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Cell lineage tracing reveals the plasticity of the hemocyte lineages and of the hematopoietic compartments in *Drosophila melanogaster*

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Abstract

Much of our knowledge on hematopoiesis, hematopoietic compartments, hematopoietic cell lineages and immunity has been derived from studies on the vertebrate immune system. The sophisticated innate immunity of insects, the phylogenetic conservation and the power of *Drosophila* genetics allowed the investigation of immune cell (hemocyte) lineage relationships in *Drosophila melanogaster*. The development of the hemocyte lineages in *Drosophila* is a result of a precisely regulated succession of intracellular and intercellular events, though the nature and extent of these interactions are not known. We describe here a cell lineage tracing system set up to analyze the development of hemocyte lineages and functionally distinct hemocyte subsets. This system allowed us to distinguish two major embryonic hemocyte lineages, the *crq* and *Dot* lineages, in two, physically separated compartments, the embryonic macrophages and the embryonic lymph gland. We followed the fate and development of these lineages in the construction of the larval hematopoietic compartments and during the cell mediated immune response, the encapsulation reaction. Our

results revealed the considerable plasticity and concerted action of the hematopoietic compartments and the hemocyte lineages in the development of the innate immune system and in the course of the cell mediated immune response in *Drosophila*.

Keywords:

Drosophila, hemocyte, plasmatocyte, lamellocyte, lineage tracing, hematopoietic, compartment

Abbreviations:

<i>crq-GAL4</i>	<i>w; croquemort-GAL4</i>
<i>Dot-GAL4</i>	<i>w; Dorothy-GAL4</i>
<i>Pxn-GAL4</i>	<i>w; Peroxidasin-GAL4</i>
<i>crq>GFP</i>	<i>w; crq-GAL4; UAS-2xEGFP</i>
<i>Dot>GFP</i>	<i>w; Dot-GAL4; UAS-2xEGFP</i>
<i>crq>Act>GFP</i>	<i>y w UAS-FLP; crq-GAL4/Act5C-FRT-y⁺-FRT-GAL4 UAS-GFP</i>
<i>Dot>Act>GFP</i>	<i>y w UAS-FLP; Dot-GAL4/Act5C-FRT-y⁺-FRT-GAL4 UAS-GFP</i>
<i>Pxn>GFP</i>	<i>w; Pxn-GAL4; UAS-2xEGFP</i>
<i>eater>GFP</i>	<i>w; eater-GAL4; UAS-2xEGFP</i>
<i>Pxn>Act>GFP</i>	<i>y w UAS-FLP; Pxn-GAL4/Act5C-FRT-y⁺-FRT-GAL4 UAS-GFP</i>
<i>eater>Act>GFP</i>	<i>y w UAS-FLP; eater-GAL4/Act5C-FRT-y⁺-FRT-GAL4 UAS-GFP</i>

1. Introduction

Innate immunity is the most ancient and common system for defense against microbes and parasites. The elements of the innate immune system have been conserved throughout evolution in the animal kingdom. In the course of the immune response vertebrate and invertebrate immune systems utilize a set of similar receptors, signaling pathways, transcription factors, humoral factors and cell mediated mechanisms. The humoral factors include antimicrobial peptides and elements of the melanization and coagulation cascades. The cell mediated mechanisms involve the phagocytosis of microbes and apoptotic tissues, and the encapsulation of larger particles, such as eggs of parasites. The evolutionary conservation of these elements and the power of *Drosophila* genetics allowed an analytical

approach to innate immunity, and in recent years *Drosophila melanogaster* has become a suitable model organism via which to investigate the principles of innate immunity (Hultmark, 1994).

