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Definition of *Drosophila* hemocyte subsets by cell-type specific antigens

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ABSTRACT

We have analyzed the heterogeneity of *Drosophila* hemocytes on the basis of the expression of cell-type specific antigens. The antigens characterize distinct subsets which partially overlap with those defined by morphological criteria. On the basis of the expression or the lack of expression of blood cell antigens the following hemocyte populations have been defined: crystal cells, plasmatocytes, lamellocytes and precursor cells. These cells are arranged in four main compartments: the circulating hemocytes, the sessile tissue, the lymph glands and the posterior hematopoietic tissue. Each hemocyte compartment has a specific and characteristic composition of the various cell types. The described markers represent the first successful attempt to define hemocyte lineages by immunological markers in *Drosophila* and help to define morphologically, functionally, spatially and developmentally distinct subsets of hemocytes.

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INTRODUCTION

The immune system of *Drosophila* responds to microbial and parasitic infections by humoral and cellular reactions. The humoral reaction involves the production of antimicrobial peptides by the fat body (7, 6). The cellular reactions, the phagocytosis, the encapsulation reaction and subsequent melanization of the encapsulated particles (5) and the tissue remodeling in eye and wing development are mediated by hemocytes (9). Although there has been some controversy over the number of different classes of hemocytes, clearly three distinct types are important for elimination of foreign and recognition of self: the plasmatocyte, the lamellocyte and the crystal cell (29, 20).

Hemocytes originate from the hematopoietic organs, the so-called lymph glands (23, 27). In *Drosophila*, three types of hemocytes have been identified by morphological criteria (23, 27). The crystal cells are round cells characterized by crystalline inclusions within their cytoplasm and are involved in melanization (24). The plasmatocytes are also round cells capable of phagocytosis, sharing many functions with vertebrate phagocytic cells (24). The lamellocytes are large, flattened cells and are involved in encapsulation of abnormally developing tissues and in killing of developing parasites. There are indications that the various hemocyte types exhibit different functions during immune reactions (4) and they recognize ‘foreign’ by specific receptors (21, 22, 14, 11).

Molecular markers have been extremely useful in the definition of cell types and cell lineages of the

mammalian immune system (10). In vertebrates, as well as in *Drosophila*, transcription factors have been used as markers for hemocytes developing in embryonic life (17, 8). From these studies it is clear that crystal cells and plasmatocytes form two lineages in embryonic development, being the former $Srp^{+}Lz^{+}$ while the latter $Srp^{+}Gcm^{+}$. For the delineation of plasmatocytes and lamellocytes two models have been proposed. One model assumes that upon immune stimulation plasmatocytes adhere to a substrate, undergo morphological changes and differentiate to lamellocytes (23, 27, 19). Another model proposes that lamellocytes do not arise directly from plasmatocytes (16). Hemocytes of *Drosophila* larvae and the adult have been characterized so far exclusively by morphological criteria and function (2). To obtain a deeper insight into heterogeneity, development and function of *Drosophila* hemocytes we have undertaken the task to define hemocyte populations by their cell surface antigens. Here we show that hemocyte subsets can be defined by the expression pattern of cell surface antigens. These cell surface antigens appear sequentially in the course of hemocyte development and are characteristic to a given subset and lineage. Their expression pattern allows a molecular and functional definition of hemocyte types, lineages in relation to their functions in the different compartments. Monoclonal antibodies have been broadly used to define blood cells in vertebrates, insects, annelids and other invertebrates. Here we describe the production and use of monoclonal antibodies to characterize *Drosophila* hemocytes on the basis of the work in our laboratories.

MATERIALS AND METHODS

Drosophila strains and isolation of hemocytes

The wild-type *Oregon-R* and the tumor suppressor mutant strain lethal(3)malignant blood neoplasm-1 [*l(3)mbn-1*; 12] of *Drosophila melanogaster* were used in the experiments. Strain *Y81* is a transgenic strain for *cecropin-A1* promoter driven *lac-Z* (25). Flies were propagated at 25°C in standard *Drosophila* medium. The age of the larvae was calculated in hours after egg laying. Larval hemocytes were prepared by dissecting the larvae in *Drosophila* Ringer's solution on ice in the wells of 96 well round bottomed tissue culture plates. Hemocytes of the adults were flushed out of the body cavity with Ringer solution. In some experiments a *Hemese-GAL-4* recombinant stock (*He-GFP*) that also caries the GFP reporter *p{UAS-GFP.nls}* has been used to visualize hemocytes (31).

Production of monoclonal antibodies

Female BALB/c mice were immunized twice with $2x10^6$ *l(3)mbn-1* hemocytes in three week intervals. One week after the second immunization the sera of the mice were tested for antibodies on *l(3)mbn-1* hemocytes by indirect immunofluorescence and immunocytochemistry. Three weeks later the mice received a final booster injection and after three days a cell suspension was prepared from the spleens in RPMI-1640 tissue culture medium supplemented with 5% Fetal Calf Serum and 10mM glutamine. The spleen cells were fused with Sp2/0 cells in the presence of polyethylene glycol (PEG-1540). Hybridomas were selected in HAT medium and maintained as described by Kohler and Milstein (13). In most cases tissue culture supernatants have been used. Antibodies recognizing different epitopes on the same molecule (for example, P1a,P1b or L1a,L1b,L1c, respectively), thus forming one single cluster, have been mixed and used for cell-type analysis.

Indirect immunofluorescence and flow cytometry

Hybridoma cells were screened for antibody production on live *l(3)mbn-1* hemocytes by indirect immunofluorescence using FITC-conjugated anti mouse immunoglobulin (Sigma) as a second antibody, at 1:100 dilution. An anti human CD45 antibody, T2/48 (30), was used as a negative control. The analysis of the cells was carried out by using a FACSTAR-PLUS (Becton Dickinson, Mountain-View, CA., USA) equipment. The selected clones were re-cloned and supernatants produced. In some experiments biotinylated anti mouse antibodies were used and visualized with Cy3-streptavidin .The samples were analyzed by a Zeiss Axioscope fluorescent microscope.

