Hemocyte-specific molecular markers in the hematopoiesis and innate immunity of *Drosophila melanogaster*

PhD thesis

István Nagy

Biological Research Centre of the Hungarian Academy of Sciences
Institute of Genetics

Szeged, 2004.

Table of contents

I. Introduction

I.1. *Drosophila melanogaster* as a model system

Molecular studies on developing embryos of *Drosophila melanogaster* have enhanced our understanding of signal transduction, differentiation and organogenesis. By the aid of the newly described molecules and genes, homologous signal transduction and differentiation pathways have been described between *Drosophila* and higher eukaryotes, including man [1-5]. The biochemical specificities of a large number of these molecules have been conserved during evolution and it has been shown that some of these have maintained analogous functions [6]. The immune system and the signal transduction pathways during immune response in *Drosophila* show remarkable similarities with the innate immunity of vertebrates, suggesting that they share a common evolutionary ancestry [7-14]. Due to the evolutionary conservation of general molecular pathways, the genetic studies performed in *Drosophila* have been very successful in the identification of homologous developmental pathways in vertebrates [15-17]. In the past few years a significant progress has been made in our understanding of the immune reactions of *Drosophila*, that are due to the use of genetic screens [18,19], the completion of the *Drosophila* genome sequence and the invention of the DNA microarray analysis [20,21]. Several genes are identified that play a crucial role in innate immune reactions. At the same time *Drosophila* is widely used as a model organism in studies of the host-pathogen interactions. Consequently, the large-scale similarities coupled with resources available for rapid genetic and molecular analyses, makes *Drosophila* as one of the best organism for analysis of the molecular mechanisms of the innate immunity [5,22,23].

Genetic evidence in *Drosophila* suggests that hemocyte proliferation and differentiation does not depend upon the same subset of signalling molecules [24]. Cellular and molecular characterization of immune system of *Drosophila* could therefore improve the analysis of the immune system and immunity of other species, including man. Additionally, hemocytes are capable of sustained cell proliferation that makes the hematopoietic system of *Drosophila* as one of the best model for analysis of the biological consequences of mutations in oncogenes and tumour suppressors as well as in detection of oncogene-specific downstream targets [20].

I.2. Defence reactions and compartments of the immune system of *Drosophila*

Host-defence reactions in *Drosophila* are generated by a variety of microorganisms and parasites. The immune system of *Drosophila* is very efficient in the protection of the host against invaders such as pathogens including viruses, bacteria and fungi or against parasitic insects. It is capable of limiting the microbial infection within hours. Like other invertebrates, *Drosophila* lacks an adaptive immune system; the defence mechanisms are therefore based on the well-developed innate immune responses that comprise of both humoral and cellular reactions. Although evidence has been presented that these defence reactions interact to fight infection [25], the nature of these interactions is still unclear. The ***humoral immune defence*** requires several hours for its full expansion, and involves the synthesis of constitutive and inducible extracellular molecules. The reactions involved in this process are the induction of the proteolytic cascades that leads to localized melanization and blood coagulation, production of reactive intermediates of oxygen [26] or nitrogen [27] and a rapid synthesis of antimicrobial peptides that are secreted directly into the hemolymph. These molecules contribute to the protection against invading microorganisms.

Two reactions are immediately triggered in insects by wounding or introduction of foreign objects: *phenoloxidase activation and hemolymph clotting* [28]. In *Drosophila* phenoloxidase catalyses the key steps in the formation of melanin. Phenoloxidase is present in the hemolymph as an inactive pro-enzyme that is converted to its active form by a serine protease cascade when a signal from the surface of the bacterial wall – such as the putative beta-1,3-glucan receptor – activates the cascade. Although it is known that the serine protease cascade activates the blood clotting in insects, the mechanism by which it acts is poorly understood.

Reactive intermediates of oxygen and nitrogen. The mammalian phagocytes generate reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) which are released into the phagosome or extracellulary, and which are toxic to a variety of microorganisms [29,30]. Some of the effects of ROI and RNI may be due to their roles in immune-related signal transduction pathways, rather than to direct cytotoxic effects on parasites or pathogens. In mammals, ROI such as superoxide anion (H_2O_2) and RNI such as NO function as second messengers in signal transduction pathways that include activation of NF-κB [31]. *Drosophila* hemocytes involved in defence reactions such as multicellular

capsule formation generate H_2O_2 [26] and NO [27]. It is anticipated that these molecules destroys the intrahemocoelic parasites. Although H_2O_2 and NO have been detected during cellular immune responses in *Drosophila* but whether these factors are related to NF-κB activation is unknown. However an NF-κB-like Relish gene is required for the induction of the humoral immune response [32,33].

Antimicrobial peptides. One possible way of a response to the infection is the rapid and transient production of different antimicrobial peptides. Hemocytes, besides playing role in regulating the antimicrobial response [34,35] also produce these peptides [36,37] however the fat body is considered as the most important source of antimicrobial peptides. When bacteria are digested, peptidoglycan fragments and lipopolysaccharide molecules are released and these molecules together with other putative endogenous signals, act to signal the fat body to begin antibacterial peptide synthesis. To date seven inducible antimicrobial peptides have been described in *Drosophila*. They fell into three main categories:

1.) cecropin [38], defensin [39], drosocin [40], attacin [41] and diptericin [42] are antibacterial peptides with known function upon bacterial induction;

2.) antifungal peptides are activated exclusively upon induction with fungi and the only known member of this category in *Drosophila* is drosomycin [43]

3.) metchnikowin [44] has both antibacterial and antifungal activity.

The ***cellular immune reactions*** are mediated by the hemocytes and are involved in normal development and in the immune response [45]. Any defect in hemocyte function exposes the organism to microbial intruders terminating in fatal diseases. One of the most important characteristics of the hemocytes is that they directly and actively interact with the invading microorganisms and parasites so it is anticipated that they play a key role in the insect immune defence. The scenario of the hemocyte-mediated responses involves the recognition of the foreign target or wound site as non-self and hemocytes must be activated to perform effector responses like phagocytosis or encapsulation. These responses are coordinated by inter- and intracellular signalling events.

Mechanisms of cellular immune reactions that occur within minutes after infection includes:

a.) phagocytosis – where the microorganisms, foreign particles and tissue debris are taken up by phagocytes. This process refers to the engulfment of entities by an individual

cell. Hemocytes phagocytose both biotic targets like bacteria, yeast and apoptotic bodies [46] as well as small abiotic targets like synthetic beads or particles of India ink. It is well demonstrated that the phagocytosing of microbes by *Drosophila* hemocytes is very similar to the phagocytosis of mammalian macrophages, so it is anticipated that the process of phagocytosing is conserved from insects to human [14]. Cell membrane receptors on the surface of the hemocytes bind to the bacterium [14], and this interaction initiates a chain reaction of events – resulting in pseudopod formation that reaches out and engulf the bacteria. In *Drosophila* plasmatocytes are the phagocytic hemocytes.

b.) nodulation and encapsulation – where tissues and other elements recognized as non-self are demarcated, enveloped by multicellular capsules and melanized [22,26]. The *nodulation* is a response reaction to bacterial infection. The process involves the direct capture of bacterial cells/aggregations in a matrix of hemocytes. It begins with the formation of microaggregates that grow by successive hemocyte adhesion. On the end of the process the nodule turns black due to the formation of melanin. The *encapsulation* reaction involves the host's immune capacity to recognize and destroy the invading parasite [47]. The concentration of the hosts circulating hemocytes and their aptitude to form a hemocytic capsule around the parasitic eggs shows a high correlation [48,49]. Beside the genetic status of both host and parasitoid, the success of the hosts encapsulation reaction depends upon the occurrence of the primary hemocytic response that gives rise to the amplification of the hemocyte population and of the presence of a hemocyte load large enough to form a cellular capsule before the parasitic egg become protected by adhering to the host tissues [47-52]. Two types of hemocytes contribute to the process of encapsulation: lamellocytes enclose the wasp egg in a capsule whereas crystal cells contribute to the melanization of this cellular capsule [52]. Upon parasitization with the wasp *Leptopilina boulardi* hemocytes in the lymph glands are also affected – there is an increase in the number of lamellocytes and crystal cells within [52].

As hemocytes are an important source of many humoral molecules and several humoral factors affect hemocyte function, it is quite subjective to divide the immune system of *Drosophila* into cellular and humoral responses. In addition processes like recognition of foreign intruders requires both humoral and cellular defences for an efficient response so it is considered that these two immunological responses are complementary, and both may be

seen in response to the same infection [53]. *Drosophila* possesses specific mechanisms to discriminate between microbes and responds to infection by inducing the appropriate reactions. One possible mechanism of recognizing the foreign invaders is the direct interaction of cell surface receptors of hemocytes with molecules on the invading organism. Alternatively indirect recognition is also possible by recognition of humoral receptors that bind to and opsonize the surface of the invader.

The humoral immune response of *Drosophila* has the ability to distinguish between Gram-negative and Gram-positive bacteria through the Imd and Toll pathways, respectively [14,54]. Diverse peptidoglycan recognition protein (PGRP) members are involved in distinguishing between invading bacteria and activation of appropriate immune reactions [14]. These proteins are pattern recognition molecules of the innate immune defence that bind to peptidoglycan, the major cell wall component of Gram-positive bacteria [55]. The PRGP-genes are expressed in the fat body, in hemocytes and in the epithelial cells of the gut. PGRP-LC is predicted to code for a membrane bound protein and is thus a major activator of the imd/Relish pathway in the *Drosophila* immune response [14,56], however in larvae, the imd-mediated antibacterial defence is also activated by peptidoglycan-recognition protein-LE [57].

The apparatus of the immune system is constituted of a physical barrier that resembles the mechanical protection in the defensive processes - comprised of the cuticle and gut and of the innate immunity with its biochemical- and cellular constituents. The main elements of the immune system are:

a.) the *fat body* – an adipose tissue that is the major site of the antimicrobial peptide synthesis, a functional homologue of the mammalian liver [58];

b.) the *hemocytes* which are - beside antimicrobial peptide production [36] - the major battery of the cellular immunity;

c.) the *lymph gland* - a hematopoietic organ, the presumed origin of larval and adult hemocytes, that is composed of paired lobes located along the dorsal vessel.

There are three components of the cellular immune system of the *Drosophila* namely the above-mentioned hemocytes and lymph gland and the so-called sessile hematopoietic tissue, which consists of randomly distributed hemocyte clusters that are nested directly

under the integument [59]. They are grouped in clusters in direct contact with the epidermal layer and contain plasmatocytes and crystal cells.

I.3. Classification and origin of *Drosophila* hemocytes

In *Drosophila melanogaster* the circulating cells or *hemocytes* represent the cellular elements of the innate immune defence. Hemocytes are primarily responsible for defence against parasites and pathogens; microbial killing results from the combined action of the phagocytic process with humoral defence factors such as various antimicrobial peptides. The hematopoietic system of the fruitfly contains several different types of cells. Each of these cell types is quite distinct in appearance and each has a specific biological function. Until quite recent the identification of hemocyte subsets has been based upon morphological, ultrastructural and functional characteristics. Based on morphological criteria the circulating hemocytes can be divided into four classes: crystal cells, plasmatocytes, podocytes and lamellocytes [60]. Crystal cells are 10-15 μm in diameter cells having crystalline inclusions in their cytoplasm. Plasmatocytes are 8-10 μm in diameter round cells having granules in their cytoplasm (these granules are staining with acid-phosphatase enzyme). Podocytes and lamellocytes are variable shaped 10-30 μm diameter cells. Podocytes have huge number of phillopodia while lamellocytes are large flattened cells with smooth cell membrane.

Circulating hemocytes of *Drosophila melanogaster* develop in two waves that take place in different tissues. Hemocytes in the developing embryo originate from the cephalic mesoderm [60,61], and the primordial function of these cells is the tissue remodeling [62]. It have been reported that two classes of hemocytes are represented in this developmental stage namely the most frequently observed cells - the plasmatocytes (also referred as macrophages) and the crystal cells. The function of the plasmatocytes in this developmental stage is the elimination of apoptotic cells and engulfing microorganisms [62]. Crystal cells in the embryo are believed to participate in blood clotting and melanization. Several embryonic markers have been identified including peroxidasin [63], Collagen type IV [64,65], scavenger receptor dSR-CI [66] and the CD36 superfamily member croquemort [62]. As hemocytes in the embryo migrate freely in the hemocoel [61] they are distributed throughout the embryo. Following embryonic development at least four classes of hemocytes have been reported in the larva [12,52,67]. They include plasmatocytes, podocytes, lamellocytes and crystal cells.

Plasmatocytes are capable of adhering to foreign surfaces, they include more than 90% of the hemocytes found in the circulation of the larval stages with similar functions to those plasmatocytes found in the embryo: phagosytosing microbes and synthesizing antimicrobial peptides. It is anticipated that these cells can terminally differentiate into podocytes that are larger cells with phillopodias with unknown function and into flattened cells called lamellocytes. These differentiated forms of plasmatocytes are also capable of attaching to foreign surfaces. Lamellocytes can form multilayer capsules around parasites or abnormally developing tissues [51]. Crystal cells are non-adhesive hemocytes that contain phenoloxidase precursors. In this developmental stage crystal cells contain crystalline inclusions, they take part in melanization and coagulation reactions and they contribute to wound healing response by participating in clot formation. It is anticipated that the pool of circulating hemocytes in adult flies is constituted exclusively of plasmatocytes [59,68].

On the end of the embryogenesis the larval hematopoietic organ called *lymph gland* differentiates along the dorsal vessel [69]. When the larva reaches the third instars stage this organ already contains several pair of lobes. The number of the lobes depends on temperature and is normally between four to six pairs. The lobes are identified by their relative position to the pericardial cells that separate the lobes and are surrounded by a basementlike material [59]. The anterior pair of lobes contains undifferentiated cells - called prohemocytes; cells containing enlarged cisternae of rough endoplasmic reticulum and numerous Golgi-vesicles – called secretory cells; cells with phagocytic capacity – plasmatocytes; cells containing crystalline inclusions – crystal cells. In larval stages posterior lobes contains only prohemocytes [59].