The cell mediated immune response of *Drosophila melanogaster* involves circulating cells in the hemolymph, known as hemocytes. Three main morphological classes of hemocytes have been identified: plasmatocytes, crystal cells and lamellocytes (Rizki and Rizki, 1980; Brehélin, 1982). The plasmatocytes, round cells with a diameter of 8-10, μm make up >95% of the circulating hemocytes. They participate in phagocytosis and encapsulation, and in the production of antimicrobial peptides (Brehélin, 1982). Crystal cells are similar to plasmatocytes in size and morphology, but are distinguished by crystal-like inclusions in the cytoplasm these inclusions containing elements of the phenoloxidase cascade, involved in melanization (Rizki and Rizki, 1959; Nappi et al., 1995). The third cell type, the lamellocytes, are large (25-40 μm) flat cells, which differentiate upon immune induction and are rarely seen in healthy wild type larvae (Brehélin, 1982; Lanot et al., 2001).

Hemocytes differentiate in spatially separated compartments (Lebestky et al., 2000). In the embryo, this differentiation proceeds in two waves (Holz et al., 2003). The procephalic mesoderm anlage gives rise to embryonic macrophages - freely moving cells in the embryo- and crystal cells which remain in the anterior midgut region (Lebestky et al., 2000). The second wave of hematopoiesis takes place just before the end of embryogenesis, when prohemocytes originating from the cardiogenic mesoderm anlage form a compact hematopoietic organ, the lymph gland (Holz et al., 2003). In the larva, the hemocytes reside in three main cellular compartments: the lymph gland, the sessile hemocyte population and the circulating blood cells. The lymph gland is a hematopoietic organ consisting of paired lobes along the anterior part of the dorsal vessel (Shestra and Gateff, 1982). The primary lobes are separated into three functional areas: the medullary zone comprises prohemocytes, the cortical zone built up by plasmatocytes and crystal cells, and the posterior signaling center (PSC), formed by a few cells on the posterior tip of the primary lobes. The PSC halts the differentiation of the prohemocytes in the medullary zone (Mandal et al., 2007; Krzemień et al., 2007). The sessile population of hemocytes is located underneath the larval cuticle (Goto et al., 2003; Lanot et al., 2001; Zettervall et al., 2004), serving as a precursor pool for effector cells, plasmatocytes and lamellocytes in the course of the cell mediated immune response (Zettervall et al., 2004; Márkus et al., 2009).

Although the hematopoietic compartments and hemocyte subsets have been defined by the expression patterns of immunological markers and *in vivo* reporter constructs (Kurucz et

al., 2003, 2007a, 2007b; Honti et al., 2009; Márkus et al., 2009; Tokusumi et al., 2009), the findings of the experiments carried out so far reflect a transient state of the immune system, and our understanding of the lineage-relationships among hemocyte subsets and the composition of the hematopoietic compartments is therefore fragmentary.

We set out to track the development and dynamics of hemocyte subsets and lineages from the early embryonic stage with the aid of the genetic lineage tracing system described by Ito et al. (1997). The key components of this tool are a tissue-specific GAL4 driver, a FLIP enzyme under the control of UAS, and an *Act5C-FRT-y⁺-FRT-GAL4* element. Expression of the FLIP enzyme in a particular tissue induces site-specific recombination between the FRT sites, thereby allowing the *Act5C* promoter to activate the GAL4 source ubiquitously. Through the introduction of a *UAS-GFP* element in this system, the cells which once expressed the tissue-specific driver, and also their descendants, are irreversibly marked, which allows tracing of a whole cell lineage throughout development.

Using a combination of genetic lineage tracing, hemocyte marker molecules and functional assays, we tracked the morphological and functional changes in the hemocytes during the immune response and elucidated the lineage origin of the functionally distinct hemocyte subsets. We additionally determined how the embryonic blood cell lineages build up the larval hemocyte compartments and traced the plasmatocytes, crystal cells and lamellocytes back to their embryonic origin.