Immunocytochemical analysis of hemocytes and the hematopoietic organs

All procedures were carried out at room temperature (20ºC). Hemocytes were adhered to a multiwell microscopic slide (SM-011, Hendley-Essex, England) for 30 minutes in Ringer solution containing 0.1%BSA and a few crystals of 1-Phenyl-2-Thiourea (PTU). The cells were fixed with acetone for 6 minutes, air dried and rehydrated with PBS solution for 10 minutes and blocked with PBS containing 0.1% BSA (PBS-BSA). The hybridoma supernatants were applied for 45 minutes. Cells of the hematopoietic tissues were visualized after dissecting the larvae in Ringer solution, fixing with 0.1% paraformaldehyde in Ringer solution containing 0.05% Triton-X for 15 minutes at room temperature and reacting with the

antibodies as described above. The samples were washed five times, three minutes each, with PBS. Biotinylated anti-mouse Ig (DAKO; Copenhagen, Denmark, 1:400 in PBS-BSA) was added and incubated for 30 minutes. This was followed by 5 washes, as above then peroxidase streptavidin (DAKO, Copenhagen, Denmark, 1:400 in PBS-BSA) was added and incubated for 45 minutes. The samples were washed twice with PBS and three times with acetate buffer, 3 minutes each. The reaction was visualized by 0.05% 3-amino-9-ethyl-carbazole (AEC, Sigma-Aldrich) dissolved in sodium acetate buffer (0.2M, pH4.6), containing 0.003% H_2O_2. The reaction was stopped by three washes with distilled water, 5 minutes each. The samples were mounted in 87% glycerol, containing 0.1% sodium azide and the staining was analyzed by Nomarski objective in a Zeiss Axioscope microscope**.**

Western blotting of proteins

Hemocytes were collected from larvae at selected stages by bleeding into *Drosophila* Ringer solution on ice and washed ones in Ringer solution. After centrifugation cells were extracted into lysis buffer (50mM Tris-HCl, pH 8.0, 150mM $MgCl_2$, 1.0% NP40, 0,5% sodium deoxycolate, 0.1% SDS) supplemented with (1mM phenyl-methyl-sulfonyl fluoride, 0.014 mg/ml pepstatin and 0.01 mg/ml leupeptin). Protein extracts were separated by SDS-PAGE. After electrophoresis the proteins were transferred onto the nitrocellulose membrane Hybond-C (Amersham) in transfer buffer (25mM Tris, 90 mM glycine, 20% methanol). Transfer was performed at a constant voltage of 18V overnight at 4^{o}C. Non specific binding sites on the nitrocellulose membrane were blocked with 7 mM Na_2HPO4, 3 mM NaH_2PO4, 150 mM NaCl, 0.1% Tween 20 (PBST) supplemented with 10% fat-free dried milk for 1 h at room temperature. The blot was incubated with the hybridoma supernatant for 3 h with agitation at room temperature. The blot was washed with PBST three times for 10 minutes each and then incubated with the secondary antibody, the HRP-conjugated anti-mouse antibody (DAKO). Following three washes of 10 minutes in PBST, the proteins were visualized by the ECL-Plus (enhanced chemiluminescence) system (Amersham).

Functional assays

Phagocytosis assay was carried out by incubating heat killed FITC-labelled *E. coli* and *Staphylococcus saprophiticus* bacteria as follows. Cells were seeded in 25 µls of Schneider medium on the spots of multiwell microscopic slides (SM-011, Hendley-Essex, England) and 2 µls of 10% FITC-labelled bacterium

suspension was added immediately. The slides were incubated for 20 minutes in a humid chamber at room temperature. After incubation 10 µls of 1% Trypan blue solution was added to quench the fluorescence of non-phagocytosed bacteria. The slides were covered with a coverslip and the fraction of the cells showing fluorescence were counted, as well as the number of the phagocytosed bacteria was determined by counting the bacteria within the cells. In each experiment 500 cells were counted and analyzed. In some experiments the phagocytic capacity of the cells was correlated with their immunological phenotype. Cecropin production was determined by using *Drosophila* strain *Y81* (25), transgenic for the inducible, *cecropin-A1* promoter driven *lac-z* gene. The larvae were induced with live *Enterobacter cloaceae* bacteria and the hemocytes were prepared for double staining with X-Gal and FITC labeled L1 antibody 4 hours later. The encapsulation reaction was carried out by infesting second instar *Oregon-R* larvae with *Leptopilina boulardi*, strain G486 (3). The larvae were dissected at different intervals following the infestation and the hemocytes or the tissues stained and the reactions were evaluated.

RESULTS

Clustering of the antibodies

The antibody production by hybridomas was tested on live *l(3)mbn-1* and *Oregon-R* hemocytes by flow cytofluorimetry and the positive supernatants were further analyzed.. The antibodies were clustered as clusters of differentiation (CDs) on the basis of their histochemical reactions on *l(3)mbn-1*, and on *Oregon-R* circulating hemocytes furthermore on the basis of the molecular mass of the proteins recognized by Western blotting. Four main clusters have been defined, as shown in Table 1. and Fig.1. Fig. 1a shows the immunohistochemical reactions of representative antibodies for each cluster, namely, H2 (14), P1 (30) now, NimrodC1 or NimC1, (15), L1 now, Atilla, (Ref.30**,** Laurinyecz et al, in preparation) and C4. Pan hemocyte antibody cluster -"H" reacts with all circulating hemocytes. Three antibodies react with the majority of round cells, marked as plasmatocyte-"P". A large set of antibodies form the lamellocyte-specific "L" cluster; the recognized markers are present on large flattened cells and on a few small round cells. The members of the forth cluster, the crystal cell cluster, marked with "C" are present on cells with crystal inclusions. Out of the three antibodies in the P cluster two react with all plasmatocytes and in Western blot analysis mark the same molecular weight protein. It was assumed therefore that the two P1 antibodies may see two different epitopes on the same molecule. This was confirmed later in gain of function and loss of function type experiments

(15). One antibody in the "H" cluster, H1, besides recognizing all circulating hemocytes react also with pericardial cells (Fig.1b). In wild type *Drosophila* strains, similarly to *l(3) mbn-1* mutant, the H-, the P-, the L- and the C-cluster antibodies react with all hemocytes, plasmatocytes, lamellocytes and crystal cells, respectively (not shown).

