EGF-like repeats in the

recognition of diverse ligands has been identified in *Drosophila*. Members of this family are also found in many insect species, *C. elegans* and mammals, in which they might have similar functions. Eater is a type I membrane protein that contains 32 characteristic EGF-like NIM repeats in the extracellular domain. NimC1 is also a single-pass transmembrane protein with 10 NIM repeats expressed by *Drosophila* phagocytic cells called plasmacytocytes (Kocks et al. 2005; Kurucz et al. 2007b). Both receptors act as phagocytic receptor; they bind bacteria directly and are also involved in cell adhesion (Kurucz et al. 2007b). The gene encoding NimC1 is part of a cluster of 10 related Nimrod genes. Similar proteins are also found in the silkworm and the beetle *Holotrichia diomphalia* (Kocks et al. 2005; Kurucz et al. 2007b; Zsamboki et al. 2013).

The ligand binding of phagocytosis receptors activates signaling pathways that together induce the rearrangement of the actin cytoskeleton, extension of the plasma membrane and engulfment of the particle. Various signaling molecules, including actin binding proteins, membrane traffic regulators, ion channels, kinases and lipases, are activated during phagocytosis; phosphoinositide 3-kinase, phospholipase C, Rho GTPases and PKC regulate this process (Stuart and Ezekowitz 2005).

During microbe internalization, several TLR family members are recruited to phagosomes, where they sample the contents of the phagosomes to determine the nature of the microbes being ingested (Underhill et al. 1999). Professional phagocytes kill pathogens by the production of reactive superoxide ions through an assembly of NADPH oxidase on phagosomal membranes. Microbe internalization by phagocytes may induce pro-inflammatory signals. The production of cytokines such as IL-1, IL-6 and TNF- α , and chemokines such as IL8, are critical in the development of an effective innate immune response and in the initiation of the adaptive immune response through maturation of antigen-presenting DCs and the activation of antigen-specific T lymphocytes to resolve the infection and induce immunological memory (Iwasaki and Medzhitov 2004).

The consequences of phagocytosis depend on the microbial target, since many pathogenic microbes regulate the mechanisms of phagocytosis to evade destruction. The evolved diversity and redundancy of phagocytic molecules and mechanisms must reflect evolutionary pressure. An emerging number of novel genes involved in the phagocytosis of bacteria and apoptotic cells were found through the use of genetically exploitable model organisms, including *Drosophila* or *C. elegans*, which have phagocytic cells (Reddien et al. 2001; Manaka et al. 2004; Kocks et al. 2005; Kurucz et al. 2007b; Fig. 5).

Phagocytes have difficulties with certain pathogens. *Listeria monocytogenes* can escape from the phagosome into the cytosol. Macrophages can usually engulf *Mycobacterium*

tuberculosis, but the bacterium prevents the lysosomes from fusing with the phagosome and the bacteria may stay alive within the macrophage. As a result, these cells will be surrounded by other macrophages, forming a type of chronic inflammation called granuloma. In response to foreign particles that are too large to be taken up by phagocytosis, a special cell may differentiate, the lamellocyte in *D. melanogaster* and the multinucleated giant cell in vertebrates. These cells differentiate from phagocytic cells (Honti et al. 2010) and are actively involved in the insulation of the particles and tumours. Recently, a so far unrecognized cell type, named as the multinucleated giant hemocyte (Márkus et al. 2015) was discovered in *Drosophila*, which, by many criteria resembles the multinucleated giant cell of vertebrates (Fig. 6).

***Drosophila* as a model system to study the differentiation of blood cells**

Even though there are a number of differences between the immune systems of insects and vertebrates, striking similarities can be observed in the differentiation of their blood cells. In both taxa, blood cells differentiate in multiple waves and localize in separate hematopoietic compartments (Dzierzak et al. 2008; Márkus et al. 2009). Although insects lack adaptive immunity, and therefore rely on their very effective innate immune system against infections and invaders, the development of their blood cell lineages shows similarities to the differentiation of the vertebrate myeloid and also the lymphoid blood cell lineages. Due to these similarities, and the well-established genetic background of *D. melanogaster*, the immune system and blood cell differentiation of the fruit fly is widely studied as a model through which to understand the basic features of hematopoiesis.