2. Materials and methods

2.1. Fly stocks and genetic crosses

Wild-type *Oregon-R*, *crq-GAL4*, *Dot-GAL4*, *Act5C-FRT-y⁺-FRT-GAL4 UAS-GFP*, *y w UAS-FLP* (Exelixis), *y w; UAS-2xEGFP* (Bloomington Drosophila Stock Center), *Pxn-GAL4* and *eater-GAL4* (gifts from Dr Jesper Kronhamn and Professor Dan Hultmark, UCMP, Umeå University, Umeå, Sweden) *Drosophila* stocks were used. For lineage tracing experiments, we established a homozygous *y w UAS-FLP; Act5C-FRT-y⁺-FRT-GAL4 UAS-GFP* line. Virgins collected from this line were crossed to males bearing the respective hemocyte-specific driver element (*crq-GAL4*, *Dot-GAL4*, *Pxn-GAL4* and *eater-GAL4*). In the GAL4-expressing cells, the FLP was activated, which resulted in excision of the *y⁺* cassette in the *Act5C-FRT-y⁺-FRT-GAL4* construct, which subsequently acted as a constantly active GAL4 source.

The flies were kept on a standard cornmeal–yeast diet at 25°C.

2.2. Antibodies

Marker molecules expressed by hemocyte subpopulations were defined by mouse monoclonal antibodies as L1a,b,c, L2, L4 or L6 (Kurucz et al., 2007b).. The plasmatocytes were detected with a mixture of P1a and P1b antibodies (Kurucz et al., 2007a). Crystal cells were detected with C1 antibody (HC12F6) a kind gift from Professor Tina Trenczek, Giessen University, Germany. Isotype-matched mouse monoclonal antibody was used as negative control. The anti-GFP antibody (Mol. Probes, 1:2000 dilution) was applied in combination with Alexa Fluor 488-conjugated anti-rabbit immunoglobulin (Mol. Probes, 1:1000 dilution), and the L1, L2, L4 and L6 antigens were visualized with Alexa Fluor 568 or Alexa Fluor 633-conjugated anti-mouse immunoglobulin (Mol. Probes, 1:1000 dilution).

2.3. Immunostaining, hemocyte imaging and counting

Larvae were dissected in 20 µl of Shields and Sang's medium (Sigma) on a multispot microscope slide (SM-011, Hendley, Loughton, U.K.); the released hemocytes were allowed to settle and adhere at room temperature for 45 min. The samples were fixed with acetone for 6 min, rehydrated and blocked for 15 min in PBS containing 0.1% BSA (Sigma), incubated with the respective monoclonal antibodies for 1 h at room temperature, washed three times with PBS and reacted with anti-mouse Alexa Fluor 568 or Alexa Fluor 633-conjugated immunoglobulin at 1:1000 dilution for 1 h. The bound antibody was detected under UV light. The GFP expression was detected either directly in paraformaldehyde-fixed (2% paraformaldehyde in PBS, 12 min) samples, or the GFP signal was enhanced with rabbit anti-GFP antibody (1:2000 dilution) in combination with Alexa Fluor 488-conjugated anti-rabbit immunoglobulin (1:1000 dilution). Nuclei were stained with DAPI (DAKO).

The microscopic analysis was performed with a Zeiss Axioskope 2MOT epifluorescent and an Olympus FV1000 confocal microscope. Differential counting of hemocytes was based on L1, L2, L4, L6 and NimC1 antigen staining. At least 200 hemocytes were counted in each sample by means of nuclear staining with DAPI.

2.4. Time-lapse confocal imaging of *Drosophila* embryos

Embryos were dechorionated in 50% bleach, mounted in 10S Voltalef oil (VWR) onto a glass-bottom culture dish and imaged with an Olympus cell[^]R microscope. Projections and

time-lapse series assembly were performed with ImageJ. The method was described in detail by Jankovics and Brunner (2006).

2.5. Immune induction

The cell-mediated immune response was induced by infestation with the parasitoid wasp *Leptopilina boulardi* strain G486 (Russo et al., 1996.). In cell lineage tracing experiments 72 h old larvae were exposed to female wasps overnight at 18 °C. Hemocytes were isolated 72 h after this exposure. In the time-lapse experiments, *Oregon-R* flies were allowed to lay eggs for 2 h in vials and 72-h-old larvae were infested with the wasps for 2 h. Hemocytes were isolated from infested and non-infested larvae after 1, 3, 5, 8, 12, 16, 24, 48 and 72 h.