Reactions of representative antibodies in blood cell compartments

Larval hemocytes and their progenitors are distributed throughout the body in morphologically well definable compartments, as the circulating hemocytes, the sessile hemocytes and the lymph glands (Fig.2). We also identified two large clumps of 100-200 hemocytes under the cuticle of the abdominal segments A8 and A9 (Figs.2A and 2C). In this organ-like structure the hemocytes are clustered and form a well-definable clump of cells in each individual, therefore we operationally define this cluster of cells as a posterior hematopoietic tissue (PHT) (Figs. 2A and 2C). The H2 –Hemese -molecule is expressed in the circulating hemocytes, in the sessile cells, in the lymph gland and in the posterior hematopoietic tissue of the larva. In the third instar the hemocytes attached to the developing imaginal discs (Fig.2D) also express this antigen. Hemocytes of the adult fly *do not* express the Hemese molecule (not shown).

Expression of the P1 molecule, NimC1, is confined to cells with plasmatocyte morphology at all stages of the larval development and in the adult life. In first stage instars a substantial fraction of plasmatocytes lack NimC1 in the circulation however they carry Hemese, confirming that these cells are hemocytes. The NimC1 negative population of hemocytes in the circulation is diminishing in the second and in the third instar and before pupation almost all cells express NimC1.. In the lymph gland and in the PHT NimC1 is expressed in small islands (15). The sessile hemocytes are heterogeneous with respect to NimC1 expression (15).Hemocytes, attached to the developing imaginal disc in the third instar also express the NimC1 (Fig 3A). In the adult more than99% of the circulating cells show strong NimC1 expression (Fig.3b). The <1% NimC1 negative cells have crystal inclusions in their cytoplasm (Fig.5C).

The "L" antigens are expressed in cells with lamellocyte morphology (Fig.1A). The L1 molecule, a transmembrane protein (now Atilla, Laurinyecz et al, in preparation), is expressed in <0.1% hemocytes (1—2 L1 positive cells in 10 larvae) of wild type larvae just before pupariation, however vigorous differentiation of L1 positive cells is initiated by, infestation with parasites (3) or by wounding of the cuticle (18). A small fraction of small round cells also carry the Atilla antigen. (Fig1A). These cells lack NimC1 but carry Hemese

confirming that they are hemocytes and may belong to the lamellocyte lineage. Atilla positive cells are seldom seen in the lymph gland of normal animals (Ref.8 and Márkus et al, unpublished) however they appear as an outer cell layer on the lymph gland following infestation with parasitic wasps (Fig. 8E). The sessile hemocytes and hemocytes attached to the imaginal disks of the larva and hemocytes of the adult also lack Atilla (not shown). The L2 antigen is expressed on all cells with lamellocyte morphology however the epitope is intracellular; L4 and L6 are present on a subset of lamellocytes as extracellular epitopes (Kurucz, Laurinyecz et al. in preparation). L5 has been defined as the high molecular-weight isoform of filamin, an acting-binding protein, presumably involved in the rearrangement of cytoskeleton accompanying the morphological changes in the course of lamellocyte development (26).

The C4 molecule is expressed in a small subset of hemocytes in the circulation (Fig.1A) and in the first lobes of the lymph gland (Fig 4), but not in the second and third lobes, nor in the posterior hematopoietic tissue or in any other tissues. The C4 antigen is present either as crystal inclusion or is distributed homogeneously in the cytoplasm of round, 8-10 μm sized cells. It is expressed in all stages of larval development, in the pupa and also in the adult (Fig.5A and 5B). The staining pattern of the cells in the adult resembles to that of the larva where the cells have crystal inclusions in their cytoplasm (Fig. 5C).

Functions of hemocyte subsets

Hemocytes of *Oregon-R* and *l(3)mbn-1* larvae and of adults were tested for their capacity to phagocytose bacteria, to produce the antimicrobial peptide Cecropin-A1 and to mount an efficient cellular immune reaction to the eggs of the parasitic wasp *Leptopilina boulardi*.

Phagocytosis and Cecropin-A production

To test the relationship between phenotype and function we have immuno-challenged hemocytes with bacteria or with parasitic wasps. Hemocytes expressing NimC1 show vigorous phagocytosis, while those expressing Atilla do not take up bacteria (Fig.7A). Likewise, Atilla positive cells do not phagocytose (Fig.7B). The cecropin production of hemocytes was determined following an “in vivo” challenge of the larvae by live *Enterobacter cloaceae* bacteria (25). Atilla^{+} cells are not involved in antimicrobial peptide production, the Cecropin-A1 production is limited to the reciprocal cell population, the NimC1 positive cells, as shown in Fig.7C, 7D.

Reactions to parasites and "tumors"

Second instars of *Oregon-R* were infested with the parasitic wasp *Leptopilina boulardi* strain G486 and the changes in the immunological phenotype of the hemocytes in the circulation, in the hematopoietic organs and on the parasitic egg were typed with anti-NimC1 and anti-Atilla antibodies. Two days after infestation Atilla positive cells appeared in the circulation (Fig.8A) as well as on the lymph gland (Fig.8E). The lymph gland also had NimC1 positive cells (Fig.8B). On the egg first NimC1- (Fig.8C), later Atilla- (Fig.8F) positive cells appeared. The "tumor" often seen in *l(3)mbn-3* larvae – a granuloma-like structure – is built up from Atilla positive cells (Fig. 8.D) with the little contribution of NimC1 positive cells (not shown).