I.4. Transcription factors controlling hemocyte differentiation

Compared to the knowledge about genes that play roles in the hematopoiesis of mammals [70], little is known about the genetic control of the hemocyte lineage differentiation, proliferation and cell fate determination in *Drosophila*. Over the past years evidence has emerged demonstrating that cross antagonism of lineage-specific transcription factors plays an important role in determining cell phenotype in the hematopoiesis of *Drosophila* [67] and hematopoietic cell differentiation can be also mapped on the basis of the patterns of transcription factor expression in each lineage [71]. The transcription factors

functions during hematopoiesis to promote progenitor cell development and regulate lineage commitment and differentiation. The same type of transcription factors controls vertebrate and invertebrate hematopoiesis. The transcription factors with known function in the hematopoiesis of *Drosophila* are the next:

-Lozenge protein shares 71% identity with protein AML-1 [72], the most frequent target of chromosomal translocation in acute myeloid leukemia [73];

-Glial cell deficient/Glial cell missing is necessary for proper differentiation of glia cells from neuroglia progenitors [74], with homologous genes from human and mouse whose products that share a highly conserved N-terminal region with *Drosophila* GCM [75,76]. In *Drosophila* Glide/Gcm is also expressed and hemocytes [74,77,78] and together with its homologue Glide2 [79,80] -which has redundant functions with Glide/Gcm- regulates the macrophage differentiation in *Drosophila* embryos;

-the function of GATA transcription factors in diverse developmental contexts depends in part on physical interaction with cofactors of the Friend of GATA (FOG) family. FOG plays a curtail role in erythroid and megakaryocytic cell differentiation [81,82]. A FOG homologue in *Drosophila* named U-shaped (Ush) [71,83] acts downstream of Srp. It is expressed in hemocyte precursors and plasmatocytes throughout embryogenesis and larval development. The ultimate role of the ush is to limit crystal cell production so it is not needed for plasmatocyte specification and migration. A presumptive model suggested that Ush and Lz functions antagonistically during crystal cell lineage commitment [83];

-in vertebrates, transcription factors members of the GATA zinc-finger family [84] have a conserved function in differentiation and organ development. GATA-1, -2 and -3 are required for different aspects of hematopoiesis. GATA-1 [85-87] is necessary for erythroid and megakaryocytic maturation, GATA-2 [88,89] for definitive hematopoieis and GATA-3 [90] for T cell development. Lebestky et al. [67] characterized hemocyte sub-populations and differentiation lineages with the help of these transcription factors. Lozenge (lz) is necessary for the development of crystal cells while transcription factor Glial cell deficient/Glial cell missing (Glide/Gcm) [74,91], together with its homologue Glide2 [79,80], two evolutionary conserved proteins, are required for the development of plasmatocytes. The expression of the GATA homolog Serpent [13,92-94], that is upstream from Glide/Gcm, Glide2 and Lozenge, is needed for lz, glide/gcm and glide2 expression in flies. Misexpression of Glide/Gcm in

crystal cells can change their fait to that of the plasmatocyte lineage. However lz expression did not change, nor did the number of crystal cells increase in glide/gcm loss-of-function mutants [67].

It is well demonstrated that Srp is expressed in both embryonic and larval hemocytes, but the expression of the glide/gcm, glide2, lz and ush is proven in the embryo but the expression in the larva and adult is still unclear.

I.5. The *Drosophila* Ras oncogene

The ras genes encode highly conserved GTP-binding proteins that regulate cell growth, proliferation, and differentiation in almost all multicellular eukaryotes [95]. In addition, ras genes have been the subjects of intensive research because they are mutated in almost 30% of human cancers [96]. Many of the oncogenic mutations lock Ras in the activated state, leading to the constitutive activation of downstream effector pathways. The mechanisms by which Ras activation leads to increased cell proliferation and impaired differentiation, as is observed in many cancers, have still not been fully elucidated.

In *Drosophila,* Ras oncogene at 85D (Ras85D, also known as Ras1 and hereafter referred to as Ras) appears to be the ortholog of H-ras, Ki-ras, and N-ras found in mammals [97]. Ras oncogene at 64B (Ras64B, also known as Ras2) is the *Drosophila* counterpart of mammalian R-ras.

Previous studies of Ras-mediated signalling pathways in *Drosophila* indicate that the properties of the activated Ras in the fruitfly differs from mammalian Ras mutations where Ras appear to sustain continued growth and proloferation [98]. The hematopoietic system of *Drosophila* can be utilized to study the proliferative effects of Ras and other oncogenes. *Drosophila* hemocytes appear to be capable of sustained cell proliferation, as a number of mutations can lead to the increased numbers of circulating larval hemocytes [12].

I.6. Regulation and specification of *Drosophila* hematopoietic lineages

The lineage specification, proliferation and differentiation of hemocytes are tightly controlled. Genes implicated in these processes are the following: the Janus kinase (JAK)/signal transducer and activator (STAT) [99-102] that regulates hemocyte development; Toll/Cactus signal transduction pathways [103,104] that plays role in

regulating hemocyte proliferation and hemocyte density in the larva; Notch signaling pathway that play role in the differentiation of crystal cells under noimmune-, and lamellocytes under immune conditions [68,105]; mxc that controls the larval plasmatocyte lineage proliferation and differentiation in lymph glands and circulating hemocytes [106].

In *Drosophila* embryos homologue for the mammalian vascular endothelial growth factor (VEGF) receptor is expressed in hemocytes and it functions in controling the migration of hemocytes [107,108]. The *Drosophila* homologue of the mammalian receptor for platelet-derived growth factor (PDGF)/vascular endothelial growth factor (VEGF) is expressed on the surface of the larval prohemocytes and plasmatocytes [109]. This receptor controls hemocytes proliferation in vitro. Dramatic increase in the circulating hemocytes can be observed when one of its putative ligands, PVF2 is overexpressed in vivo [109].

Numerous scenarios have been proposed for the lineage relationships among different hemocyte types. Based upon morphological criteria hemocytes are classified into two differentiation lineages (Figure 1.), namely the plasmatocyte-lamellocyte- and the crystal

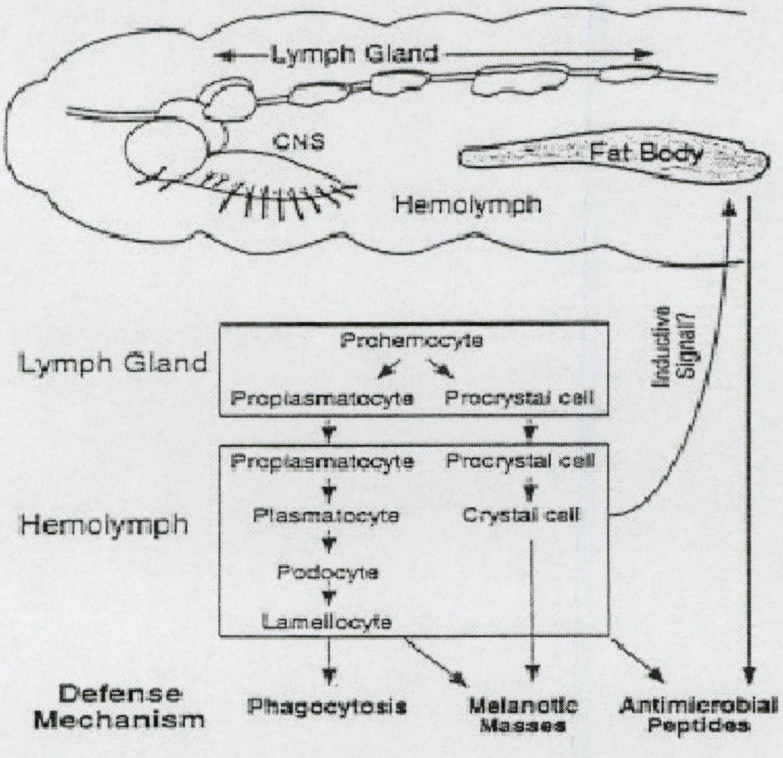

Figure 1. Biology of the *Drosophila* blood cell system and immune response (Perrimon et al. (1998), Cell 92(6), 697-700

cell-lineage [60,102]. In this model the podocytes and lamellocytes develops directly from the plasmatocytes.current model of *Drosophila* hematopoiesis proposes a genetic hierarchy with Srp - that is expressed in hematopoietic stem cells- acting upstream of Glide/Gcm, its homologue Glide2, Lz and Ush. Glide/Gcm together with Glide2 and Lz are required for plasmatocyte and crystal cell lineage development, respectively [67] (Figure 2.). In addition Ush functions to limit crystal cell lineage commitment [83]. Misexpression of Glide/Gcm in crystal cells cause transformation of these cells into plasmatocytes based upon changes in morphology and expression of the plasmatocyte-specific receptor Croquemort [62]. However, expression of Lz in plasmatocytes does not convert them into crystal cells. A small population of Lz-expressing precursor cells appear to develop into plasmatocytes instead of crystal cells. Upon maturation these plasmatocytes no longer express Lz but do express Glide/Gcm. The exact fate of these cells is still unclear: whether they represent a plasmatocyte sub-population, or form a new hemocyte lineage. This theory also suggests a two-lineage differentiation model where the lamellocytes develop from the plasmatocytes, and the crystal cells form a separate lineage.

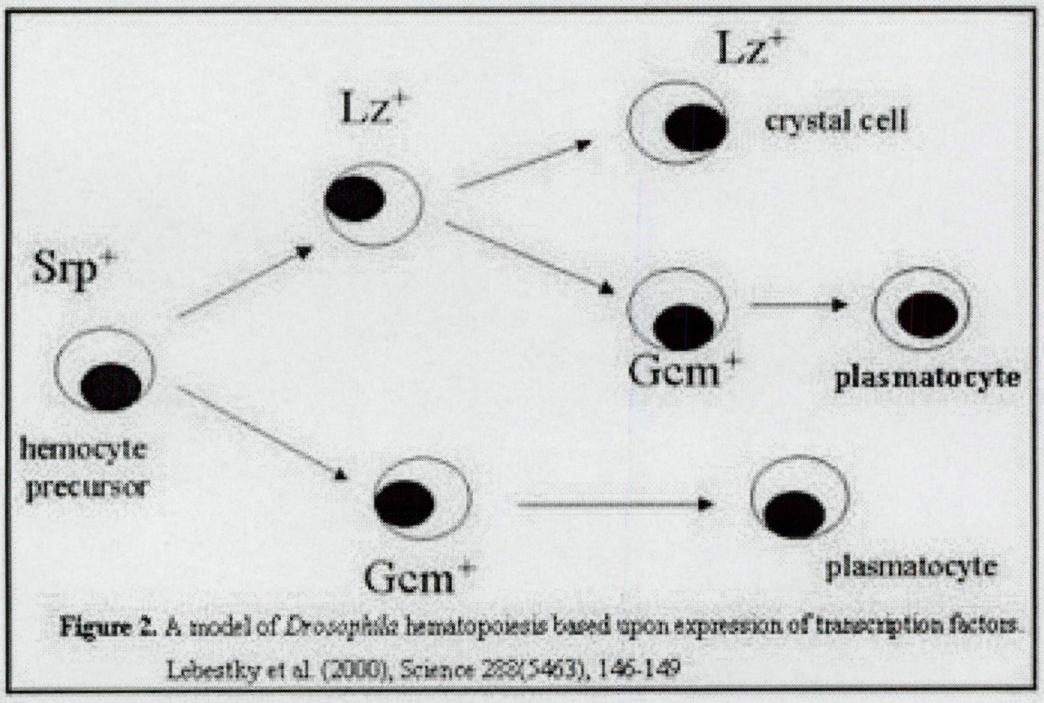

Figure 2. A model of *Drosophila* hematopoiesis based upon expression of transcription factors. Lebestky et al. (2000), Science 288(5463), 146-149

I.7. Aim of the study

Great progress has been made over the past several years in identifying antimicrobial peptides and the signalling pathways that regulate their synthesis; there has been progress in understanding the genetic control of blood cell differentiation as well [67], but still much less is known about the cellular defence responses mediated by hemocytes and about the genetic and molecular mechanisms controlling the developmental migrations of hemocytes.

Until recently a very few hemocyte-specific molecules were identified in *Drosophila*. However a hierarchy of molecular markers whose overlapping expression patterns subdivide and specify various cell lineages could characterize hematopoiesis. Understanding the development and function of hemocyte subsets would be further facilitated by availability of method for separation of subsets of hemocytes in sufficient amount, purity and viability.

We believe that the identified molecules specifically expressed on hemocytes could help us in understanding the molecular level of the hemocyte functions; these molecules could be used as molecular markers for definition of the main differentiation lineages; could be used to define relationships between various hematopoietic cells and might be exploited as markers for characterization of different stages of *Drosophila* hemocyte development. This kind of analysis could highlight familial relationships between cell lineages that might be overlooked if the terminally differentiated phenotypes and the expression of different transcriptional factors are used as the exclusive basis of comparison as it is proposed that morphologically similar hemocytes may have different patters of gene expression.

The conservation of immune responses and mechanisms controlling these processes, coupled with resources available for genetic analysis validates *Drosophila* as an important system for the study of innate immunity. Finally, genetic and molecular analyses could help in checking up the interactions between different immune cells, and could help in understanding the molecular evolution of the innate system.

II. Materials and methods

II.1. Chemicals and reagents

- AEC stock solution - 1% 3-amino-9-ethyl-carbazole (Sigma) in DMF
- Acetate buffer – 0.2 M Na-acetate, pH 4.6
- BBS - PBS containing 0.1% BSA

- Phosphate buffered-saline (PBS) – 0.13 M NaCl, 7 mM Na_2HPO_4, 3 mM Na_2HPO_4, pH 7.4
- PBT – 1xPBS containing 0.1% Triton-X-100
- Ringer solution – 7.5g NaCl, 0.35g KCl, 0.21g $CaCl_2$, 1000ml dW, pH 7.0
- 0.15M NaCl solutions
- $CrCl_3$ x $6H_2O$ (B.D.H. Chemicals Ltd., England)
- Shields and Sang M3 insect medium (Sigma-Aldrich)
- Stabilized glutamin (200 mM) (Pansystems, Aidenbach)
- Heat-inactivated fetal calf serum (FCS; Gibco)
- Ficoll-hypaque solution, r=1.077 (Pharmacia)
- Sodium azide (Sigma)
- Alsever's solution (24.6g glucose, 9.6g sodium citrate, 5.04g sodium chloride dissolved in 1200 ml of distilled water and the pH adjusted to 6.1 with citric acid)
- Rabbit anti-Mouse Immunoglobulin (DAKO, Copenhagen) dialyzed against 0.9% NaCl solution and adjusted to 1 $mgml^{-1}$
- Anti-phospho-Histone H3 (PhHH3) antibody produced in rabbit (Upstate Biotechnology, Lake Placid)
- T2/48 antibody directed against the CD45 human leukocyte antigen [110] as a negative control, 0.5 $mgml^{-1}$ in 0.15 M NaCl solution
- Goat anti-Mouse Immunoglobulin, FITC conjugate (DAKO, Copenhagen)
- Goat anti-Mouse Immunoglobulin, biotin conjugate (DAKO, Copenhagen)
- Goat anti-Rabbit Immunoglobulin, FITC conjugate (DAKO, Copenhagen)
- Peroxidase conjugated streptavidine (DAKO, Copenhagen)
- HRPO-conjugated anti-Mouse antibody (DAKO, Copenhagen)
- Fluorolink Cy3 labelled streptavidine (Amersham Pharmacia)
- 4',6-Diamidino-2-phenylindole (DAPI) (Sigma-Aldrich)

II.2. *Drosophila melanogaster* stocks

Flies were kept on standard *Drosophila* medium at 25°C. We used Oregon-R as a wild-type stock. The *l(3)mbn-1* stock is an EMS induced tumour suppressor mutant with proliferating tumorous hemocytes in the circulation [111]. A bipartite expression system

based on the yeast GAL4 transcription factor and its target sequences (Upstream Activating Sequences or UAS) provide the opportunity to express transgenes in a spatially restricted mode [112]. To overexpress constructs in hemocytes, transgenic flies carrying the target genes under the control of GAL4 responsive elements (UAS) were crossed to transgenic flies carrying the CgGAL4 [18]. With the help of this system we overexpressed the Ras^{wt} and Ras^{V12} oncogenes in tissue specific manner. The hemocytes in the larval progeny that specifically carried either UAS-Ras^{wt}/CgGAL4 or UAS-Ras^{V12}/CgGAL4 were analysed. Larvae expressing Ras^{wt} are hereafter referred to as Ras-wt and larvae expressing Ras^{V12} are referred to as Ras-act.