2.6. In vivo phagocytosis assay

Escherichia coli bacteria were conjugated with TRITC (Sigma) as described previously for FITC-conjugation (Hedengren et al., 1999). Immune induced larvae were injected with TRITC-labeled *E. coli* bacteria 1 h before dissection (Kurucz et al., 2007a). The hemocytes were isolated 16 and 24 h after the mean time point of the infestation and were fixed and reacted with antibodies as described in section 2.3. The phagocytic capacity of the hemocytes was correlated with their immunological phenotype.

3. Results

3.1. Definition of embryonic hemocyte drivers for lineage tracing

To study the *Drosophila* hemocyte lineages, we first used time-lapse confocal microscopy to investigate the expression pattern of the embryonic hemocyte compartment specific *croquemort-GAL4* (*crq-GAL4*) driver [active in embryonic macrophages (Olofsson et al., 2005 and Fig. 1A)] and the *Dorothy-GAL4* (*Dot-GAL4*) driver [expressed in the embryonic lymph gland (Kimbrell et al., 2002 and Fig. 1B)] with the *UAS-GFP* reporter system. We succeeded in detecting the *crq-GAL4*-driven GFP expression at the germ band retraction stage (Supplementary Video S1), whereas the *Dot-GAL4*-driven GFP expression occurred later, after dorsal closure (Supplementary Video S2). At that time, *crq*>GFP was not expressed in the embryonic lymph gland, and we likewise detected no *Dot*>GFP signal in the embryonic macrophages. The lack of overlap in *crq*>GFP and *Dot*>GFP expression meant

that the *crq-GAL4* and *Dot-GAL4* drivers mark distinct embryonic lineages, situated in anatomically separated compartments.

3.2. Identification of the components of the *crq* and *Dot* lineages in the larva

To shed light on the descendants of the substantiated two embryonic lineages in the larval hematopoietic tissues, we investigated the GFP expression patterns of *crq*>GFP and *Dot*>GFP in *Drosophila* larvae. We found *crq*>GFP expression in <1% of the circulating and sessile hemocytes, but use of the *crq-GAL4* driver in lineage tracing (*crq*>*Act*>GFP) revealed the labeling of at least 7% of the circulating and 15% of the sessile hemocytes (Fig. 2A). We found that >98% of the hemocytes of the *crq* lineage took up *E. coli* both in the circulation and in the sessile tissue, indicating that these hemocytes preserved their embryonic phagocytic function (Fig. 2B). In the larval circulation, we also found GFP-expressing crystal cells, defined by the crystal cell-specific marker C1 (Tina Trenczek personal communication, Kurucz et al., 2007b) (Fig. 2B), indicating that at least a fraction of the crystal cells comprise part of the *crq*-lineage. At the same time, we did not detect *crq*>*Act*>GFP-expressing cells in the lymph gland.

The *Dot-GAL4* driver is known to be active in the larval lymph gland and in the pericardial cells (Kimbrell et al., 2002). By using this *Dot-GAL4* driver in our lineage tracing studies (*Dot*>*Act*>GFP), we found GFP-expressing hemocytes in the cortical zone of the primary lymph gland lobes and in the secondary lobes (Fig. 2A). We also observed GFP expression in the pericardial cells situated along the dorsal vessel (data not shown), but we detected no hemocytes of the *Dot* lineage either in the circulation or in the sessile tissue (Fig. 2A), as an indication that hemocytes of the *Dot* lineage do not participate in the production of circulating hemocytes or the sessile hematopoietic tissue.