Expression of blood cell antigens during ontogenesis

The blood cell composition undergoes characteristic changes during the ontogenesis of the fly (Fig.6). In the larva plasmatocytes and crystal cells are present carrying H, P and C antigens respectively. Following immune induction by the parasitic wasp *Leptopilina boulardi* a new class of hemocytes emerge, the lamellocyte, which carry the Atilla- (Ref.14 and Fig.6), the L2-, the L4-, the L5- and the L6 antigens (Kurucz, Laurinyecz et al., in preparation). In the pupa all cell types (H-, P--, L-, C- antigen positive cells) are present as marked with the representative antibodies (Fig. 6). During the transition from the pupa to the adult fly a characteristic change in the pattern of blood cell antigens occur: Hemese disappears from the hemocytes with plasmatocyte morphology and there are no large lamellocytes in the adult. Likewise, none of the lamellocyte antigens are expressed by adult hemocytes. The adult hemocytes with plasmatocyte morphology carry NimC1 and NimC1 negative cells with crystal inclusions all express C4.

DISCUSSION

Here we report for the first time the use of cell surface antigens as molecular markers for characterization of subsets, compartments and lineages of *Drosophila* hemocytes. Previous studies have demonstrated the presence of several classes of hemocytes in *Drosophila*: the prohemocyte, the proplasmatocyte, the plasmatocyte, the podocyte, the lamellocyte, the procrystal cell, the crystal cell (23, 27, 16) and the secretory cell (16). Prohemocytes, proplasmatocytes, plasmatocytes, procrystal cells, crystal cells, secretory cells and lamellocytes are resident or develop in or under the influence of the lymph gland

(27). Plasmatocytes circulate in the hemolymph (23, 27,) and are attached to the integuments (16). Although these diverse classes of hemocytes have been characterized by light- and electron-microscopy and with respect to function, their origin and the relationship between the lineages are far from clear. One or another cell type/types may also represents morphological variants of the same cell type or lineage. Here we describe the main blood cell types, the hematopoietic compartments and function and classify hemocytes of *Drosophila melanogaster* in the course of ontogeny by the expression pattern of hemocyte specific antigens by using a panel of monoclonal antibodies developed in our laboratory.

Immunization of mice with whole hemocytes resulted several monoclonal antibodies. The antibodies and consequently the defined antigens fell into four main clusters on the basis of their reactivity's to cell -type specific molecules: the panhemocyte (H)- the plasmatocyte-(P), the lamellocyte- (L) and the crystal cell-(C) specific markers. The expression or the lack of expression of these molecules allows the definition of four main classes of hemocytes arranged in four main anatomical compartments. The main cell types in the larva are the plasmatocytes, the lamellocytes, the crystal cells and the stem cells. The plamatocytes are characterized by the expression of the H and the P antigens, the lamellocytes carry the H and the L1-L6 antigens with heterogeneous expression of L4-L6 antigens, the crystal cells are distinguished by the expression of the H and C2-C4 antigens. The stem cells –we propose – express variable amounts of H antigens but they lack P-, C- and L-markers. These latter cells – lacking the markers - are located in three main anatomical sites as the sessile tissue, the lymph glands and the posterior hematopoietic tissue (PHT). The P1 (NimC1) positive cells are phagocytic and are involved in the production of antimicrobial peptides but also contribute to the demarcation of parasites and tumors. The cells carrying the L antigens are involved in the demarcation of large foreign particles. The L1-L5 positive cells fell into two morphologically distinct subsets, the small round NimC1-negative plasmatocyte-like cells and the large flat cells, termed as lamellocytes. As the small L1 positive cells are non-dividing cells (Kurucz et al. and Laurinyecz et al. manuscripts in preparation), they do not take up bacteria and are not melanized, we propose that these L1-L5 positive small cells are the immediate precursors of the large, flat lamellocytes, which undergo morphological changes during the terminal differentiation of this cell type. The C-antigen expressing cells have crystal inclusions in their cytoplasm, are melanized upon "in vitro" manipulation and are involved in melanization processes therefore we conclude that they are te crystal cells.

The hemocytes of the adult differ in their phenotype from that of the larva in that they all express

either the P (NimC1) or the C antigens and all of them lack Hemese. Adult cells with lamellocyte morphology or those carrying L-antigens have never been observed either. This shows that the adult hemolymph contains plasmatocytes and crystal cells and no lamellocytes. The characteristic plasmatocyte phenotype – e.g. the lack of the Hemese antigen -suggests that this antigen has a specific function in the larval life. The markers described in this paper are expressed in a cell-type specific manner therefore they may be related to the development or the function of the corresponding cell type. Cloning of the genes for the markers suggests that this is indeed the case: molecular cloning and down regulation of the Hemese antigen by RNAi show that the molecule is involved in the regulation of lamellocyte development (14), NimC1 was found to be involved in regulation of phagocytosis (Kurucz et.al., submitted for publication). The L5 antigen is the high molecular weight isoform of filamin (26, 28) expressed in lamellocytes regulating lamellocyte development.