Hematopoietic compartments, the hematopoietic niche

In vertebrates, blood cells first differentiate in the extra-embryonic blood islands of the yolk sac. Early intra-embryonic hematopoiesis takes place in the aorta-gonad-mesonephros (AGM) region of the embryo. Later in development, hematopoiesis occurs in the spleen, the liver and the lymph nodes. After the formation of the bone marrow, it serves as the source of most of the blood cells, but secondary lymphoid organs, such as the thymus and the lymph nodes, are also involved in the proliferation and maturation of certain blood cell types: the T cells and B cells, respectively (Hartenstein 2006). In the bone marrow, hematopoietic stem cells (HSCs) are localized in a special microenvironment, the hematopoietic

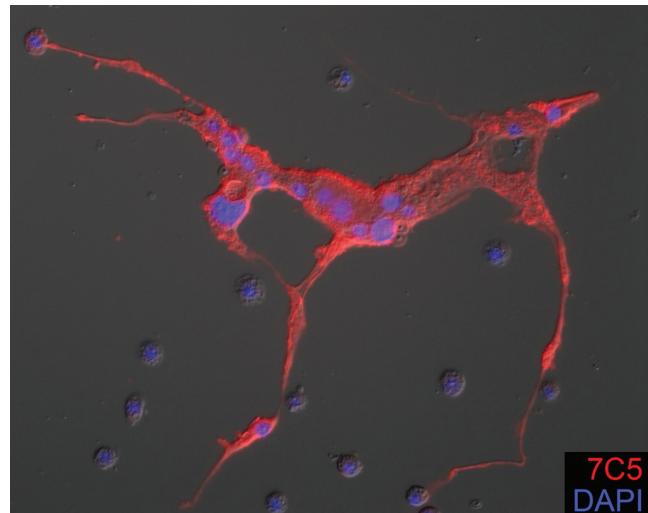


Figure 6. The multinucleated giant hemocyte of *Drosophila ananassae*. The red staining indicates the giant hemocyte, blue staining indicates the nuclei (Artwork by Dr Róbert Márkus).

niche, in which stromal cells affect the multipotency and self-renewal of the HSCs (Wilson and Trumpp 2006).

In the *Drosophila* embryo, two mesodermal layers, the procephalic and the cardiogenic mesoderm anlagen, take part in hemocyte differentiation. The procephalic mesoderm anlage gives rise to macrophages and crystal cells, while the cardiogenic mesoderm anlage produces hemocytes that build up one of the larval hematopoietic compartments, the lymph gland (Holz et al. 2003; Honti et al. 2010). Genetic lineage tracing experiments revealed that the other two hematopoietic compartments of the larva, the circulation and the sessile tissue (a compartment of hemocytes attached to the sub-epithelial layer of the body wall), originate from the embryonic macrophage cell lineage, and do not mix with lymph gland cells without immune challenge (Márkus et al. 2009; Honti et al. 2010). The lymph gland of the *Drosophila* larva is the best-characterized hematopoietic compartment in insects. It consists of three regions: the cortical zone containing differentiated effectors, the medullary zone, in which prohemocytes proliferate, and the posterior signaling center (PSC), which has been described as a hematopoietic niche in *Drosophila*. The cells of the PSC, which are determined by the expression of Antennapedia and Collier (Mandal et al. 2007; Krzemien et al. 2007), emanate long filopodia to the medullary zone of the lymph gland and block the differentiation of prohemocytes, thereby maintaining their precursor state. Genetic studies have shown that Hedgehog and Decapentaplegic and the JAK/STAT pathway play major roles in the function of the lymph gland (Fossett 2013). However, less is known about the regulation of cell differentiation events in the sessile tissue and in the circulation. Neurons were recently demonstrated to be

involved in the hemocyte homing in the sessile compartment (Makhijani et al. 2011). In the circulation, many factors are known to affect blood cell proliferation and differentiation, but the regulatory networks have been characterized only tentatively (Zettervall et al. 2004).

Transcriptional and epigenetic regulation of hematopoiesis

Similarly to other developmental processes, hematopoiesis is mainly regulated by the formation and maintenance of gene expression patterns, which define the morphology and function of each blood cell type. Genes that are indispensable for a certain function are activated, whereas other genes are inactivated; as an example, in the B cells, B cell receptor genes are active, while the phagocytic receptor genes are inactive (Parra 2009). Gene expression patterns are formed by transcription factors that specifically bind *cis*-regulatory target sequences on the DNA, thereby promoting the transcription of target genes (Latchman 1997). To ensure the proper gene expression patterns, transcription factors are expressed cell-type specifically.