3.3. Marker and functional analysis of the *crq* and *Dot* lineages following immune induction

To reveal the contributions of the *crq* and *Dot* lineages to the cell mediated immune response, we analyzed the expression of the L1 and L6 lamellocyte-specific molecules (Kurucz et al., 2007b), and the phagocytic capacity of the hemocytes. At 72 h after parasitic immune induction of the *Dot*>*Act*>GFP larvae we detected 9% GFP-expressing hemocytes in the circulation (Fig. 3B), indicating that hemocytes of the *Dot* lineage entered the circulation from the lymph gland. These GFP-expressing hemocytes comprise three subpopulations: half of them are small, non-phagocytic cells residing in small clusters (Fig. 3B) that failed to

express the plasmatocyte-specific NimC1 or the lamellocyte specific L1, L2, L4, L6 markers; 23% percent of them which took up *E. coli* were defined as plasmatocytes by the expression of the NimC1 molecule; the third population (27%) of GFP-positive hemocytes exhibited no phagocytic activity, displayed lamellocyte morphology and expressed the L1 and L6 molecules (Fig. 3B).

Our investigations of the *crq>Act>GFP* larvae 72 h after immune induction revealed 13% GFP-expressing hemocytes in the circulation; these consisted of two subpopulations: 86% of them were round hemocytes with phagocytic activity, and the remaining 14% were non-phagocytic with lamellocyte morphology and expressed the L1 and L6 markers.

These results demonstrate that the *Dot* and *crq* lineages both contribute to the production of plasmatocytes and lamellocytes.

3.4. Cell shape and marker analysis of the hemocytes following immune induction

Our marker and functional analyses indicated that the hemocytes of the *crq* lineage may also differentiate to lamellocytes, implying that hemocytes with phagocytic capacity are able to transform into non-phagocytic lamellocytes. To study this conversion and to assess the circulation as the third hematopoietic compartment contributing to lamellocyte production, we investigated the cell shape and marker molecule expression changes in the circulating hemocytes upon immune induction. We monitored the expression of the lamellocyte-specific L1, L2, L4 and L6 and the plasmatocyte-specific NimC1 marker molecules by means of immunostaining at different time points after immune induction (Fig. 4 and Supplementary Fig. S3).

At 3 h after immune induction, all circulating hemocytes were small, round cells, 95% of them expressing the NimC1 marker (Supplementary Fig. S3A5) and none of them expressing any of the lamellocyte markers (Figs. S3A1-A4 and Fig. 4B).

At 5 h after infestation, a subpopulation of round cells had started to express the L1 and L4 markers (Figs. S3B1 and B3).

At 8 h after infestation, we observed a marked (80%) increase in the proportion of hemocytes expressing the L4 marker molecule, but at this time point only 25% of the hemocytes were L1-positive. Some of the L1 and L4-expressing hemocytes were slightly flattened, but their size was unchanged (Supplementary Figs. S3C1 and C3).

At 16 h, a new hemocyte subset, the discoidal lamellocytes (Supplementary Figs. S3E1 and E3), appeared in the circulation, most of them expressing the L4 antigen, while a fraction of them were positive for L1. The small discoidal lamellocytes were morphologically

indistinguishable from spread plasmatocytes. L2 and L6 antigen-expressing hemocytes (10% and 2%, respectively) appeared at this time (Supplementary Figs. S3E2 and E4 and Fig. 4B). The L2 antigen was expressed (between 12 and 16 h after immune induction) on small, elongated lamellocytes probably committed to terminal differentiation. Between 16 and 24 h after immune induction, the proportion of elongated lamellocytes increased dramatically (Fig. 4B). At 24 h they all expressed the L1, L4 and L2 antigens, while L6 antigen expression was restricted to only a subset of these cells (Supplementary Figs. S3F1-F4).

Between 24 and 48 h the proportions of L1 and L4-expressing discoidal lamellocytes decreased significantly (Fig. 4B). At this stage, all the elongated lamellocytes expressed the L2 antigen, while L6 expression remained confined to a subset of these cells (Supplementary Figs. S3G1-G4).