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Table 1. *Drosophila* blood cell antigens

CD	Cell type	MW[+]	Clone
H1	all hemocytes and pericardial cells	150	H11
H2	all circulating hemocytes	30-60	1.2
H3	all circulating hemocytes	16	4A12
P1a	plasmatocytes	95	N1
P1b	plasmatocytes	95	N47
P3	plasmatocyte subpopulation	*	8B1
L1a	lamellocytes	16	H10
L1b	lamellocytes	16	7A6
L1c	lamellocytes	16	29D4
L2	lamellocytes	20,50,110	31A4
L4	lamellocyte subpopulation	86	1F12
L5	lamellocyte subpopulation	90, 240	4B8
L6	lamellocyte subpopulation	*	H3
C2	crystal cells	*	21D3
C3	crystal cells	*	10D2
C4	crystal cells	*	9C8
C5	crystal cells	*	1.19

[+] molecular mass in kDa, under non-reducing conditions
* not done

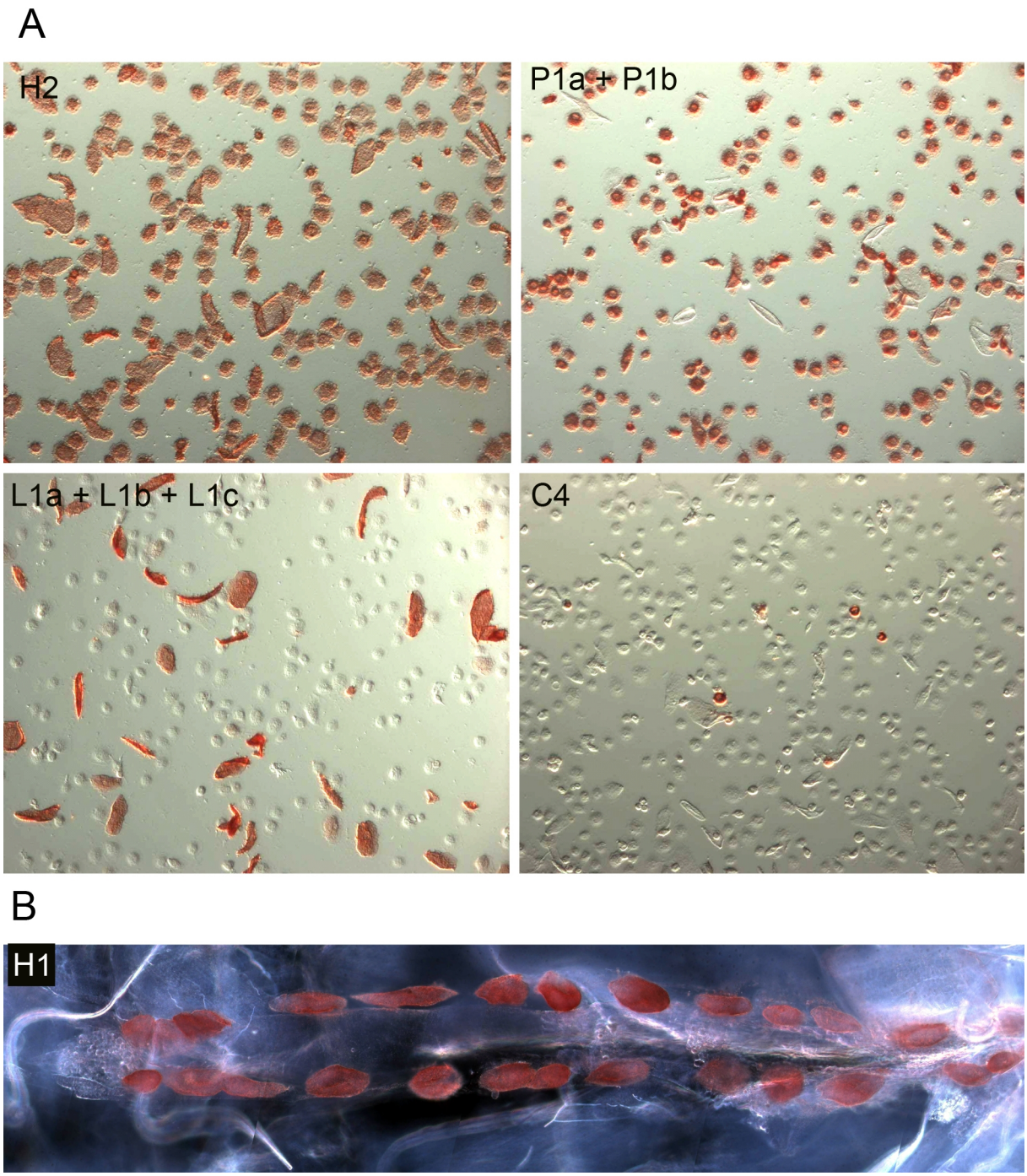

Figure1. Immunohistochemical reactions of representative H-, P-, L- and C – cluster antibodies

A.) Reactions of the antibodies on *l(3)mbn-1* larval hemocytes.

B.) Rection of the H1 antibody on *Oregon-R* pericardial cells.