II.3. Hybridoma production

Mouse monoclonal antibodies (mAbs) to cell surface and intracellular antigens of different hemocyte subsets of *Drosophila* were produced in our laboratory and are described in [20,113,114 and Kurucz, E., Vilmos, P., Nagy, I., Cartoon, Y., Ocsovszki, I., Hultmark, D., Gateff, E. and Ando, I. manuscript in preparation].

II.4. Immunglobulin purification and conjugation of the antibodies

A Protein-G Sepharose column (Pharmacia) was used to purify the immunglobulin fraction from the hybridoma supernatants, according to the manufacturer's instructions. The purified antibodies were conjugated with FITC or biotin.

II.5. Isolation of circulating larval hemocytes

The larval hemolymph from wandering third instars of the appropriate genotype was collected after careful piercing the larvae by a sharp tungsten wire and the hemolymph was rinsed in *Drosophila* Ringer solution. The samples were used for immunostaining, functional experiments and microarray analysis.

II.6. Rosetting

II.6.1. Sensitization of sheep erythrocytes

To prepare a stock suspension of anti-mouse Ig-sensitized SRBC a 300 µl aliquot of packed SRBC was washed four times with 0.9% NaCl (w/v) solution. After addition of 0.15

mg rabbit anti-mouse Ig in 150 µl the sample was mixed and 150 µl of 0.1% $CrCl_3$ solution was added drop by drop while constant shaking. The suspension was gently layered under 2 ml of ice-cold 0.9% NaCl solution. After incubation overnight at 4^oC the reaction was stopped by 5.0 ml PBS. The cells were then washed four times with PBS and resuspended in 1.5 ml of PBS containing 10% FCS. For each coupling 0.1 mg of anti-hemocyte monoclonal antibody was added to 200 µl of SRBC suspension and incubated on ice for one hour. Finally, the sensitized SRBCs were washed four times with PBS and resuspended in 400 µl PBS containing 10% FCS and 0.1% NaN_3. The sensitised SRBC are stable for 10 days at 4^oC.

II.6.2. Formation of rosettes and separation

The rosetting and separation were carried out at 4^oC in CSSM3. For each rosetting reaction, twenty 3^{rd} instars larvae were dissected, hemocytes collected in 200 µl CSSM3, washed once, resuspended in 200 µl of CSSM3 and counted. The sensitised SRBCs were washed tree times in CSSM3, counted and added at a hemocyte:erythrocyte ratio of 1:100. Samples were incubated in flat-bottomed, 96 well tissue culture plates on ice for 90 minutes. After incubation 2 ml of ice-cold CSSM3 was added the cells were resuspended and layered on the top of 2 ml of Ficoll solution in a 10 ml glass test tube. The sample was spun with 500g for 30 minutes. The non-rosetting cells (negative population) were harvested from the interface, while the rosettes (the hemocytes covered by sensitised SRBC, the positive population) were collected from the bottom of the tube as the pellet. The volume of both samples was adjusted to 2 ml each, and centrifuged with 350g for 10 minutes. The pelleted cells of the rosetting fraction were exposed to a hypotonic shock of 200 µl of dW for one second and immediately 5 ml of complete CSSM3 was added to re-adjust ionic strength and osmolarity. Both the rosetting and the non-rosetting hemocytes were washed once with CSSM3 and used.

II.7. Immunocytochemistry

All the steps were carried out on room temperature (20^oC). A total of 30 µl of hemocyte suspension in *Drosophila* Ringer solution containing phenil-thiourea (PTU), 0.1%

BSA and 5mM EDTA was placed in each spot of a multispot microscope slide (SM-011, Hendley-Essex, Loughton, U.K.). The hemocytes were allowed to adhere for 45 min at room temperature and then fixed in acetone for 6 min and air-dried. The samples were then rehydrated and blocked in PBS containing 0.1% BSA for 20 minutes. Samples were incubated with the primary antibody (hybridoma supernatant or purified immunglobulins) for 1 h, washed three times with PBS for 5 min each, and incubated with biotinilated goat anti-mouse immunglobulins for 45 minutes. After three washes with PBS, 5 minutes each, the samples were incubated with peroxidase-conjugated streptavidine for 45 minutes. The slides were washed three times with PBS and three times with acetate-buffer (pH 4.6), three minutes each. The bound antibody was visualized with 0.5% 3-amino-9-ethyl-carbazole (AEC) as a chromogen, diluted in acetate-buffer containing 0.003% H_2O_2. The reaction was stopped by three washes with distilled water, 5 minutes each. The samples were and analysed.

II.8. Staining of whole larvae

Late 3rd instar larvae were dissected on room temperature in PBS and fixed in PBS containing 2% paraformaldehyde for 20 minutes. Samples were rehydrated and blocked in PBS containing 0.1% BSA for 20 minutes. Prepared samples were placed one by one in a 96-well U-form multiwell plate and incubated overnight on +4°C with hybridoma supernatant containing 0.1% Triton-X. The following steps were carried out on room temperature. The samples were washed three times with PBT, 10 minutes each and incubated with biotinilated goat anti-mouse immunglobulins (1:500 in PBT). After one hour the samples were washed as before and incubated with peroxidase-streptavidine (1:300 in PBT) for one hour followed by 3 washes with PBT and three washes with acetate buffer, three minutes each. The reaction was visualized as described in II.7. The samples were placed on microscope slides covered and analysed.

II.9. Fluorescens stainings

II.9.1. Staining the hemocytes using FITC labeled antibodies

Hemocytes were isolated and treated as described in II.7. After saturation the samples were incubated for 45 minutes with the optimal dilution of the FITC labeled anti-hemocyte

antibodies and with DAPI (1:300 in BBS) to visualize nuclei's of hemocytes. After 3 washes in PBS, 5 minute each the slides were covered and analysed.

II.9.2. Staining the hemocytes using biotin labeled antibodies

Hemocytes were isolated and treated as described in II.7. After saturation the samples were incubated with the optimal dilution of the biotin-labeled purified anti-hemocyte antibodies for 45 minutes, followed by three washes in PBS, 5 minutes each. Cy3-streptavidine (1:3000 in BBS) and DAPI (1:300 in BBS) were added to the samples for 45 minutes. After washing steps the samples were covered and analysed as described.

II.10. Functional tests

II.10.1. Phagocytosis

Phagocytosis of the FITC-labeled E.coli bacteria by hemocytes was recorded, as follows. Bacteria were heat-killed and labeled with FITC [115]. Hemocytes were seeded in 25 µl medium on the sports of multivell microscopic slides, and 2 µl of 10% FITC-labeled bacterium suspension was added immediately. Bacteria were added in saturated amounts, so that all the hemocytes capable of phagocytosis took up bacteria. Following a 15-minute incubation of hemocytes with FITC-labeled bacteria in a humid chamber at room temperature the hemocytes were washed free of bound bacteria and fixed with acetone. In some experiments the phagocytic capacity of the hemocytes was correlated with their immunological phenotype. For this purpose the hemocytes were saturated with BBS and reacted with hybridoma supernatants recognizing P1 and/or L1 antigen expression. After 3 washes with PBS, the cells were incubated for 45 minutes with 1:500 dilution of biotin labeled goat anti-mouse immunoglobulins as secondary antibody. Following 3 washes in PBS, cells were incubated for 45 minutes with 1: 3000 dilution of Cy3 labeled streptavidine, and 1:300 dilution of DAPI. After 3 washes in PBS the slides were covered and analysed.

II.10.2. Mitosis

Mitotic cells were visualized by immunostaining for phospho-Histone H3 (PhHH3) [116], using a rabbit anti-PhHH3 antibody (1:100 in BBS) following 3 washes with PBS, 5 minutes each. The samples were then incubated with a FITC labeled goat anti-rabbit

immunoglobulin (1:100 in BBS) and DAPI (1:300 in BBS). After washing steps the slides were covered and analysed.

II.10.3. Encapsulation

One-week-old females of the parasitic wasp, *Leptopilina boulardi* strain G486 were used to infest 2nd instars larvae. Fifty larvae were exposed to five *Leptopilina* females and kept at 18°C overnight. The wasp females were than removed, and three days after infestation, the phenotype of the hemocytes was determined using anti-hemocyte antibodies.

II.11. Injection of hemocytes into adult flies

The hemolymph of third instars larvae was collected in ice-cold *Drosophila* Ringer solution and the hemocyte concentration was determined in Bürker chamber. Wild type, adult virgin females were anaesthetized with diethyl-ether. Cell suspensions with the appropriate hemocyte concentrations or control buffer were injected into the abdomen of adult flies using a thin glass needle. The flies were transferred to fresh food, and the number of surviving flies were determined daily. For each genotype, the experiment was repeated at least three times and a total of 50-139 wild type adult flies were injected with hemocytes. In each experiment at least 50 adult flies were injected with the control buffer. To retrieve the hemocytes from the injected adults, the flies were anaesthetized and perfused with ice-cold *Drosophila* Ringer solution. Cells were collected on a glass slide, stained and analysed as described above.

II.12. Image processing

The samples were analyzed with AxioVision 2.0.5. (Axioscope MOT2, Zeiss) microscope, using either Nomarski optics or epifluorescent illumination. Images were recorded with an AxioCam camera and AxioVision 3.1 software.

III. Results

III.1. Separation of the hemocytes

III.1.1. Rosette forming specificity of the antigens

For the separation of hemocytes we applied antibodies that react with the extracellular epitope of the transmembrane proteins. For this purpose three antibodies were used. Antibody that identifies H2 - an antigen that is expressed on all hemocyte sub-populations of circulating hemocytes was used as a positive control. Also two discriminative antibodies that are directed against antigens that mark two different hemocyte subpopulations were applied. These subpopulations are easily distinguishable from each another even by morphological criteria. One of the antibodies recognizes antigen P1 a molecular marker that is expressed exclusively on plasmatocytes and proplasmatocytes. The other one is the antibody directed to the antigen L1, which is specific for lamellocytes – big, flattened fully matured cells and their direct precursors that are little round cells of plasmatocyte morphology. As antibody T2/48 is directed against the CD45 human leukocyte antigen it was used as the negative control.

Rosettes were formed around the small, round plasmatocytes by the P1-SRBC and after gradient centrifugation the rosette forming plasmatocytes were recovered from the pellet. The non-rosetting cells - the lamellocytes, small round cells having crystalline inclusions and small round cells without crystals - were recovered from the interface. The rosette formation of the L1-SRBC was restricted to the large flattened lamellocytes and a few small round cells with plasmatocyte morphology and these cells can be recovered from the pellet, while hemocytes with plasmatocyte morphology and cells with crystals in their cytoplasm were collected from the interface. In the positive control H2-SRBC forms rosettes around all hemocytes and rosettes can be recovered exclusively from the pellet, while using the negative control T2/48 antibody for sensitisation of the SRBC, the plasmatocytes, lamellocytes and cells containing crystalline inclusions were recovered from the non-rosetting fraction from the interface. There were no hemocytes in the pellet.

These results indicate that the rosette formation of the antibodies is restricted to their sub-population specificity: H2-SRBC reacted with all hemocytes sub-populations while the

discriminative P1-SRBC and L1-SRBC reacted with plasmatocytes and lamellocytes, respectively. Neither P1-SRBC nor L1-SRBC formed rosettes with cells containing crystals.

III.1.2. Molecular characterization of separated subsets

The immunological phenotype of hemocytes in each separated subset was determined by immunohistochemistry and indirect immunofluorescence. Antibodies mentioned above and antibody that recognizes antigen C1 (Dr. Tina Trenczek, unpublished data) - specific for crystal cells and their precursors were used to determine the precise localization of the given sub-populations within the separated fractions.

All recovered cells, regardless of their origin, reacted with the pan-hemocyte H2 antibody. After separation with L1-SRBC the majority of the rosette-forming cells were reacting with the L1 antibody (82,9±9,0%). This population comprised of cells with lamellocyte morphology and a minor population of small, round cells with plasmatocyte morphology; the former being the terminally differentiated large lamellocytes while the latter may be their precursors. In the L1-positive population few P1-positive small round cells were also detected (13,2±5,8%) but no hemocytes expressing the crystal cell specific antigen was observed. In the fraction of non-rosetting hemocytes P1-positive round cells (90,3±6,4%) as well as hemocytes lacking the P1 marker were detected (data not shown). The latter population is composed of the crystal cells determined by the expression of C1 antigen and of a hemocyte subpopulation that lack the expression of all the three sub-population specific antigens.

After rosette formation with P1-SRBC the rosette forming hemocytes were small round cells with plasmatocyte morphology and 95,7±3,6% of them stained with the P1 antibody (Figure 3.). A small portion (2,8±1,6%, Figure 3.) of L1 positive cells was also detected within this fraction. As $L1^+$ fraction this fraction also lack crystal cells. In the non-rosetting fraction, hemocytes with lamellocyte, crystal cell and plasmatocyte morphology were found (Figure 3.). The lamellocytes and a minor population of small round cells, the presumed lamellocyte precursors reacted with the L1 antibody (78,6±8,1%). We also detected a minor subset, which expressed neither the P1, nor the L1 marker these cells represent the crystal cells expressing C1 (data not shown) and a sub-population, lacking the P1, L1 and the C1 antigens.