The transcriptional regulation of blood cell differentiation is based on evolutionarily well conserved regulatory factors (Williams 2007). In mammals, 3 GATA factors (GATA-1, -2 and -3) control various aspects of hematopoiesis and lineage commitment (Fossett and Schulz 2001). Interestingly, one of the 5 *Drosophila* GATA factors, Serpent, is expressed by all hemocyte types, and its role is essential for hematopoiesis (Rehorn et al. 1996). Another *Drosophila* GATA homolog, *pannier*, regulates the differentiation of both hemocytes and the dorsal vessel, the *Drosophila* heart tube (Minakhina et al. 2011). Two other *Drosophila* proteins, which are also homologs of mammalian hematopoietic factors, U-shaped (the *Drosophila* homolog of the mammalian FOG (Friend Of GATA) and lozenge (a RUN-X homolog) play key roles in the differentiation of plasmacytes and crystal cells, respectively (Waltzer et al. 2002, 2003; Bataillé et al. 2005; Muratoglu et al. 2006). The plasmacyte fate also requires the expression of two other transcription factors, Gcm and Gem2 in *Drosophila*, but the homologs of these proteins are not known to play a role in hematopoiesis in mammals (Alfonso and Jones 2002). Little is known about the target genes of these transcription factors, although a few genes have been found, which contain GATA binding sites in their regulatory regions (Tokusumi et al. 2009).

To exert their action, transcription factors must bind to their target sequences, which requires accessible DNA. The accessibility of a locus is dependent on the density of the higher-order chromatin structure. In the interphase, dense

chromatin regions (heterochromatin) and less compacted regions (euchromatin) can be observed in the cell nuclei; euchromatic chromatin regions are the subject of intensive transcription, while heterochromatic genes are generally repressed. A distinction between active and inactive chromatin domains can therefore be formally made. The role of epigenetic regulation is to set up and maintain the active and inactive states of the higher-order chromatin structure. The structure of chromatin enables or inhibits the accessibility of the chromatin for the transcription factors and the transcription machinery, and thus maintains gene expression patterns in cell lineages throughout cell divisions. Various molecular signals, ranging from histone modifications to regulatory RNAs, are involved in the regulation of the higher chromatin structure (Grimaud et al. 2006).

Most epigenetic factors involved in gene regulation have been identified in *Drosophila* as the regulators of homeotic genes. These factors are formally grouped into two categories: the Polycomb group of proteins involved in gene silencing, and the antagonistic trithorax group of proteins, which are activators of homeotic gene expression (Kennison 1995; Pirrotta 1998). Their DNA targets have been identified, and named PRE (Polycomb Response Element) (Simon et al. 1993) and TRE (Trithorax Response Element), respectively (Rozovskaia et al. 1999). The members of the Polycomb and the trithorax group are conserved in mouse and human. These proteins have been shown to regulate not only homeotic genes, but many others, which are involved in development, tumorigenesis, ageing, maintenance of the stem cell state and blood cell differentiation (Grimaud et al. 2006).

Polycomb proteins form multimeric repressor complexes, which are responsible for maintaining the inactive chromatin state. Three of these complexes have so far been identified, and biochemically characterized. These are PRC1 (Polycomb Repressive Complex 1), containing the Posterior Sex Combs, Polyhomeotic and Polycomb proteins (Kingston et al. 1996; Shao et al. 1999; Saurin et al. 2001), PRC2 (Polycomb Repressive Complex 2) containing the Enhancer of Zeste and Extra Sex Combs proteins (Ng et al. 2000; Tie et al. 2001; O'Connell et al. 2001) and phoRC, containing Pho and dSfmbt (Klymenko et al. 2006). These complexes work agonistically both in *Drosophila* and in vertebrates, and their sequential action is precisely regulated (Spivakov and Fisher 2007). The antagonistic trithorax proteins possess much more diverse functions. Some trithorax group genes encode transcription factors (e.g. *kohtalo*; Treisman 2001), while others encode components of nucleosome remodeling complexes (e.g. *brahma*; Tamkun et al. 1992).