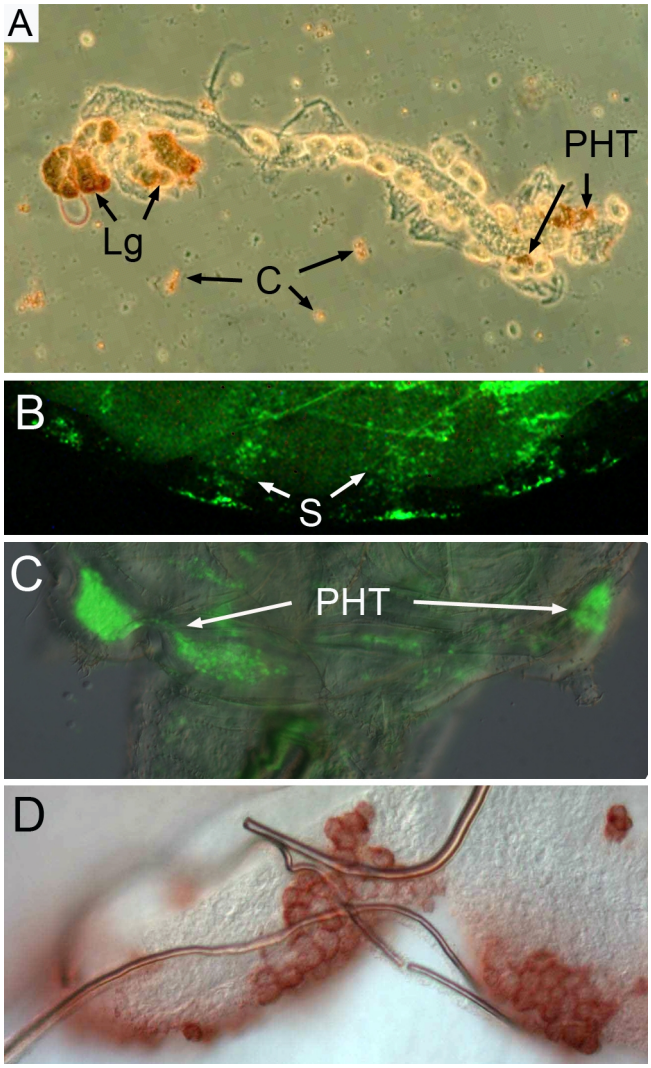

Figure 2. Hemocytes and the hematopoietic compartments expressing the H2 (Hemese) antigen

A.) Expression of the Hemese (H2) antigen in circulating hemocytes (C), in the lymph gland (Lg) and in the posterior hematopoietic tissue (PHT) as visualized by immunohistochemistry.

B.) The sessile hemocytes (S) in a larva „in vivo" as detected by GFP fluorescence in a *He-GFP* larva.

C.) Visualization of the posterior hematopoietic tissue (PHT) by expression of GFP driven by a Hemese-specific driver in the He-GFP stock.

D.) Expression of the Hemese antigen on hemocytes attached to an imaginal disk in *Oregon-R*.

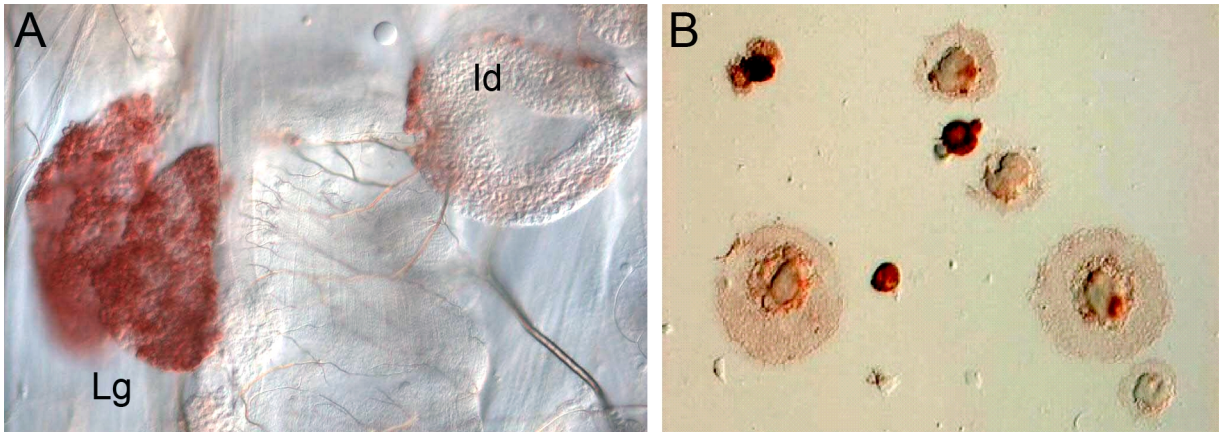

Figure3. Reaction of the P1a+P1b antibodies on hemocytes in *Oregon-R*.

32.) Hemocytes expressing the NimC1 antigen of the lymph gland (Lg) and those attached to an imaginal disk (Id) were visualized by immmunohistochemistry using a mixture of the P1a and the P1b antibodies.

33.) Circulating hemocytes of the adult fly express the NimC1 antigen as visualized by a mixture of the P1a and the P1b antibodies.

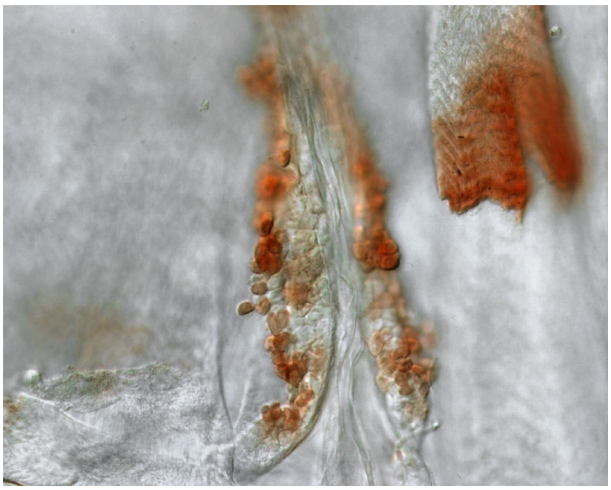

Figure 4. Expression of the C4 antigen in the lymph gland of *Oregon-R* larvae as visualized by immunohistochemistry.