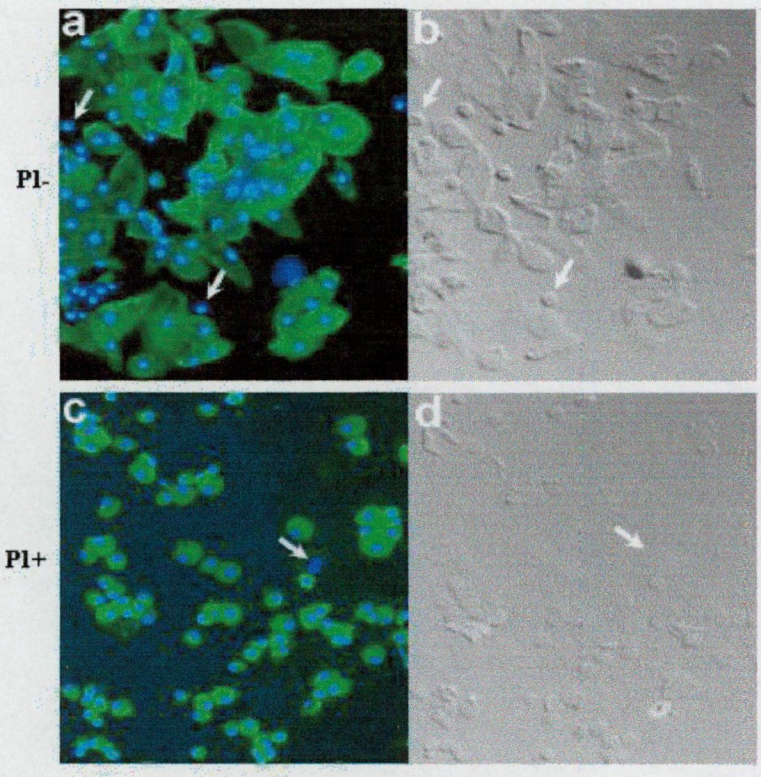

Figure 3. Indirect immunfluorescense staining of the recovered hemocyte fractions after separation with the P1-SRBC. Magnification 20X. (a) Merged pictures of the non-rosetting fraction stained for the expression of the lamellocyte-specific L1 marker visualised by the FITC fluorochrome (green) and DAPI staining (blue), (b) Nomarski image of the same population. White arrows point to contaminating L1- hemocytes. (c) Merged picture of the resetting hemocytes which were analyzed for the expression of the plasmatocyte-specific P1 marker visualized by FITC fluorochrome (green) and DAPI staining (blue), (d) Nomarski image of the same population. White arrow points to a contaminating P1- cell.

These data show that the immunological phenotype of the cells separated with the mAbs corresponds to the phenotype of the main hemocyte subsets in *Drosophila*. The recovery exceeded 90% in each group.

III.1.3. Double staining

To elucidate the relationship between the plasmatocytes and lamellocytes we have performed double staining experiments on the hemocytes of the separated fractions of both P1 and L1 separations and also on circulating hemocytes of the l(3)mbn-1 stock as the control. Using simultaneously the P1 and L1 antigens visualised with two different fluorochromes we were able to identify two new sub-populations, that we named double negative ($P1^-L1^-$) and double positive ($P1^+L1^+$) pools. The sub-population of the double negatives is considered as the immature precursor pool as they lack the expression of the sub-population specific antigens and also lack crystalline inclusions. This subpopulation is present in the non-rosetting fraction of both separations. Both of the rosetting fractions contain the double positives. These cells expresses both plasmatocyte and lamellocyte specific antigens. This pool of double positives contains less then 3% of the circulating hemocytes of the l(3)mbn-1 mutant.

These data indicate that additional pools of hemocytes are present in the circulation of l(3)mbn-1 mutant larvae lacking or expressing both of the sub-population specific antigens.

III.1.4. Functional characterization of the separated subsets

We next determined whether the separated subsets were capable of performing functions that they possess before separation. For this purpose we compared the functional activities of the hemocytes before and the activities of sub-populations after the separation. First we tested the functional activity of the hemocytes in an *in vitro* phagocytosis assay, where hemocytes were examined for their ability to engulf FITC-labeled E.coli. In the control pool – isolated 3rd stage larval hemocytes before separation – a sub-population of hemocytes expressing the P1 antigen, and hemocytes lacking P1 and L1 antigens engulfed bacteria, while hemocytes expressing L1 antigen doesn't take up any particle. When analysing the separated fractions we concluded that hemocytes from the P1 rosetting fraction expressing P1 antigen engulfed bacteria (Figure 4a,b.). In the $P1^-$ fraction hemocytes

expressing L1 antigen lack phagocytosis while a 1-3% of the cells lacking the expression of L1 engulfed bacteria particles. Only a small proportion of the L1 rosetting hemocytes phagocytosed. All of the hemocytes showing phagocytic activity of this fraction expressed the P1 antigen. The majority of hemocytes from the L1 non-rosetting cells expressed the P1 antigen and engulfed bacteria. We could never observe a hemocyte - regardless of its origin - having crystalline inclusion that phagocytosed E.coli.

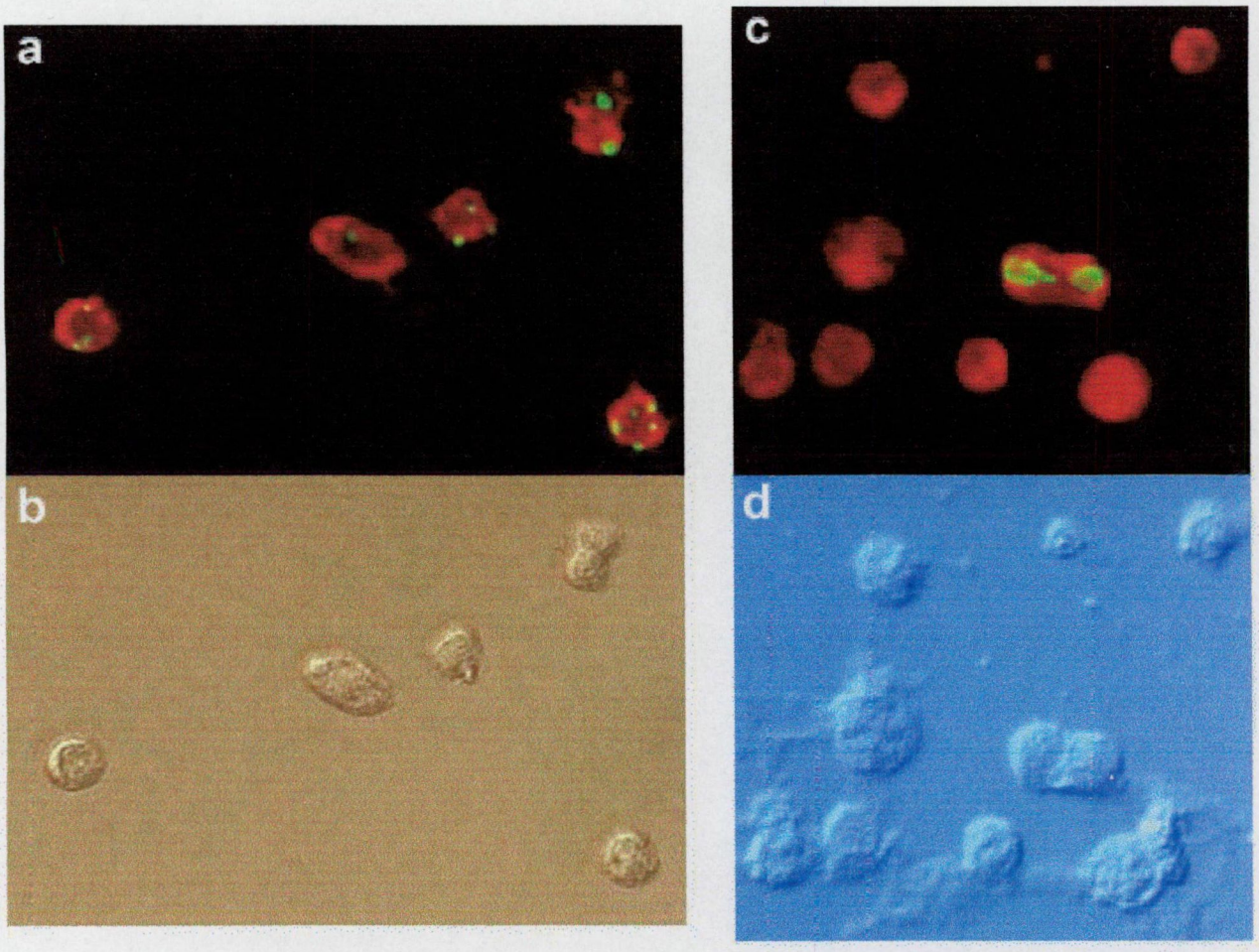

Figure 4. Phagocytosis and mitotic activity of the separated hemocytes. (a,b) All hemocytes in the P1-positive fraction show phagocytic activity; (c,d) hemocyte in mitosis expressing the P1 antigen from the P1-positive fraction. 40X Magnification. (a) Hemocytes from the pellet of P1-SRBC separation express the P1 antigen (visualized with Cy3 fluorochrome) and they take up FITC labelled E.coli. (b) Phase-contrast image of the hemocytes represented on (a). (c) Hemocytes from the pellet of P1-SRBC separation showing mitotic activity (detected with the aPhHH3 and visualized with FITC express the P1 antigen. (d) Nomarski image of hemocytes represented on (c).

Second, the mitotic activity of the hemocytes was tested using the anti-PhHH3 antibody, a marker that stains chromosomes in cells undergoing mitosis. Cell proliferation was detected in the control pool and also hemocytes of the P1 rosetting and the L1 and the P1 non-rosetting fractions showed mitotic activity suggesting that there is a dividing pool in the $P1^+$, $P1^-$ and in the $L1^-$ fractions. When we analysed the antigen expression of the dividing pools we observed that only hemocyte expressing P1 antigen (Figure 4c,d.) and cells lacking both P1 and L1 antigens showed mitotic activity. This in a good agreement with results observed from the control pool where hemocytes expressing L1 antigen and cells containing crystalline inclusions lack mitotic activity.

These data show that the separated subsets of *Drosophila* hemocytes retain their functions that they disposed before separation.

III.2. Overproliferation of hemocytes caused by activation of Ras

Ras has been implicated in regulating cell proliferation and differentiation in many organisms. To examine the effects of increasing Ras activity in hemocytes, we expressed the activated form of Ras oncogene, Ras^{V12} in hemocyte specific manner, using the CgGal4 driver. In this driver the GAL4 is expressed in all embryonic and larval hemocyes.

III.2.1. Expression of hemocyte-specific antigens by cells of the hematopoietic lineage of the Ras-act mutant

Late third instars of Ras-act display a dramatic 40-fold increase in the number of circulating hemocytes. To determine the antigen expression profile of the hemocytes we stained the cells for the expression of the panhemocyte and sub-population specific antigens. All of the circulating hemocytes as well as hemocytes of the lymph gland express the H2 marker (Figure 5., Table 1.). By morphological criteria the majority (>95%) of the circulating hemocytes are of plasmatocyte morphology. These cells however lack the expression of the plasmatocyte specific P1 antigen (Figure 5., Table 1.). As the expression profile of these hemocytes is $H2^+P1^-L1^-C1^-$ we speculate that these cells represent the multipotent progenitor cell population. Lamellocytes expressing the L1 antigens are always present in the circulation (Figure 5., Table 1.) and the number of these cells is higher (3%) than those in the wild type. Both little, round and big, flattened $L1^+$ hemocytes are detectable. By morphological criteria

we could not observe a significant increase in the number of cells containing crystalline inclusions (~1%). However these cells contain much more crystals compared to wild type. When stained with the C1 marker (Figure 6., Table 1.) a significant increase in the C1 expressing cells is detectable compared to wild type. As the antigen C1 is present on the hemocytes containing crystalline inclusions in their cytoplasm as well on the proposed precursors of the crystal cells that are round cells lacking crystals we suggest that this change in number of $C1^+$ cells is due to the increased number of procrystal cells in the mutant.

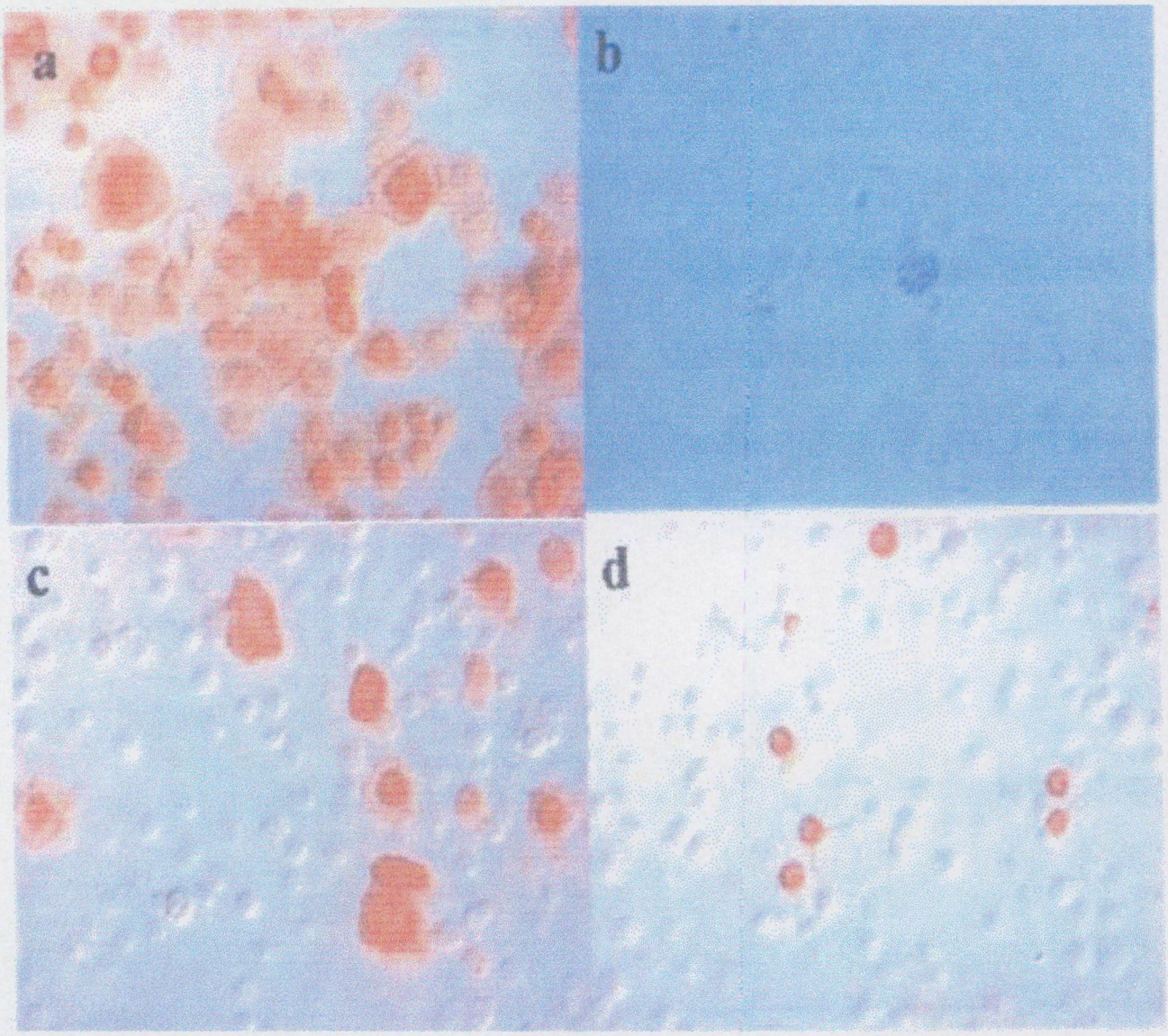

Figure 5. Immunostaining of the Ras-act circulating hemocytes. Magnification 40X. (a) All hemocytes express the pan-hemocyte antigen H2. (b) Hemocytes lack the plasmatocyte-specific P1 expression. Hemocytes expressing lamellocyte-specific (c) and crystal cell-specific C1 (d) antigens.