The epigenetic maintenance can be regarded as a battle between activation and inactivation. This battle begins with signals that mark chromatin domains as prone to activation or silencing. DNA itself can be marked, since methylation of the

CpG islands in DNA is itself a repressive mark (Gruenbaum et al. 1981). However, the most important subject as concerns activation or silencing marking is the N-terminal tail of the histone proteins. There are several modifications that can be performed on the N-terminal domains of the core histone proteins. Some of these modifications serve as activation signals (*e.g.* acetylation at various sites by histone acetyl transferase (HAT) complexes), while others are repressive marks (*e.g.* H3 lysine 27 trimethylation by histone methyl transferases). According to the histone code theory, the combination of these epigenetic marks renders a chromatin domain active or inactive (Strahl and Allis 2000; Turner 2000; Gan et al. 2007). Certain chromatin domains have been found to be “bivalent”, *i.e.* simultaneously exhibiting repressive and active histone marks, and associated with low levels of gene expression. The genes in bivalent domains are “poised” or “primed” to be expressed or silenced when a cell is committed to a certain fate or lineage (Bernstein et al. 2006; Mikkelsen et al. 2007).

The question as to how the differentiation of blood cells is achieved through regulation of the chromatin state cannot yet be completely answered, though the concerted action of the transcriptional and epigenetic regulatory levels has been convincingly demonstrated in the differentiation of B cells in mammals.

Transcriptional and epigenetic events in the regulation of the B cell fate

The lineage commitment of B cells is a multistep process, which has been well characterized. The earliest progenitor in the lymphoid lineage is the lymphoid-primed multipotent progenitor (LMPP), which is derived from long-term hematopoietic stem cells. The more differentiated common lymphoid progenitor (CLP) has the potential to differentiate into the terminally differentiated T- and B lymphocytes. The overall differentiation process consists of three main parts: the commitment, the maintenance and the terminal differentiation. Several transcription factors are involved in the regulation of B cell differentiation (Fig. 7). The concentration of these factors in the nucleus is characteristic of each step of the differentiation process, and hence a transcription factor activity pattern can be attributed to each developmental stage (Parra 2009).

The early cellular choice toward lymphoid development is dependent on two transcription factors: PU.1 and Ikaros. Ikaros is itself neither an activator nor a repressor, but it can cooperate with repressor and chromatin remodeling complexes to activate or repress its target genes (Ng et al. 2007).

In the maintenance phase, Pax5 “preserves the fate” of blood cells committed to the B cell lineage. Pax5 is activated

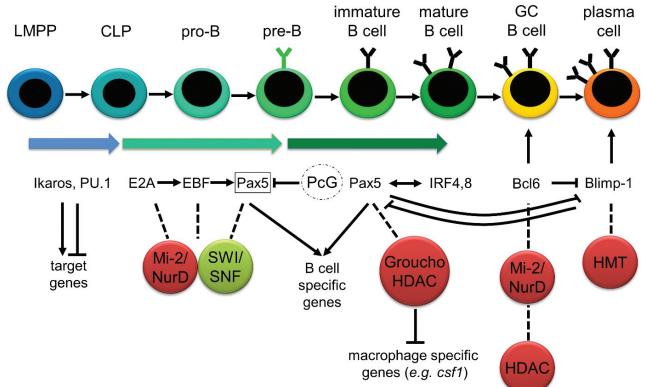


Figure 7. Transcriptional and epigenetic regulation of B cell fate. Transcription factors and epigenetic regulators that determine the B cell fate are displayed under each differentiation stage.

by E2A and EBF (Early B cell factor). EBF is the mammalian homolog of Knot/Collier, which plays a key role in the maintenance of lymph gland integrity in *Drosophila* (Krzemien et al. 2007). E2A and EBF are considered to be the initiators of the commitment process. The epigenetic events during the maintenance phase are coordinated via positively and negatively acting chromatin remodeling complexes, such as SWI/SNF and Mi-2/NuRD, respectively. Pax5 expression is maintained by auto-regulation; IRF4 and IRF8 are positively regulated by Pax5, and both upregulate Pax5. In non-B cell lineages, Polycomb proteins play the major role in repressing the *Pax5* locus (Decker et al. 2009).