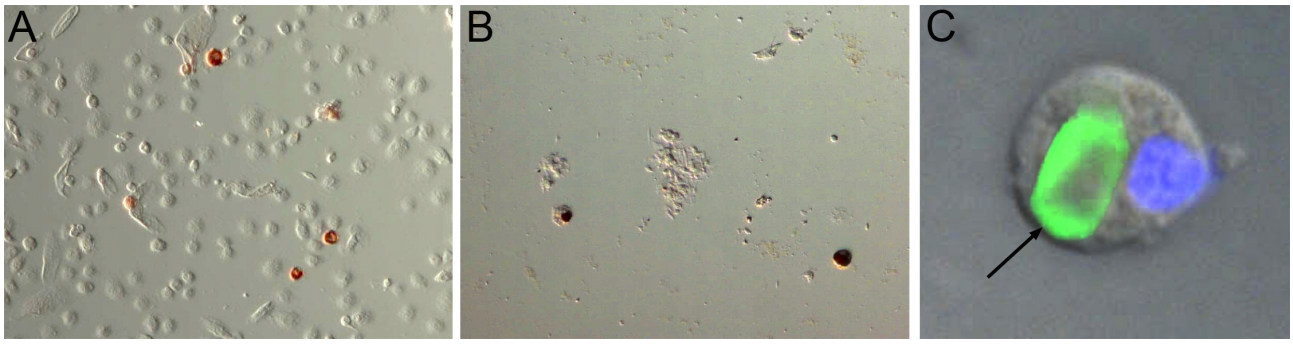

Figure 5. Expression of the C4 antigen in circulating hemocytes

A.) Expression of the C4 antigen in *l(3)mbn-1* larval hemocytes as visualized by immunohistochemistry.

B.) Expression of the C4 antigen in *Oregon-R* adult hemocytes as visualized by immunohistochemistry.

C.) Expression of the C4 antigen in the crystal (arrow) of a crystal cell in the adult by indirect immunofluorescence (green). Nucleus is visualized by DAPI (blue) in *Oregon-R*.

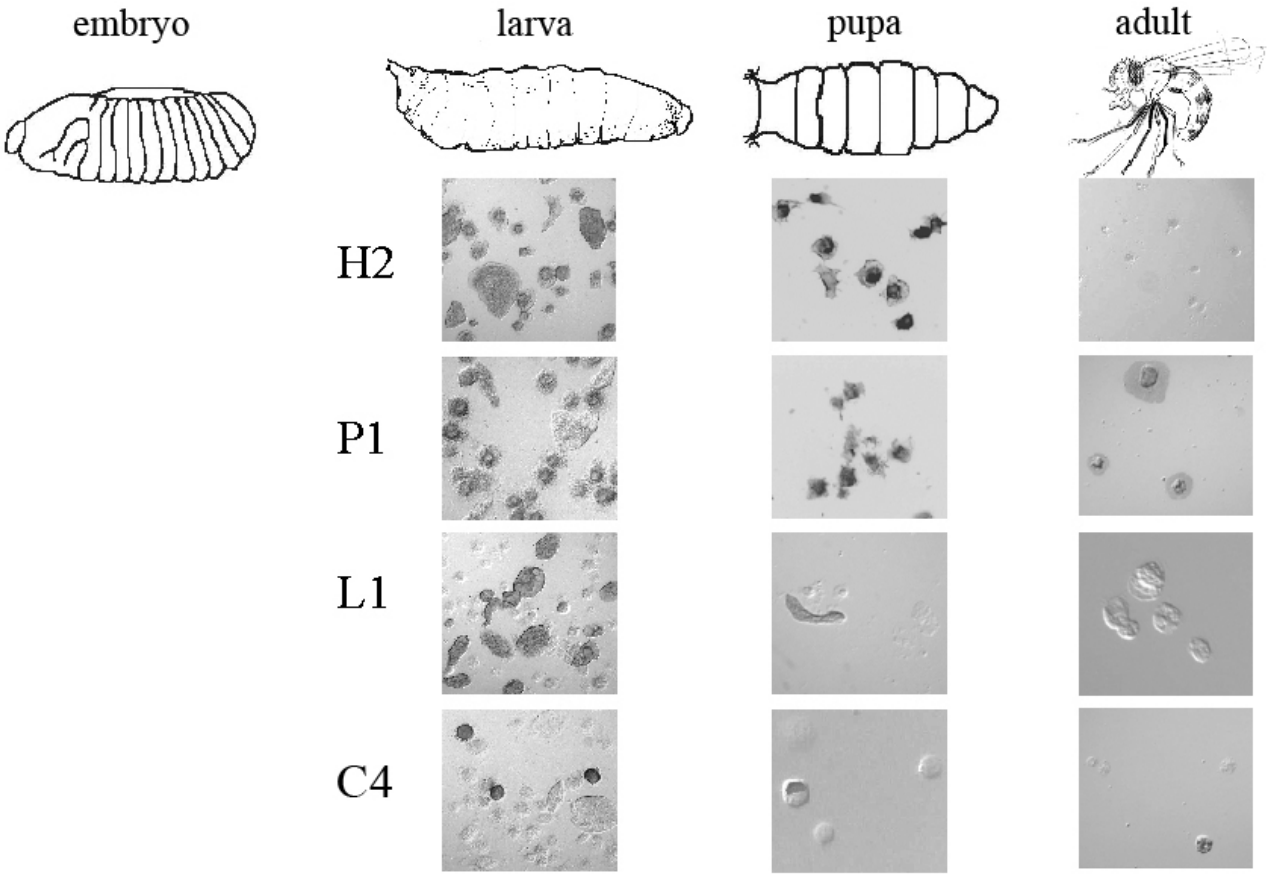

Figure 6. Expression of the Hemese (H2), the Nimrod-C1 (NimC1, P1) the Atilla (L1) and the C4 antigen in the course of ontogeny.