Antigens	Stem cells	Phagocytes	Lamellocytes	Crystal cells
H2	+	+	+	+
P1	-	-	-	-
L1	-	-	+	-
C1	-	-	-	+

Table 1. Hemocyte sub-populations of the Ras-act mutant determined on the basis of the expression of hemocyte-specific antigens.

These data indicates that the circulating hemocytes of the Ras-act mutant differ from their wild type counterparts in their antigen expression.

III.2.2. Mitotic activity of the Ras-act mutant cells

We next examined whether the large increase in Ras-act larval hemocyte numbers is due to an increased proliferation of hemocytes. We obtained two lines of evidence that suggest that the mutant cells do indeed overproliferate (see also III.2.3.). First, to ascertain if cell division is involved in the proliferation of the hemocytes of Ras-act mutant, circulating larval hemocytes were adhered to multispot slides, fixed and stained simultaneously with anti-PhHH3 and sub-population specific antigens. In a control experiment circulating larval hemocytes from wild type and l(3)mbn-1 mutant were analysed under same conditions, and the results were compared.

	Oregon-R	l(3)mbn-1	Ras-act
Dividing cells[1]	0.32%(±0.49)	1.10%(±0.82)	3.93%(±1.27)
Stem cells and precursors	+	+	+
Plasmatocytes	+	+	-[2]
Lamellocytes	-	-	-
Crystal cells	+	+	+

[1] Number of hemocytes showing mitotic activity at any given time
[2] No plasmatocyte specific antigen expressing hemocytes

Table 2. Mitotic activity of the hemocyte subsets defined by the molecular markers.

We found that only a small proportion of hemocytes of the wild type undergo mitosis at any given time and the division is restricted to $P1^{-}L1^{-}C1^{-}$, $P1^{+}L1^{-}C1^{-}$ and to the $P1^{-}L1^{-}C1^{+}$ populations of circulating hemocytes (Table 2.). Although the subpopulations undergoing mitosis in the l(3)mbn-1 mutant correspond to those of the wild type a larger proportion of the circulating hemocytes stained with anti-PhHH3 is detectable (Table 2.). In the Ras-act mutant the proportion of mitotically active hemocytes is even higher (Table 2.), indicating that Ras-act hemocytes actively divide while in circulation.

Following expression of L1 antigen, $L1^{+}$ cells do not divide. As little round lamellocytes already lack mitotic activity the terminal differentiation from round cells to large, flattened cells does not require cell division. Cells, carrying C1 marker diffusely distributed in the cytoplasm lacking crystalline inclusions divide in the circulation. Cells expressing C1 antigen and having crystalline inclusions don't divide.

These results suggest that the mutant hemocytes actively proliferate in the circulation of the mutant larvae and also that immature precursor cells, plasmatocytes and crystal cell precursors are hemocytes with susceptible mitotic activity.

III.2.3. The Ras-act larval hemocytes kill transplanted hosts

As Ras-act hemocytes actively divide in the circulation, we cultured the Ras-act hemocytes in the abdomen of wild type adult flies. Previous studies have shown that cells from tumorous lymph glands can be serially passaged in the abdomen of adult flies [117]. Following injection with Ras-act hemocytes, the abdomen of the injected flies becomes enlarged, and 64% of the injected flies died within 3 days (Figure 6.). These changes are associated with the overproliferation of cells within the host [117]. A similar result was obtained when hemocytes from l(3)mbn-1 mutant larvae were injected into the abdomen of adult flies (Figure 6.). In control animals injected with the same number of wild-type hemocytes, only 24% of the host animals died and the abdomen of the injected flies showed no change in size (Figure 6.). In control animals injected with the injection buffer, only 10% of the host animals died (Figure 6.). The Ras-act hemocytes can be repeatedly passaged in the abdomen of adult flies, and during this process they retain their original size and morphology and continue to express the pan-hemocyte marker H2.

These results suggest that the mutant hemocytes injected in the abdomen of adult flies cause the death of the host animal.

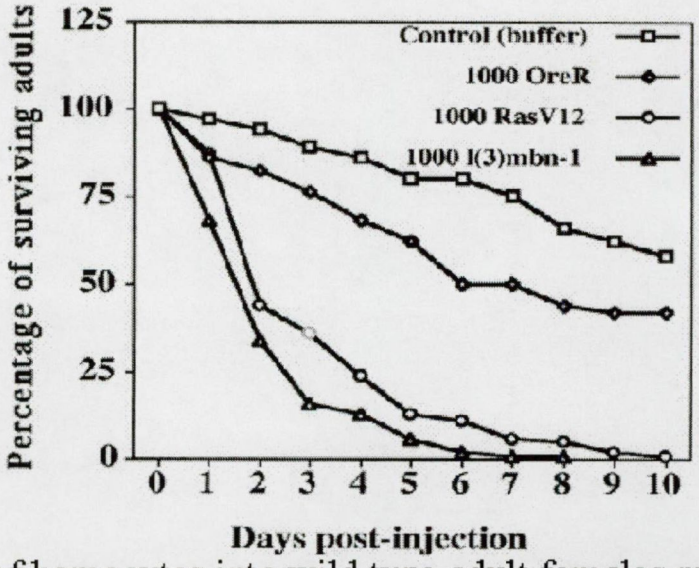

Figure 7. Injection of hemocytes into wild type adult females reduces survival. Survival curves of female flies injected with either control buffer or 1000 hemocytes each from Ore-R, Ras^{V12} or l(3)mbn-1 lervae. A total of 50, 131 or 139 wild-type female flies were injected with hemocytes from Ore-R, Ras^{V12} or l(3)mbn-1, respectively. At least 200 wild-type flies were injected with the control buffer. Hemocytes fom l(3)mbn-1 or Ras^{V12} larvae reduce the survival of wild-type hosts compared to buffer or hemocytes from Ore-R larvae.

III.2.4. Functions of hemocyte subsets of Ras-act mutant

Oregon-R larval hemocytes were used as a control in determination of the ability of Ras-act mutant hemocytes to phagocytose bacteria and to mount an efficient cellular immune reaction to the eggs of the parasitic wasp *Leptopilina boulardi*. Simultaneously the phagocytic capacity of the l(3)mbn-1 mutant hemocytes was verified.

III.2.4.1. Reactions to bacteria

To test the reaction of hemocytes to bacteria *in vitro*, hemocytes were collected from intact larvae on slides and their capability of phagocytosing was tested using FITC-labeled bacteria. The antigen expression of the hemocytes was also determined. The number of cells showing green fluorescence (actively phagocytosing hemocytes), the number of phagocytosed bacteria per cell and cells showing red fluorescence (hemocytes expressing the given antigen) were counted.

We have found out that circulating hemocytes of the wild type and l(3)mbn-1 mutant expressing P1 antigen actively phagocytose while those expressing L1, including small, round cells of plasmatocyte morphology, don't take up bacteria. L1 expressing hemocytes of the Ras-act mutants also lack phagocytic activity. With simultaneous use of the P1 and L1 antigen we concluded that some little round cells of the $P1^-L1^-$ population also take up bacteria although in a less proficient manner. These cells lack crystalline inclusions. To compare the phagocytic ability of different sub-populations we determined the phagocytic capacity and antigen expression of the individual cells. As the activated Ras cause a dramatic increase of the $P1^-L1^-$ circulating hemocytes (see III.2.1.) first we determined the phagocytic capacity of the immature precursors. These cells phagocytose less than 5 particles. In wild type and in the hemocyte-overproducing mutant the $P1^+L1^-$ cells took up >10 particles, while the phagocytic activity of the $P1^-L1^-$ was significantly lower (<5 particles) - similar to that in the Ras-act mutant. We could never observe crystal cells that take up bacteria.

These data show that the phagocytic capacity and the molecular phenotype of the hemocytes are in correlation, and that the Ras-act hemocytes are capable of phagocytosing bacteria, but are slightly less proficient than wild-type hemocytes.

III.4.2.2. Reactions to parasites

Second instars were infested with the parasitic wasp *Leptopilina boulardi*. Reactions to infestation such as the changes in number and immunological phenotype of hemocytes in the circulation were followed. When wild type larvae are immuno-challenged by the wasp *L. boulardi*, the lamellocyte differentiation is induced and the number of lamellocytes is increased in number. They functions in encapsulating the wasp egg.

Twenty-four hours after infestation the number of $L1^+$ cells with various morphological features increases in the circulation. These $L1^+$ cells vary from little round cells to large flattened lamellocytes. Two days after infestation the fraction of $L1^+$ hemocytes still increases and 72 hours after infestation it reaches a plateau.

These data indicate that lamellocyte differentiation occurs in Ras-act larvae.

III.4.3. Microarray analysis of Ras-expressing hemocytes

To identify those genes whose expression correlates with oncogenic Ras-induced cell overproliferation, we examined expression profiles of genes using oligonucleotide microarrays. There is a 10-fold increase in the number of Ras-act hemocytes ($192x10^3/\mu l$) compared with that of Ras-wt hemocytes ($18x10^3/\mu l$). Therefore, a comparison of the patterns of gene expression between these two populations of hemocytes is likely to identify those genes whose expression is altered by increasing Ras activity.

Using Affymetrix *Drosophila* oligonucleotide microarrays, we compared the expression profiles of >13,000 genes between the two populations of hemocytes. In a comparative analysis of Ras-act to Ras-wt hemocytes there is at least a threefold increase in the expression of 279 genes (for details see [20]). The expression of 76 genes is decreased threefold or more in Ras-act compared with that in Ras-wt (for details see [20]).

Among the genes known to regulate hemocyte fates in *Drosophila*, expression of lozenge is increased 5.7-fold in Ras-act hemocytes. *lz* expression is necessary for crystal cell fate specification and lz mutants lack crystal cells [67]. Although lz is induced in Ras-act hemocytes, we do not observe a significant increase in morphologically recognizable crystal cells in Ras-act larvae (see III.2.1.). However, these cells appear to express much higher levels of both a putative prophenoloxidase activator (128-fold) and Black cells (Bc; 3.6-fold), which encodes monophenol oxidase. Both these enzymes are believed to function in crystal cells. Thus, increased Ras activity may increase the expression of a subset of crystal-cell-specific genes. The transcription factor, serpent, is essential for the development of all embryonic hemocytes *srp* expression is increased in Ras-act hemocytes by 2-fold. The transcription factor, *gcm*, has been shown to be necessary for plasmatocyte fate specification [74]. Expression of *gcm* is not detected in either Ras-act or Ras-wt hemocytes. This may correlate with our finding that these cells do not express the plasmatocyte-specific marker,

P1. However, croquemort and peroxidasin, genes that are expressed in normal plasmatocytes 62,63] are expressed to similar levels in both Ras-wt and Ras-act hemocytes.

These data show that lozenge and serpent expression is increased while the expression of gcm is not detected in Ras-act hemocytes, and also that increasing Ras activity does not cause a general increase in the expression of genes normally associated with plasmatocytes.

IV. Discussion

Most of the insect species contains several types of blood cells that are - based on morphological criteria - easily distinguishable from each another. In *Drosophila* however only four types of hemocytes are distinguishable using morphological criteria [60]. Hemocytes of different lineages - plasmatocytes, lamellocytes, podocytes, crystal cells and the progenitors of these cells - must be produced continuously throughout the ontogenesis of the *Drosophila melanogaster*. We propose that the number of distinct cell types that differs from one another in origin or in function is much higher, ever though certain subpopulations resembles same morphological phenotype. How the hematopoietic system develops and the fate of individual blood lineages is chosen is topic of this work.

An antibody-based resetting technique for the physical separation of *Drosophila* hemocyte-subpopulations: In order to better understand the differentiation process and function of different hemocyte sub-populations we have separated the circulating hemocytes using an antibody-based rosetting technique exploiting the expression pattern of cell surface antigens. With this method we have been able to isolate distinct subsets of *Drosophila* hemocytes.

After separation with the P1 antigen and in the presence of growth factor/s [118] both of the fractions can be maintained *in vitro* as they both contains hemocytes that retains mitotic activity. The molecular phenotype of the hemocytes of the $P1^+$ fraction indicates that the majority of the hemocytes are plasmatocytes as demonstrated with P1 staining. Still a small fraction of hemocytes also expresses the L1 antigen. This is probably due to the contamination of the fraction with lamellocytes and also because some double positive cells are present as demonstrated with simultaneous staining for P1 and L1 antigens. Cells

expressing the P1 marker actively phagocytose. The lifetime of the culture is short and this is probably due to the lack of immature precursors that could uphold the culture. We propose that hemocytes committed to the plasmatocyte lineage expressing the plasmatocyte-specific marker are capable only for a few additional divisions, and for this reason this culture dies out. The $P1^-$ fraction comprises the mitotically active $P1^-L1^-$ that phagocytose, the lamellocytes and crystal cells both lacking phagocytosis. Preliminary data indicates that the double negatives elongate the lifetime of this culture.

Following separation with the L1 antigen the lifetime of the $L1^+$ fraction - that is composed exclusively of lamellocytes lacking both mitotic activity and phagocytosis - is within days. As in the case of $P1^-$ fraction double negatives in the $L1^-$ fraction are able to maintain this culture.

We concluded that the separated subsets retain their functionality as they phagocytose and show mitotic activity. The high recovery and the functional activity of the separated hemocytes make them suitable for molecular analysis. Cultured *in vitro* these sub-populations can differentiate that makes them appropriate for further *in vitro* studies of hemocyte development.

Ras can induce an overproliferation of functional yet abnormal hemocytes: We have shown that the overexpression of activated Ras in *Drosophila* hemocytes can induce hemocyte overproliferation. This one-step model of Ras-induced leukemia in *Drosophila* may thus represent a valuable system to characterize the link between increased Ras activity and uncontrolled cell proliferation.