The continuous expression of Pax5 in the B cell lineage is essential, since it has both activator and repressor functions. It activates B cell-specific genes, while it represses unnecessary genes, such as *csf1* (*colony stimulating factor-1*), which is normally expressed in macrophages. This repressive function is achieved via interactions with epigenetic regulatory factors (Tagoh et al. 2004).

The terminal differentiation of B cells is regulated by the Bcl6 and Blimp-1 transcription factors. B cells in which Bcl6 is active migrate to the germinal centers, while B lymphocytes expressing Blimp-1 become plasma cells. Bcl6 exerts its effect through interactions with HDAC and nucleosome remodeling complexes, while Blimp-1 acts via cooperation with histone methylating complexes. Blimp-1 and Bcl6 are the repressed targets of each other (Martins and Calame 2008; Parra 2009).

Studies on B cell commitment and differentiation have made it clear that transcriptional and epigenetic regulatory processes cannot be separated during blood cell differentiation. Different levels of regulation act in a coordinated manner to control lineage commitment and the differentiation of blood cells (Yokota et al. 2013).

Epigenetic regulation of blood cell differentiation in *Drosophila*

Several signal transduction pathways have been identified as regulators of the hemocyte fate in *Drosophila*. They are associated either with the maintenance of the progenitor hemocyte pool in the lymph gland or with the differentiation of effector blood cells, and especially lamellocytes during the immune response to parasites (Fossett 2013). The transcriptional targets of some of these pathways, e.g. the JAK/STAT pathway have also been identified (Bina et al. 2010). A large-scale genetic screen led to the identification of several genes, including transcription and epigenetic factors, cell cycle regulators, signal transduction pathway components and their regulatory networks that are required for the proper organization and function of the hematopoietic niche of the lymph gland (Tokusumi et al. 2012). However, our knowledge on the regulatory connections between epigenetic factors and signal transduction pathway targets is still fragmentary, despite the fact that most of the Polycomb and trithorax group genes in *Drosophila* have been identified.

Although the Polycomb and trithorax group genes have an indispensable function in the regulation of homeotic genes, surprisingly, few of the classical Polycomb and trithorax group genes have been investigated and proven to be involved in the epigenetic regulation of hemocyte differentiation in *Drosophila*. However, extensive screens have never been carried out to identify the Polycomb and trithorax group genes on the basis of the regulation of hemocyte differentiation.

One member of the Polycomb group that has been shown to have a function in hematopoiesis is *multiple sex combs* (*mxc*), which has been described as a regulator of proliferation and plasmacytoid differentiation (Remillieux-Leschelle et al. 2002). In *mxc* mutant larvae, the number of plasmacytoid cells is higher, due to overproliferation, and lamellocytes appear in the circulation. A few of the Polycomb group genes tested {(*Sex comb on midleg* (*Scm*), *Polycomb-like* (*Pcl*), *Polycomb* (*Pc*), *Posterior sex combs* (*Psc*) and *extra sex combs* (*esc*)} did not display a synergistic effect with *mxc* in the regulation of the hemocyte fate. Later, two other Polycomb group genes, *polyhomeotic proximal* (*ph-p*) and *Enhancer of Polycomb* (*E(Pc)*) were shown to affect lamellocyte differentiation as potential targets of the ROS-activated JNK signaling pathway in the lymph gland (Owusu-Ansah and Banerjee 2009; Theopold 2009).

Similarly as observed for the Polycomb group genes, only a few trithorax group genes are known to regulate the differentiation of hemocytes. The genes *domino* and *brahma* have been shown to be indispensable for normal hemocyte differentiation (Braun et al. 1998; Remillieux-Leschelle et al. 2002). However, several other factors previously identified as chromatin modifiers and epigenetic regulators were

recently shown to be the key regulators of the hemocyte fate in *Drosophila*. One of them is the nucleosome remodeling factor (NURF) complex, which plays a role in the silencing of JAK/STAT activated genes when the ligand is absent. In the case of activation, NURF dissociates from the chromatin of the regulated genes, and hemocyte differentiation can take place (Kwon et al. 2008).

These data imply that epigenetic factors play a major role in the regulation of lamellocyte differentiation. Lamellocytes are normally absent from the circulation, but they differentiate rapidly in the event of immune induction or certain tumorous conditions. It was recently recognized that not only do lamellocytes differentiate from precursors of the lymph gland and the sessile tissue, but circulating plasmacytoid cells, which were previously believed to be terminally differentiated blood cells, can also convert into lamellocytes upon immune induction (Honti et al. 2010). This transition is most probably regulated by an interaction of several epigenetic factors and may serve as a key to the understanding of the molecular events that lead to the lamellocyte fate.