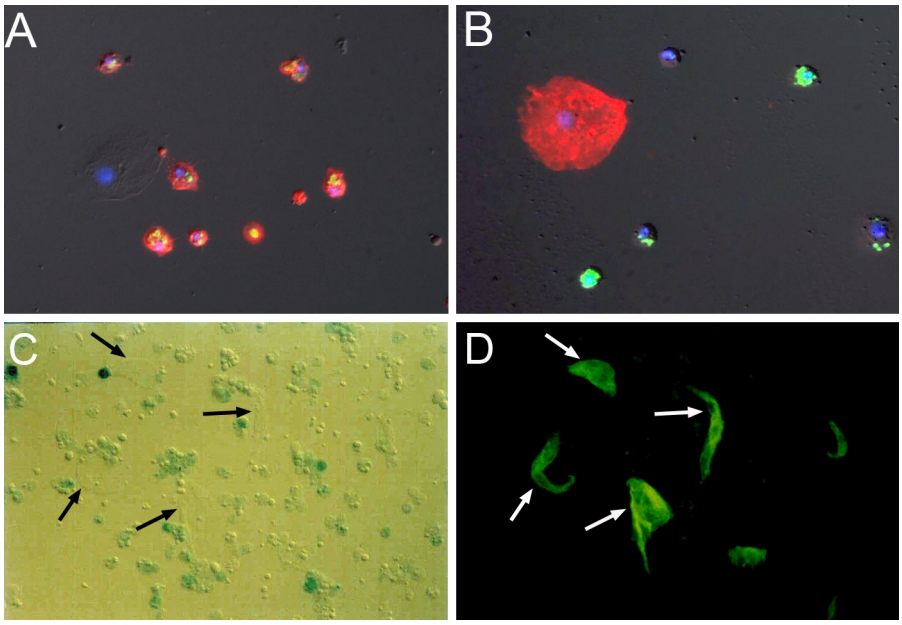

Figure 7. Phagocytosis and antimicrobial peptide (cecropin-A) production by P1- antigen positive cells.

A and B.) *Oregon-R* larvae were induced by the parasitic wasp *Leptopilina boulardi* and the larvae were injected with FITC-labelled *E. coli* bacteria (green). The hemocytes were stained with a mixture of P1a and P1b antibodies to visualize NimC1 positive cells (A) or alternatively, stained with a mixture of L1a+L1b+L1c antibodies to visualize Atilla positive cells (B).

C and D.) Larvae of *Y81* stock were injected with live *Enterobacter cloaceae* bacteria and the recovered hemocytes were stained for β-galactosidase (arrows show positive, cecropin-A producing cells) (C), and simultaneously, for the Atilla antigen by a mixture of L1a+L1b+L1c antibodies and FITC-anti-MIg(D). Arrows show lamellocytes on panel - D.

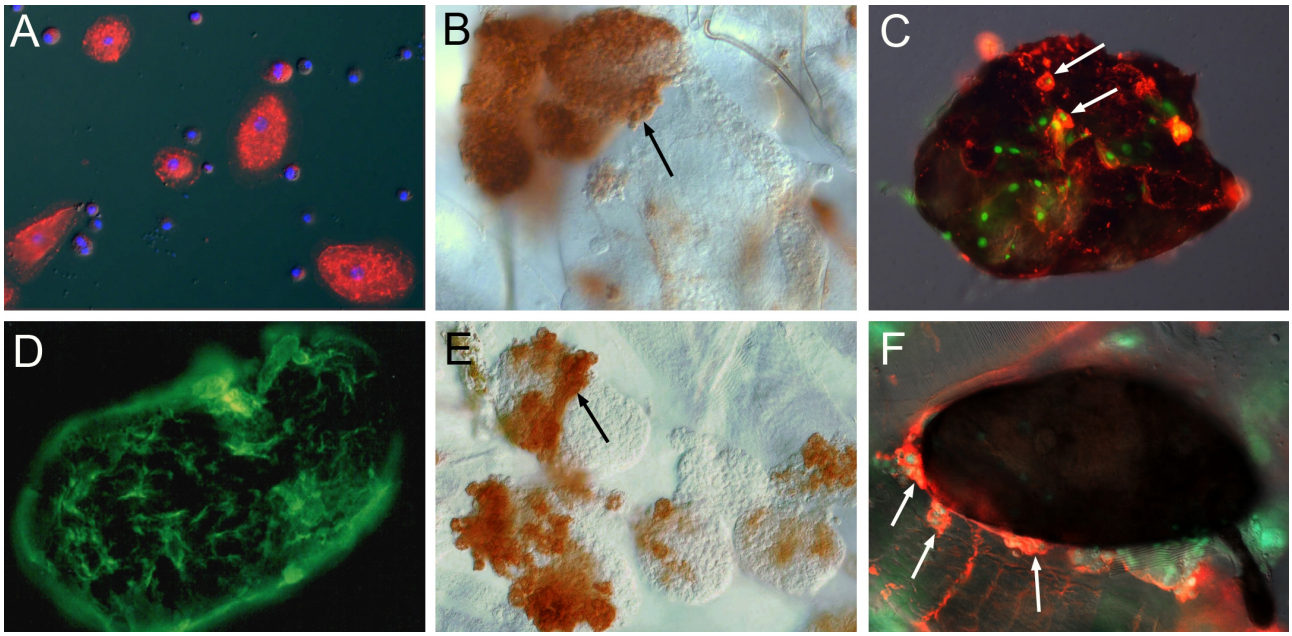

Figure 8. Visualization of hemocyte subsets in the course of the cellular immune reaction. Following induction with the parasitic wasp *Leptopilina boulardi* Atilla antigen positive cells appear in the circulation (A) as detected by a mixture of the L1a+L1b+L1c antibodies and biotinylated anti-mouse-Ig, developed by Cy3-Streptavidin (red). Nuclei were visualized by DAPI (blue). After infestation with the parasitic wasp NimC1 antigen positive cells appear (arrow) in the lymph gland (B) and on the egg of the parasite (arrows) (C) as visualized by immunohistochemistry (B) and fluorescence microscopy (C), respectively. Atilla positive cells appear (arrow) after infestation on the lymph gland (E) as well as on the parasitic egg (arrows) (F) as visualized by immunohistochemistry (E) and fluorescence microscopy (F), respectively. The granuloma-like body, „tumor" of the *l(3)mbn-1* strain is covered by Atilla-antigen positive cells as visualized by inirect immunofluorescence using a mixture of L1a+L1b+L1c antibodies in combination with FITC-anti-MIg. Figures A-B-E in wild type larva, C, F in *He-GFP*, D in *l(3)mbn-1* larva.