Previous studies by others have shown that activated Ras can induce growth and cell proliferation in *Drosophila* [98,119]. Expression of activated Ras in *Drosophila* imaginal discs results in disc overgrowth. Analysis of patterns of BrdU incorporation and apoptosis showed that some of the consequences of Ras overexpression were the result of nonautonomous mechanisms [119]. These nonautonomous mechanisms have not yet been elucidated. Expression of activated Ras in clones of cells in the wing imaginal discs showed that increased Ras activity results in increased cell growth (mass accumulation). Increased Ras activity also results in cell death in the intervein regions of the wing when cells have to undergo terminal differentiation [98]. In contrast, the hemocyte overproliferation that we

describe generates a more uniform cell population, most likely resulting from a cell-autonomous effect of Ras in the hemocytes. Therefore, *Drosophila* hemocytes might represent a simpler system to elucidate the molecular mechanisms underlying Ras-induced neoplastic cell overproliferation.

Although the number of the mutant hemocytes shows a remarkable 40 fold increase in the number of circulating hemocytes as the CgGAL4 driver is expressed in all embryonic and larval hemocytes it is unclear which hemocyte subpopulation is effected by the leukaemia-like overexpression of the Ras-act hemocytes. The mutant hemocytes differ from their wild type counterparts in their expression pattern of plasmatocyte-specific antigens as they completely lack the P1 antigen. Since more than 95% of the hemocytes lack any of the hemocyte sub-population specific antigen expression we concluded that these cells represent the multipotent progenitor cell population. The number of both $L1^+$ lamellocytes and $C1^+$ crystal cells is also increased, though not in such an emphatic manner than the $P1^-L1^-$. This suggests that the activation of the Ras affects these subpopulations as well.

The circulating hemocytes of the mutant appear to be normal by functional criteria's. The majority of overproliferating hemocytes are capable of phagocytosis, though in reduced manner. Moreover lamellocyte differentiation may be induced with exposing to parasitic wasp infestation. The expression of the plasmatocyte markers croquemort and peroxidasin by these hemocytes is also normal.

By comparing the RNA samples from Ras-act and Ras-wt hemocytes, we have been able to assess the transcriptional changes over the entire predicted genome that result from an increase in Ras activity in a specific lineage. One overall finding is that many of the genes that are upregulated in Ras-act cells include genes that function in cell cycle regulation and DNA replication. These genes include both positive and negative regulators of cell proliferation [20].

A second finding is that the circulating hemocytes of the Ras-act mutant show different expression patters of genes with known function in hemocyte determination. Lozenge, a gene known to regulate crystal cell lineage determination shows a significant increase in the expression; on the other hand Ras-act hemocytes lack the expression of gcm, a fundamental regulator of the plasmatocyte lineage.

Lineage commitment and antigen expression of the hemocyte sub-populations: Recent evidence suggests that all hemocyte lineages, consisting the plasmatocyte/lamellocyte- and the crystal cell lineage are generated from the hematopoietic stem cell through a common progenitor [59,67]. The pluripotent hematopoietic stem cell gives rise to all mature cell types of the blood by differentiating through intermediate progenitor cells that undergo lineage commitment and subsequent development along a single pathway. The expression pattern of these hematopoietic stem cells is $H2^{+}P1^{-}L1^{-}C1^{-}$ and they show mitotic activity and phagocytose foreign objects although less proficient than mature plasmatocytes. Stem cells are anchored in specific locations as the lobes of the lymph gland and sessile tissue [59] where decisions concerning proliferation and differentiation/migration pathways are made. They give rise to lineage precursors that transform into transit cells and sequentially express lineage specific features during their differentiation program. Morphologically and functionally mature cells in the circulating pool of the hemocytes are heterogeneous.

Larval hemocytes, regardless of their origin and state of differentiation, express the panhemocyte marker H2 and are scattered throughout the body and also in hematopoietic compartments. These compartments are morphologically well definable and include the lobes of the lymph gland and the circulating hemocytes [113]. P1 antigen is exclusively expressed on the plasmatocytes; subpopulations of small round hemocytes that phagocytose bacteria and other foreign objects. In wild type this class of hemocytes comprise more than 98% of the circulating hemocytes. The antigen L1 is specifically expressed on the terminally differentiated lamellocytes and their direct precursors. Antigen C1 is expressed in a small subset of circulating hemocytes of all stages of larval development. The number of cells expressing these antigens is the highest in the late third stage larvae. The antigens are present either in crystal inclusions or distributed homogeneously in the cytoplasm of the cells regardless of the developmental stage.

Functional activity of the hemocyte sub-populations: Even though the role of the hemocyte subpopulations in innate immune response is indubitable the knowledge about the function of these cells is fragmentary. We have unravelled the function of the subpopulations with the help of the newly identified molecular markers. Cells expressing the plasmatocyte-

specific antigens phagocytose microorganisms and other particles. Lamellocytes do not phagocytose but they take part in the encapsulation reactions such as demarcation and demolition of the invading organisms. The double negatives - hemocytes lacking the expression of both plasmatocyte and lamellocyte markers – are also phagocytic cells however the phagocytosing rate of these cells is much lower (around 30%) than those of the fully matured plasmatocytes. The exact role of the double positives is still unclear. Indirect evidences suggest that these cells phagocytose as all the P1 expressing cells engulf bacteria, but their role in encapsulation reaction as well as their mitotic activity is unknown. Hemocytes presumably play an important role in the coagulation reaction too, though this still has to be confirmed. On the other hand the crystalline inclusions in the cytoplasm of the crystal cells are proposed to contain enzymes that catalyses malanization reactions.

Definition of the mitotic activity of the subpopulations typified on the basis of the expression of molecular markers: On the basis of the experiment carried on the circulating hemocytes we concluded that not all of the subpopulations preserved the ability of division. The mitotic activity of the wild type circulating hemocytes is low. Immature precursors (double negatives), plasmatocytes and crystal cell precursors shows mitotic activity. We never observed lamellocyte - including little round cell of plasmatocyte morphology - in division that expressed the lamellocyte-specific antigens. This means that the terminally differentiation of lamellocytes, from their direct precursors does not require cell division.

In the case of the l(3)mbn-1 mutant 2% of the plasmatocyte-specific antigen expressing hemocytes divides. We propose that only a fraction of plasmatocytes shows mitotic activity and in the process of maturation fully matured plasmatocytes looses the ability of dividing. As there is no molecular marker for this pool this is only a hypothesis. The direct precursors of the crystal cells shows mitotic activity; this support the speculation that crystal cells together with their direct precursors forms a detached differentiation lineage.

A model for hematopoiesis in *Drosophila*: Hematopoiesis begins early during embryogenesis and the process undergoes many changes during embryonic and larval development. The number of the various cell types in the circulation is normally kept in a

fairly constant ranges and the production process of the various cell types in distinct life stages are highly regulated.

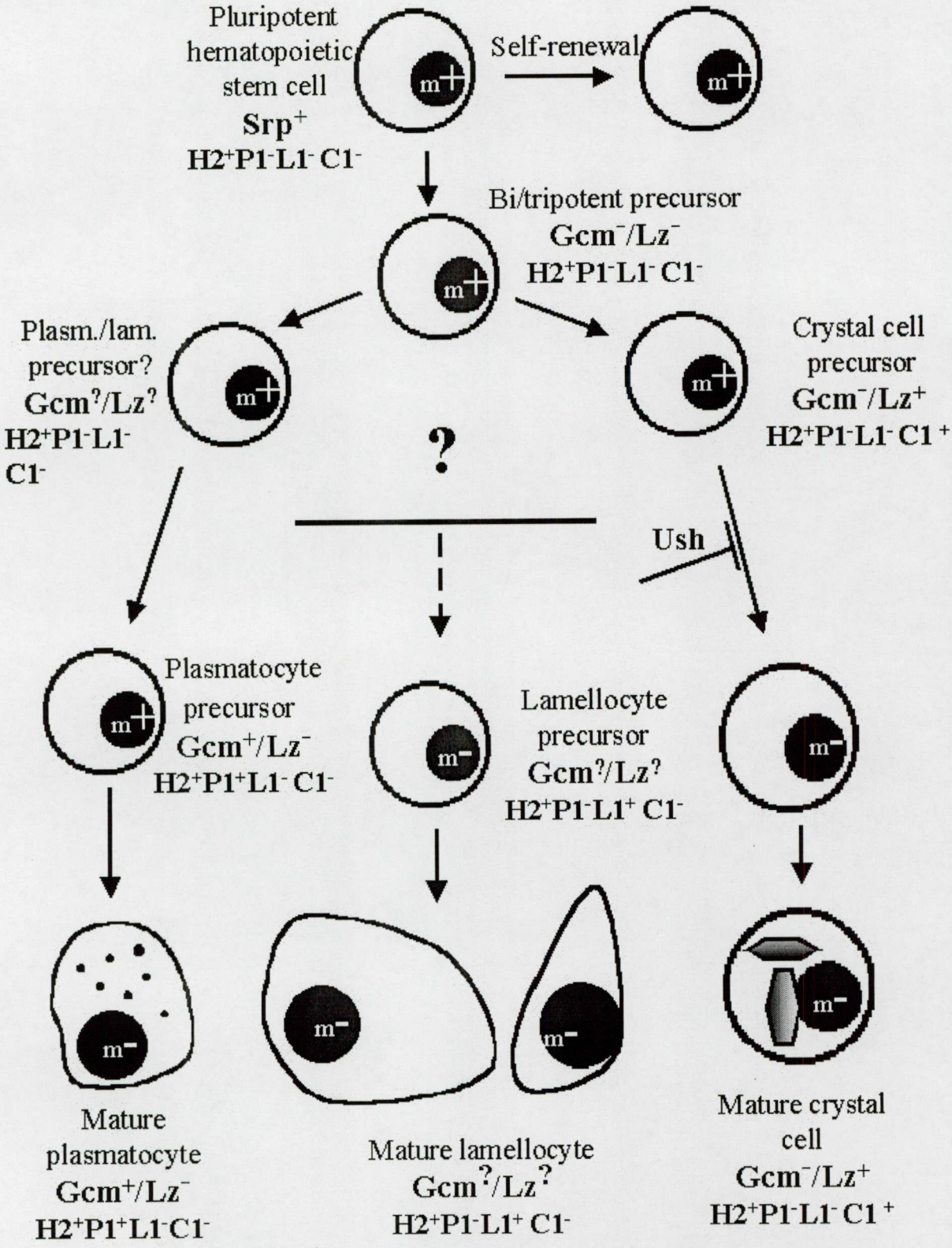

Figure 8. Hematopoiesis of *Drosophila*

Based on the results discussed above we have created a model of the hematopoiesis in *Drosophila* (Figure 7.). According to this hypothesis the pool of the Srp^+ hemocytes expresses the pan-hemocyte antigen H2 but lack the expression of the subpopulation-specific antigens ($H2^+P1^-L1^-C1^-$). These cells are considered as uncommitted pluripotent hematopoietic stem cells that have limited phagocytic capacity. As these cells show mitotic activity and are always present in the embryos and larvae we propose that this pool is able for a self-renewal. Furthermore this pool gives rise to all mature hemocyte subpopulations through extensive proliferation and multiple differentiation steps. We propose that the committed hematopoietic progenitor cells are progeny of a hematopoietic stem cell that have begun to differentiate. As stem cells these hemocytes expresses H2 but lack the expression of the subpopulation-specific antigens ($H2^+P1^-L1^-C1^-$) and still shows mitotic activity. We suggest that these cells are no longer able to give rise to stem cells but are bi/tripotent hematopoietic precursors. The descendants of the multilineage precursors are the cells that are ultimately restricted to a single-lineage potential. These cells beside expression of the pan-hemocyte antigen already express subpopulation-specific antigens. Cells committed to a crystal cell lineage - Lz^+ hemocytes - express C1 but lacks P1 and L1 ($H2^+P1^-L1^-C1^+$) expression. Crystalline inclusions are missing from the crystal cell precursors but they show mitotic activity. These cells undergo terminally differentiation – the final step of the hematopoiesis, and the fully matured crystal cells contain crystalline inclusion in their cytoplasm and lack mitotic activity. Hemocytes that undergo plasmatocyte commitment - Gcm^+ cells - express P1 but lacks L1 and C1 ($H2^+P1^+L1^-C1^-$). The plasmatocyte precursors show mitotic activity but the fully matured plasmatocytes looses the ability for division upon terminally differentiation. It is still unclear whether the lamellocyte precursors ($H2^+P1^-L1^+C1^-$) are direct descendants of the multilineage progenitors or they develop from the plasmatocyte/lamellocyte or even from lamellocyte/crystal cell precursors. However we have demonstrated that these cells form a separated lineage. The terminal phase of lamellocyte differentiation does not require cell divisions as the lamellocyte precursors lack mitotic activity, yet lamellocyte precursors undergoes dramatic specialized changes during maturation associated with morphological phenotype.

Even if the model is incomplete in terms of deciphering the links between expression of distinct transcription factors and hemocyte specific antigens it provides a theory for

understanding the basis of the hematopoiesis in *Drosophila*. In general hemocytes undergo multiple divisions and changes in antigen and transcription factor expression between the stages shown in Figure 4. A small number of stem cells give rise to a greater number of the earlier committed progenitors, which are amplified through cell division at each subsequent point in the differentiation process. According to these, each successive stage has a more restricted differentiation potential. We propose that the fully matured cell types loose the ability of mitotic activity. The molecular processes that determine whether a stem cell undergoes self-renewal or commitment to differentiate are not understood, nor are the events that lead to commitment steps leading to a specific lineage.

With the help of the discussed results it will be possible to unravel some elements of the innate immune defence of vertebrates that share analogous mechanisms with the innate immunity of the *Drosophila*. It will be also possible to identify vertebrate homologues of the *Drosophila* hemocyte-specific molecules and this may provide novel insights into the function of innate immunity of vertebrates, including man. Finally, definition of the *Drosophila* hemocyte-specific receptors and their vertebrate homologues will help in better understanding of the hematopoiesis and innate immunity.

V. Summary

Hemocytes represent the cellular elements of the innate immune defence in insects. They recognize, attack and inactivate the microorganisms and parasites invading the body cavity of the insect larvae. Although remarkable advances have been made in the past few years in understanding the hematopoiesis of *Drosophila*, little is known about the origin of hemocytes and mechanisms by which these cells act.

We have described a rosetting technique for separation of hemocyte sub-populations of *Drosophila* that relies on applying anti-hemocyte antibodies coupled to sheep erytrocytes. Hemocytes after separation retain their functions so the method can be used for functional and molecular characterization of hemocyte-subpopulations.