U-shaped is believed to be the transcription factor that suppresses lamellocyte differentiation (Sorrentino et al. 2007), and it may therefore be a candidate master gene of the conversion event, together with *Serpent*, which is an activator of the transition (Kroeger et al. 2012). The epigenetic regulatory events of the transition are mostly unknown. Recent data indicated that one of the main factors responsible for the plasticity of macrophages is *Charlatan* (Stofanko et al. 2010). Since *Charlatan* is a member of the CoREST complex, which is an epigenetic repressor, the silencing of plasmacytoid-specific genes may be essential for lamellocyte differentiation.

Several genes, such as *Peroxidasin*, *eater* and *nimC1*, become silenced during lamellocyte differentiation (Honti et al. 2010; Kroeger et al. 2012). *Eater* and *NimC1* are phagocytosis receptors (Kocks et al. 2005; Kurucz et al. 2007a), and their expression is therefore not necessary for the lamellocyte function. This finding parallels the observation that macrophage-specific *csf1* is repressed in B cells (Tagoh et al. 2004), since plasmacytoid cells also lose their phagocytic capacity while converting into lamellocytes (Honti et al. 2010).

There is evidence that signal transduction pathways are directly linked to the epigenetic regulation of differentiation processes: the epigenetic regulator *Split ends* (*spen*) serves as an inducible switch. In uninfected larvae, *spen* is involved in the activation of genes responsible for maintenance of “stem cell-ness”. In cases of bacterial or fungal infection, the down-regulation of Notch activity results in the repression of *spen*, which leads to hemocyte proliferation and differentiation (Jin et al. 2009). *Spen* may therefore be considered as one of the main epigenetic regulators of hemocyte differentiation.

The reason why most of the well-known Polycomb and trithorax group genes do not seem to be involved in hemocyte

differentiation may be that certain differences exist between homeotic and blood cell fate determination. Homeotic genes are regulated epigenetically throughout the whole course of ontogenesis; the chromatin state of the regulated genes becomes fixed early in the embryo, and the actual state is maintained throughout development (Mihály et al. 2006). However, in the case of hemocytes, the epigenetic regulatory mechanism must act rapidly to enable the lamellocyte cell fate upon immune induction. This ability to switch between different epigenetic states can be attributed to the poised chromatin state, which may require different epigenetic factors.

Conservation and convergent evolution of blood cell differentiation in *Drosophila* and vertebrates

Drosophila obviously offers a versatile system in which to study the regulatory events via which blood cell fates are achieved. The similarities in hematopoiesis and blood cell fate determination between *Drosophila* and vertebrates are impressive, but it is important to note that only a few features of these differentiation events share a true evolutionary origin. The homolog gene products, and especially those that possess the same function in different organisms, such as phagocytic receptors and transcriptional and epigenetic regulators, are obviously conserved. Similarly, signal transduction pathways which are involved in the innate immune response, such as the Toll and the Imd pathways, are typical examples of evolutionary conservation (Gilmore and Wolenski 2012). However, other characteristics of the hematopoietic system, such as the compartmentalization of blood cells or the structure and function of the hematopoietic niches, most likely evolved independently by convergent evolution in insects and vertebrates. The hypothetical last common ancestor of protostomes and deuterostomes is the urbilaterian (Valentine 2006), and the hematopoietic systems of insects and vertebrates are therefore derivatives of that of the urbilaterian. However, as no representative of urbilaterians has yet been identified in the fossil record, no information is available on the blood cells and blood cell compartments of this organism. It can be assumed that urbilaterians had functionally distinct blood cell populations, which were the primitive forms of the terminally differentiated blood cell types of insects and vertebrates. This may be a plausible explanation of the similarities of blood cell differentiation and its regulatory factors in these evolutionarily distant taxa. However, the ‘functional conservation’ of hematopoietic niches and compartments seems to be a product of convergent evolution, and is not based on conserved regulatory factors and signals (Krzemien et al. 2010). The similarity of the organization of the hematopoietic system

between distant taxa is a proof that compartmentalization of blood cells furnishes a great evolutionary advantage.

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