We have used molecular markers with specific expression on *Drosophila* hemocytes to understand the differentiation and function of these cells. By the aid of these markers we have also characterized the differentiation and function of hemocytes. The hematopoietic

stem cells show mitotic activity and they phagocytose foreign objects. The plasmatocytes phagocytose bacteria and other foreign objects. Both plasmatocytes and their immediate precursors - proplasmatocytes - show mitotic activity. The function of the terminally differentiated flattened lamellocytes and their precursors is to encapsulate foreign objects and abnormally developing tissues, which are too large to be phagocytosed. This subpopulation of hemocytes does not phagocytose and does not show mitotic activity. In contrast to the previous hypothesis we have demonstrated that the plasmatocyte and the lamellocyte differentiation lineage are separated from each another. The procrystal- and the crystal cells, which are involved in the melanization and coagulation reactions - do not phagocytose. There are no crystalline inclusions in the procrystal cells, which show mitotic activity, while mature crystal cells contain crystals but they don't divide. The procrystal- and crystal cells form a separate differentiation lineage from the plasmatocyte and the lamellocyte lineages.

Our model to study cell proliferation demonstrates the utility of the *Drosophila* hematopoietic system as a model for the genetic and functional study of oncogenic cell overproliferation. We showed that overexpression of activated Ras in *Drosophila* hemocytes results in sustained cell proliferation in a manner analogous to that observed in human leukemias.

VI. Abbreviations

AEC	3-Amino-9-ethyl-carbazole
BSA	Bovine Serum Albumin
CSSM3	Shield and Sang M3 insect medium containing stabilized glutamine and 5% fetal calf serum
DAPI	4`,6-Diamidino-2-phenylindole
dW	distilled Water
EDTA	Ethylenediamine Tetraacetic acid
EMS	Ethyl-Methyl-Sulfonate
FACS	Fluorescein Activated Cell Sorter
FCS	Fetal Calf Serum
FITC	Fluorescein Isothyocianate
Ig	Immunoglobulin

L1-SRBC	sheep red blood cells coated with the L1 antibody
mAb	monoclonal antibody
P1-SRBC	sheep red blood cells coated with the P1 antibody
PBS	Phosphate Buffered Saline
PhHH3	Phospho-Histone H3
PTU	phenil-thiourea
SRBC	Sheep Red Blood Cells
UAS	Upstream Activating Sequences

VIII. Acknowledgements

First of all I whish to thank my supervisor dr. István Andó for the possibility to join his group, as well as for supporting my ideas and progression of my work.

I am thankful to dr. Éva Kurucz (BRC HAS, Szeged) for continuous help and to her critical remarks relative to my work.

I also whish to thank to all the colleagues: Gábor Kovács, Florentina Rus, Róbert Márkus, Barbara Laurinyecz, dr. Péter Vilmos from the group of molecular immunology (BRC HAS, Szeged); dr. Asha Harakrishnan and dr. Charles Dearolf (Boston); dr. Elisabeth Gateff (Mainz) and dr. Dan Hultmark (Umea).

I whish to thank Szilvia Tápai, Ágnes Árva, Olga Kovalcsik for technical help and Edit Kotogány (BRC HAS, Szeged) for the assistance with handling the FACS equipment.

I am also grateful to dr. Péter Putnoky and dr. Miklós Erdélyi (BRC HAS, Szeged) for tutorial supervising of my progress.

This work was supported with OTKA grants T035074 and T035249.

VIII. References

[1] Keller, R. (2002) Shaping the vertebrate body plan by polarized embryonic cell movements. Science 298(5600), 1950-1954

[2] Pandur, P., Maurus, D. and Kuhl, M. (2002) Increasingly complex: new players enter the Wnt signalling network. Bioessays 24(10), 881-884

[3] Hombria, J.C. and Brown, S. (2002) The fertile field of *Drosophila* Jak/STAT signalling. Curr Biol. 12(16), 569-575

[4] Heisenberg, C.P. and Tada, M. (2002) Wnt signalling: a moving picture emerges from van gogh. Curr Biol. 12(4), 126-128

[5] Khush, R.S., Leulier, F. and Lemaitre, B. (2002) Immunology. Pathogen surveillance--the flies have it. Science 296(5566), 273-275

[6] Ingham, P.W. and McMahon, A.P. (2001) Hedgehog signalling in animal development: paradigms and principles. Genes Dev. 15(23), 3059-3087

[7] Brey, P.T, and Hultmark, D. (1989) in Molecular mechanisms of immune responses in insects, Chapman&Hall, London

[8] Hoffmann, J.A. and Reichhart, J.M. (19xx) Trends Cell Biol. 7, 307-316

[9] Hultmark, D. (1993) Immune reactions in *Drosophila* and other insects: a model for innate immunity. Trends Genet. 9(5), 178-183

[10] Hultmark, D. (1994) Insect immunology. Ancient relationships. Nature 367(6459), 116-117

[11] Medzhitov, R., Preston-Hurlburt, P., and Janeway, C.A.Jr., (1997) A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. Nature 388(6640), 394-397

[12] Dearolf, Ch.R. (1998). Fruit fly "leukemia". Biochim. Biophys. Acta 1377 (1): 13-23

[13] Rehorn, K.P., Thelen, H., Michelson, A.M. and Reuter, R. (1996) A molecular aspect of hematopoiesis and endoderm development common to vertebrates and *Drosophila*. Development 122(12), 4023-4031

[14] Ramet, M., Manfruelli, P., Pearson, A., Mathey-Prevot, B. and Ezekowitz, R.A. (2002) Functional genomic analysis of phagocytosis and identification of a *Drosophila* receptor for E. coli. Nature 416(6881), 644-648

[15] Tonkin, E., Hagan, D.M., Li, W. and Strachan, T. (2002) Identification and characterization of novel mammalian homologues of *Drosophila* polyhomeotic permits new insights into relationships between members of the polyhomeotic family. Hum. Genet. 111(4-5), 435-442

[16] Levine, S.S., Weiss, A., Erdjument-Bromage, H., Shao, Z., Tempst, P. and Kingston, R.E. (2002) The core of the polycomb repressive complex is compositionally and functionally conserved in flies and humans. Mol. Cell. Biol. 22(17), 6070-6078

[17] Shulman, J.M., Shulman, L.M., Weiner, W.J. and Feany, M.B. (2003) From fruit fly to bedside: translating lessons from *Drosophila* models of neurodegenerative disease. Curr. Opin. Neurol. 16(4), 443-449

[18] Rodriguez, A., Zhou, Z., Tang, M.L., Meller, S., Chen, J., Bellen, H. and Kimbrell, D.A. (1996) Identification of immune system and response genes, and novel mutations causing melanotic tumor formation in *Drosophila melanogaster*. Genetics 143(2), 929-940

[19] Braun, A., Lemaitre, B., Lanot, R., Zachary, D. and Meister, M. (1997) *Drosophila* immunity: analysis of larval hemocytes by P-element-mediated enhancer trap. Genetics 147(2), 623-634

[20] Asha, H., Nagy, I., Kovács, G., Statson, D., Andó, I. and Dearolf, CH.R. (2003) Analysis of Ras-induced overproliferation in *Drosophila* hemocytes. Genetics 163(1), 203-215

[21] De Gregorio, E., Spellman, P.T., Rubin, G.M. and Lemaitre, B. (2001) Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microarrays. PNAS 98(22), 12590-12895

[22] Khush, R.S. and Lamaitre, B. (2000) Genes that fight infection: what the *Drosophila* genome says about animal immunity. Trends Genet. 16(10), 442-449

[23] Tzou, P., De Gregorio, E. and Lemaitre, B. (2002) How *Drosophila* combats microbial infection: a model to study innate immunity and host-pathogen interactions. Curr Opin Microbiol. 5(1), 102-110

[24] Luo, H., Rose, P., Barber, D., Hanratty, W.P., Lee, S., Roberts, T.M., D'Andrea, A.D. and Dearolf, C.R. (1997) Mutation in the Jak kinase JH2 domain hyperactivates *Drosophila* and mammalian Jak-Stat pathways. Mol. Cell Biol. 17(3), 1562-1571

[25] Elrod-Erickson, M., Mishra, S. and Schneider, D. (2000) Interactions between the cellular and humoral immune responses in *Drosophila*. Curr Biol. 10(13), 781-784

[26] Nappi, A.J. and Vass, E. (1998) Hydrogen peroxide production in immune-reactive *Drosophila melanogaster*. J Parasitol 84(6), 1150-1157

[27] Nappi, A.J., Vass, E., Frey, F. and Cartoon, Y. (2000) Nitric oxide involvement in *Drosophila* immunity. Nitric Oxide 4, 423-430

[28] Li, D., Scherfer, C., Korayem, A.M., Zhao, Z., Schmidt, O. and Theopold U. (2002) Insect hemolymph clotting: evidence for interaction between the coagulation system and the prophenoloxidase activating cascade. Insect Biochem Mol Biol. 32(8), 919-928

[29] Nathan, C.F., and Hibbs, J.B.Jr. (1991) Role of nitric oxide synthesis in macrophage antimicrobial activity. Curr. Opin. Immunol. 3(1), 65-70

[30] Robinson, J.M. and Badwey, J.A. (1994) Production of active oxygen species by phagocytic leukocytes. Immunology 60, 159-178

[31] Kaul, N. and Forman, H.J. (1996) Activation of NF-κB by the respiratory burst of macrophages. Free Rad. Biol. Med. 21, 401-405

[32] Ip, Y.T. and Levine, M. (1994) Molecular genetics of *Drosophila* immunity. Curr Opin Genet Dev. 4(5), 672-677

[33] Hedengren, M., Asling, B., Dushay, M.S., Ando, I., Ekengren, S., Wihlborg, M. and Hultmark, D. (1999) Relish, a central factor in the control of humoral but not cellular immunity in *Drosophila*. Mol Cell. 4(5), 827-837

[34] Basset, A., Khush, R.S., Braun, A., Gardan, L., Boccard, F., Hoffmann, J.A. and Lemaitre B. (2000) The phytopathogenic bacteria Erwinia carotovora infects *Drosophila* and activates an immune response. PNAS 97(7), 3376-3381

[35] Meister, M., Lemaitre, B. and Hoffmann, J.A. (1997) Antimicrobial peptide defense in *Drosophila*. Bioessays. 19(11), 1019-1026

[36] Samakovlis, C. Kimbrell, D.A., Kylsten, P., Engström, A., and Hultmark, D. (1990) The immune response in *Drosophila*: pattern of cecropin expression and biological activity. EMBO J. 9(9), 2969-2976

[37] Meister, M., Braun, A., Kappler, C., Reichhart, J.M. and Hoffmann, J.A. (1994) Insect immunity. A transgenic analysis in *Drosophila* defines several functional domains in the diptericin promoter. EMBO J. 13(24), 5958-5966

[38] Kylsten, P., Samakovlis, C. and Hultmark, D. (1990) The cecropin locus in *Drosophila*; a compact gene cluster involved in the response to infection. EMBO J. 9(1), 217-224

[39] Dimarcq, J.L., Hoffmann, D., Meister, M., Bulet, P., Lanot, R., Reichart, J.M. and Hoffmann J.A. (1994) Characterization and transcriptional profiles of a *Drosophila* gene encoding an insect defensin. A study in insect immunity. Eur. J. Biochem. 221(1), 201-209

[40] Bulet, P., Dimarcq, J.L., Hetru, C., Lagueux, M., Charlet, M., Hegy, G., Van Dorsselaer, A. and Hoffmann J.A. (1993) A novel inducible antibacterial peptide of *Drosophila* carries an O-glycosylated substitution. J. Biol. Chem. 268(20), 14893-14897

[41] Asling, B., Dushay, M.S. and Hultmark, D., (1995) Identification of early genes in the *Drosophila* immune response by PCR-based differential display: the Attacin A gene and the evolution of attacin-like proteins. Insect Biochem. Mol. Biol. 25(4), 511-518

[42] Wickler, C., Reichhart, J.M., Hoffmann, D., Hultmark, D., Samakovlis, C. and Hoffmann, J.A. (1990) Insect immunity. Characterization of a *Drosophila* cDNA encoding a novel member of the diptericin family of immune peptides. J. Biol. Chem. 265(36), 22493-22498

[43] Fehlbaum, P., Bulet, P., Michaut, L., Lagueux, M., Broekaert, W.F., Hetru, C. and Hoffmann, J.A. (1994) Insect immunity. Septic injury of *Drosophila* induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. J. Biol. Chem. 269(52), 33159-33163

[44] Levashina, E.A., Ohresser, S., Bulet, P., Reichhart, J.M., Hetru, C. and Hoffmann, J.A. (1995) Metchnikowin, a novel immune-inducible proline-rich peptide from Drosophila with antibacterial and antifungal properties. Eur. J. Biochem. 233(2), 694-700

[45] Lavine, M.D. and Strand, M.R. (2002) Insect hemocytes and their role in immunity. Insect Biochem Mol Biol. 32(10), 1295-1309

[46] Kiger, J.A.Jr., Natzle, J.E. and Green, M.M. (2001) Hemocytes are essential for wing maturation in *Drosophila melanogaster*. PNAS 98(18), 10190-10195

[47] Carton, Y. and Nappi, A.J. (2001) Immunogenetic aspects of the cellular immune response of *Drosophilia* against parasitoids. Immunogenetics 52(3-4), 157-164

[48] Prevost, G. and Eslin, P. (1998) Hemocyte load and immune resistance to Asobara tabida are correlated in species of the *Drosophila* melanogaster subgroup. J Insect Physiol. 44(9), 807-816

[49] Eslin, P. and Prevost, G. (2000) Racing against host's immunity defenses: a likely strategy for passive evasion of encapsulation in Asobara tabida parasitoids. J Insect Physiol. 46(8), 1161-1167

[50] Moreau, S.J., Eslin, P., Giordanengo, P. and Doury, G. (2003) Comparative study of the strategies evolved by two parasitoids of the genus Asobara to avoid the immune response of the host, *Drosophila melanogaster*. Dev Comp Immunol. 27(4), 273-282

[51] Vass, E. and Nappi, A.J. (2000) Developmental and immunological aspects of *Drosophila*-parasitoid relationships. J Parasitol. 86(6), 1259-1270

[52] Sorrentino, R.P., Carton, Y. and Govind, S. (2002) Cellular immune response to parasite infection in the *Drosophila* lymph gland is developmentally regulated. Dev Biol. 243(1), 65-80

[53] Braun, A., Hoffmann, J.A. and Meister, M. (1998) Analysis of the *Drosophila* host defense in domino mutant larvae, which are devoid of hemocytes. PNAS 95(24), 14337-14342

[54] Lemaitre, B., Kromer-Metzger, E., Michaut, L., Nicolas, E., Meister, M., Georgel, P., Reichhart, J.M. and Hoffmann, J.A. (1995) A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the *Drosophila* host defense. PNAS 92(21), 9465-9469

[55] Werner, T., Liu, G., Kang, D., Ekengren, S., Steiner, H. and Hultmark, D. (2000) A family of peptidoglycan recognition proteins in the fruit fly *Drosophila melanogaster*. 97(25), 13772-13777

[56] Werner, T., Borge-Renberg, K., Mellroth, P., Steiner, H. and Hultmark, D. (2003) Functional diversity of the *Drosophila* PGRP-LC gene cluster in the response to lipopolysaccharide and peptidoglycan. J Biol Chem. 278(29), 26319-26322

[57] Takehana, A., Katsuyama, T., Yano, T., Oshima, Y., Takada, H., Aigaki, T. and Kurata, S. (2002) Overexpression of a pattern-recognition receptor, peptidoglycan-recognition protein-LE, activates imd/relish-mediated antibacterial defense and the prophenoloxidase cascade in *Drosophila* larvae. PNAS 99(21), 13705-13710

[58] Sondergaard, L. (1993) Homology between the mammalian liver and the *Drosophila* fat body. TIG 9(6), 193

[59] Lanot, R., Zachary, D., Holder, F. and Meister, M. (2001) Postembryonic hematopoiesis in *Drosophila*. Dev Biol. 230(2), 243-257

[60] Rizki,T.M. (1978) in, The genetics and biology of *Drosophila* (M.Ashburner and R.T.F.Wright, Eds.), Vol.2b,pp.397-452. Academic Press, London

[61] Tepass, U., Fessler, L.I., Aziz, A. and Hartenstein, V. (1994) Embryonic origin of hemocytes and their relationship to cell death in *Drosophila*. Development 120(7), 1829-1837

[62] Franc, N.C., Dimarcq, J.L., Lagueux, M., Hoffmann, J.A. and Ezekowitz, R.A. (1996) Croquemort, a novel *Drosophila* hemocyte/macrophage receptor that recognizes apoptotic cells. Immunity 4(5), 431-443

[63] Nelson, R.E., Fessler, R.I., Takagi, Y., Blumberg, B., Keene, D.R., Olsson, C.G. and Fessler, J.H. (1994) Peroxidasin: a novel enzyme-matrix protein of *Drosophila* development. EMBO J. 13(15), 3438-3447

[64] Abrams, J.M., Lux, A., Steller, H. and Krieger, M. (1992) Macrophages in *Drosophila* embryos and L2 cells exhibit scavenger receptor-mediated endocytosis. PNAS 10375-10379

[65] Yasothornsrikul, S., Davis, W.J., Cramer, G., Kimbrell, D.A. and Dearolf, CR. (1997) viking: identification and characterization of a second type IV collagen in *Drosophila*. Gene 198(1-2), 17-25

[66] Pearson, A., Lux, A. and Krieger, M. (1995) Expression cloning of dSR-CI, a class C macrophage-specific scavenger receptor from *Drosophila melanogaster*. PNAS 92(9), 4056-4060

[67] Lebestky, T., Chang, T., Hartenstein,V. and Banerjee,U. (2000) Specification of *Drosophila* hematopoietic lineage by conserved transcription factors. Science 288(5463), 146-149

[68] Duvic, B., Hoffmann, J.A., Meister, M. and Royet, J. (2002) Notch signaling controls lineage specification during *Drosophila* larval hematopoiesis. Curr Biol. 12(22), 1923-1927

[69] Rugendorff, A., Younossi-Hartenstein, A. and Hartenstein, V. (1994) Embryonic origin and differentiation of the Drosophila heart. Roux's Arch. Dev. Biol. 203, 266-280

[70] Orkin, S.H (1996) Development of the hematopoietic system. Curr Opin Genet Dev. 6(5): 597-602

[71] Fossett, N. and Schulz, R.A. (2001) Functional conservation of hematopoietic factors in *Drosophila* and vertebrates. Differentiation 69(2-3), 83-90

[72] Daga, A., Karlovich, C.A., Dumstrei, K. and Banerjee, U. (1996) Patterning of cells in the *Drosophila* eye by Lozenge, which shares homologous domains with AML1. Genes Dev. 10(10), 1194-1205

[73] Lutterbach, B. and Hiebert, S,W. (2000) Role of the transcription factor AML-1 in acute leukemia and hematopoietic differentiation. Gene 245(2), 223-235

[74] Bernardoni, R., Vivancos, V. and Giangrande, A. (1997) glide/gcm is expressed and required in the scavenger cell line, Dev Biol, 191(1), 118-130

[75] Altshuller, Y., Copeland, N.G., Gilbert, D.J., Jenkins, N.A. and Frohman, M.A. (1996) Gcm1, a mammalian homolog of *Drosophila* glial cells missing. FEBS Lett. 393(2-3), 201-204

[76] Kim, J., Jones, B.W., Zock, C., Chen, Z., Wang, H., Goodman, C.S. and Anderson, D.J. (1998) Isolation and characterization of mammalian homologs of the *Drosophila* gene glial cells missing. PNAS 95(21), 12364-12369

[77] Egger, B., Leemans, R., Loop, T., Kammermeier, L., Fan, Y., Radimerski, T., Strahm, M.C., Certa, U. and Reichert, H. (2002) Gliogenesis in *Drosophila*: genome-wide analysis of downstream genes of glial cells missing in the embryonic nervous system. Development 129(14), 3295-3309

[78] Freeman, M.R., Delrow, J., Kim, J., Johnson, E. and Doe, C.Q. (2003) Unwrapping glial biology: Gcm target genes regulating glial development, diversification, and function. Neuron 38(4), 567-580

[79] Alfonso, T.B. and Jones, B.W. (2002) gcm2 promotes glial cell differentiation and is required with glial cell missing for macrophage development in *Drosophila*. Dev Biol, 248(2), 369-383

[80] Kammerer, M. and Giangrande, A. (2001) Glide2, a second glial promoting factor in *Drosophila melanogaster*. EMBO J. 2001 20(17), 4664-4673

[81] Tsang, A.P., Visvader, J.E., Turner, C.A., Fujiwara, Y., Yu, C., Weiss, M.J. Crossley, M. and Orkin, S.H. (1997) FOG, a multitype zinc finger protein, acts as a cofactor for transcription factor GATA-1 in erythroid and megakaryocytic differentiation. Cell 90, 109-119

[82] Crispino, J.D., Lodish, M.B., MacKay, J.P. and Orkin, S.H. (1999) Use of altered specificity mutants to probe a specific protein-protein interaction in differentiation: the GATA-1:FOG complex. Mol. Cell 3, 219-228

[83] Fossett, N., Tevosian, S.G., Gajewski, K., Zhanh, Q., Orkin, S. and Schulz, RA. (2001) The friend of GATA proteins U-Shaped, GOG-1, and FOG-2 function as negative regulators of blood, heart, and eye development in *Drosophila*. PNAS 98, 7342-7347

[84] Orkin, S.H. (1992) GATA-binding transcription factors in hematopoietic cells. Blood 80,575-581

[85] Rylski, M., Welch, J.J., Chen Y.Y., Letting, D.L., Diehl, J.A., Chodosh, L.A., Blobel, G.A. and Weiss, N.J. (2003) GATA-1 mediated proliferation arrest during erythroid maturation. Mol Cell Biol. 23(14), 5031-5042

[86] Elagib, K.E., Racke, F.K., Mogass, M., Khetawat, R., Delehanty, L.L. and Goldfarb, A.N. (2003) RUNX1 and GATA-1 coexpression and cooperation in megakaryocytic differentiation. Blood. 101(11), 4333-4341

[87] Briegel, K., Bartunek, P., Stengl, G., Lim, K.C., Beug, H., Engel, J.D. and Zenke, M. (1996) Regulation and function of transcription factor GATA-1 during red blood cell differentiation. Development 122(12), 3839-3850

[88] Grass, J.A., Boyer, M.E., Pal, S., Wu, J., Weiss, M.J. and Bresnick, E.H. (2003) GATA-1-dependent transcriptional repression of GATA-2 via disruption of positive autoregulation and domain-wide chromatin remodeling. PNAS 100(15), 8811-8816

[89] Tsai, F.Y. and Orkin, S.H. (1997) Transcription factor GATA-2 is required for proliferation/survival of early hematopoietic cells and mast cell formation, but not for erythroid and myeloid terminal differentiation. Blood. 89(10), 3636-3643

[90] Ting, C.N., Olson, M.C., Barton, K.P. and Leiden, J.M. (1996) Transcription factor GATA-3 is required for development of the T-cell lineage. Nature 384(6608), 474-478

[91] Miller, A.A., Bernardoni, R., Hindelang, C., Kammerer, M., Sorrentino, S., Van de Bor, V. and Giangrande, A. (1999) Role and mechanism of action of glial cell deficient/glial cell missing (glide/gcm), the fly glial promoting factor. Adv Exp Med Biol. 468, 33-46

[92] Waltzer, L., Bataille, L., Peyrefitte, S. and Haenlin, M. (2002) Two isoforms of Serpent containing either one or two GATA zinc fingers have different roles in *Drosophila* haematopoiesis. EMBO J. 21(20), 5477-5486

[93] Petersen, U.M., Kadalayil, L., Rehorn, K.P., Hoshizaki, D.K., Reuter, R. and Engstrom, Y. (1999) Serpent regulates *Drosophila* immunity genes in the larval fat body through an essential GATA motif. EMBO J. 18(14), 4013-4022

[94] Tingvall, T.O., Roos, E. and Engstrom, Y. (2001) The GATA factor Serpent is required for the onset of the humoral immune response in *Drosophila* embryos. PNAS 98(7), 3884-3888

[95] McCormick, F. (1994) Activators and effectors of ras p21 proteins. Curr. Opin. Genet. Dev. 4, 71-76

[96] Bos, J.L. (1989) ras oncogenes in human cancer: a review. Cancer Res. 49, 4682-4689

[97] Neuman-Silberberg, F.S., Schejter, E., Hofmann, F.M. and Shilo, B.Z (1984) The *Drosophila* ras oncogenes: structure and nucleotide sequence. Cell 37, 1027-1033

[98] Prober, D.A. and Edgar, B.A (2000) Ras1 promotes cellular growth in the *Drosophila* wing. Cell 100, 435-446

[99] Dearolf, C.R. (1999) JAKs and STATs in invertebrate model organisms. Cell. Mol. Life Sci. 55, 1578-1584

[100] Myrick, K.V. and Dearolf, C.R. (2000) Hyperactivation of the *Drosophila* Hop Jak kinase causes the preferential overexpression of the eIF1A transcripts in larval blood cells. Gene 244, 19-25

[101] Myrick, K.V. and Dearolf, C.R. (2000) Hyperactivation of the *Drosophila* Hop jak kinase causes the preferential overexpression of eIF1A transcripts in larval blood cells. Gene 244(1-2), 119-125

[102] Mathey-Prevot, B. and Perrimon, N. (1998) Mammalian and *Drosophila* blood: JAK of all trades? Cell. 92(6), 697-700

[103] Govind, S. (1999) Control of development and immunity by Rel transcription factors in *Drosophila*. Oncogene 18, 6875-6887

[104] Qiu, P., Pan, P.C. and Govind, S. (1998) A role for the *Drosophila* Toll/Cactus pathway in larval hematopoiesis. Development 125(10), 1909-1920

[105] Lebestky, T., Jung, S.H. and Banerjee, U. (2003) A Serrate-expressing signaling center controls *Drosophila* hematopoiesis. Genes Dev. 17(3), 348-353

[106] Remillieux-Leschelle, N., Santamaria, P. and Randsholt, N.B. (2002) Regulation of larval hematopoiesis in *Drosophila melanogaster*: a role for the multi sex combs gene. Genetics. 162(3), 1259-1274

[107] Cho, N.K., Keyes, L., Johnson, E., Heller, J., Ryner, L., Karim, F. and Krasnow, M.A. (2002) Developmental control of blood cell migration by the *Drosophila* VEGF pathway. Cell 108(6), 865-876

[108] Heino, T.I., Karpanen, T., Wahlstrom, G., Pulkkinen, M., Eriksson, U., Alitalo, K. and Roos, C. (2001) The *Drosophila* VEGF receptor homolog is expressed in hemocytes. Mech Dev. 109(1), 69-77

[109] Munier, A.I., Doucet, D., Perro, dou E., Zachary, D., Meister, M., Hoffmann, J.A., Janeway, C.A.Jr. and Lagueux, M. (2002) PVF2, a PDGF/VEGF-like growth factor, induces hemocyte proliferation in *Drosophila* larvae. EMBO Rep. 3(12), 1195-1200

[110] Oravecz, T. Monostori, É., Kurucz, É., Takács, L. and Andó, I. (1991) CD3-Induced T- cell proliferation and interleukin-2 secretion is modulated by the CD45 antigen. Scand. J. Immunol. 34, 531

[111] Konrad, L., Becker, G. , Schmidt, A. , Klockner, T. , Kaufer-Stillger, G. , Dreschers, S., Edström, J. E. & Gateff, E. (1994) Cloning, structure, cellular localization, and

possible function of the tumor suppressor gene lethal(3)malignant blood neoplasm-1 of *Drosophila melanogaster*. Dev. Biol. 163, 98-111

[112] Brand, A.H. and Perrimon, N. (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401-415

[113] Kurucz, E., Zettervall, C.J., Sinka, R., Vilmos, P., Pivarcsi, A., Ekengren, S., Hegedus, Z., Ando, I. and Hultmark, D. (2003) Hemese, a hemocyte-specific transmembrane protein, affects the cellular immune response in *Drosophila*. PNAS, 100: 2622-2627

[114] Vilmos, P. (1999) An immunological approach towards the characterisation of *Drosophila melanogaster* hemocytes. Annals of Albert Szent-Györgyi Medical University 22

[115] Hudson, L. and Hay, F.C. (1976). Practical Immunology (Oxford: Blackwell Scientific Publications pp. 11 -12)

[116] Hendzel, M.J., Wei, Y., Mancini, M.A., Van Hooser, A., Ranalli, T., Brinkley, B.R., Bazett-Jones, D.P. and Allis, C.D. (1997) Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma, 106, 348-360

[117] Hanratty, W.P. and Ryerse, J.S. (1981) A genetic melanotic neoplasm of *Drosophila melanogaster*. Dev. Biol. 83:238-249

[118] Kawamura, K., Shibata, T., Saget, O., Peel, D. and Bryant, P.J. (1999) A new family of growth factors produced by the fat body and active on *Drosophila* imaginal disc cells. Development 126(2), 211-219

[119] Karim, F.D. and Rubin G.M. (1998) Ectopic expression of activated Ras1 induces hyperplastic growth and increased cell death in *Drosophila* imaginal tissues. Development 125, 1-9

Publications