At 72 h discoidal lamellocytes were absent from the circulation. At this time, the L1, L2, L4 and L6 markers were expressed by elongated lamellocytes (Supplementary Figs. S3H1-H4), while the cells with typical plasmatocyte morphology were all negative for these molecules.

At 8 h after infestation, we observed a notable overlap between the L4 and NimC1-expressing hemocyte populations, further underlining our notion that lamellocytes may originate from a pool of plasmatocytes, the typical phagocytic cells of the *Drosophila* larva (Fig. 4B).

To analyze whether the discoidal lamellocytes were able to phagocytose bacteria, we injected parasitoid wasp-induced larvae with TRITC-labeled *E. coli in vivo* and tested the circulating hemocytes for the expression of the L1, L2, L4 and L6 markers and their phagocytic capacity. At 16 h after wasp infestation, both the small round hemocytes expressing the L1 and L4 markers and the small discoidal L1, L2 and L4-expressing hemocytes (possibly committed to differentiate into lamellocytes) were able to phagocytose TRITC-labeled *E. coli* (Fig. 4C). On the other hand, the terminally differentiated large elongated lamellocytes defined by the L1, L2, L4 and L6 markers did not take up any bacteria at any investigated time point (Fig. 4C).

3.5. Tracing of the plasmatocyte lineage

To shed light on the validity of our hypothesis that a fraction of the lamellocytes are derived from plasmatocytes, we traced the hemocyte lineages in immune-induced larvae by using two drivers with plasmatocyte-specific expression: *Peroxidasin-GAL4* (*Pxn-GAL4*) (Stramer et al., 2005) and *eater-GAL4* (Tokusumi et al., 2009). More than 98% of the

plasmatocytes expressed these drivers (not shown). A large proportion of L1-expressing lamellocytes gave a GFP signal when lineage-traced with the plasmatocyte-specific *Pxn-GAL4* (Fig. 5A) or *eater-GAL4* (Fig. 5B) drivers: 46% and 36%, respectively. Similar results were obtained with the L6 lamellocyte-specific antigen (Fig. 5A,B). In the absence of the *UAS-FLP* construct (non-lineage traced), a small fraction of L1-expressing lamellocytes were detected in *Pxn>GFP* (13%, Fig. 5C) and *eater>GFP* (6%, Fig. 5D) larvae, which implies that these drivers had been expressed at some stage of differentiation and were later switched off. These findings indicate that the plasmatocytes of the *Pxn* and the *eater* lineages may change their morphology and function after immune induction, and a fraction of these cells may serve as a precursor pool for the lamellocyte population.

DISCUSSION

This study, involved an analysis of the origin and fate of hemocyte lineages and cell types in *Drosophila melanogaster*. For this, we applied a cell lineage tracing tool, whereby it was possible to mark hemocyte lineages and subpopulations permanently. Embryonic hemocytes were marked with the *crq* and the *Dot* drivers and their descendants were followed throughout larval development and the immune response. The *crq>GFP* and *Dot>GFP*-marked hemocytes separated from each other in two, physically isolated compartments, in agreement with the findings of the cell transplantation experiments of Holz et al. (2003).

Our lineage tracing experiments revealed that the descendants of the marked *crq* and *Dot* hemocytes remain separated in the larva; the *crq* lineage-marked cells are present in the sessile hematopoietic tissue and in the circulation. The presence of *crq* lineage hemocytes in both the sessile tissue and the circulation suggests a flow of hemocytes between these two compartments. Hemocytes originating from the *Dot* lineage are present exclusively in the cortical zone of the lymph gland (Fig. 6A). These results reveal that hemocytes of the *crq* lineage never enter the lymph gland; instead, they settle down to form the sessile hematopoietic tissue. A small fraction of hemocytes, the crystal cells, are also present in the circulation of the larvae. In the embryo, these cells are located in the primordial lymph gland, and in a small, immobilized cluster in the anterior midgut region (Lebestky et al., 2000). In the larval circulation, we found GFP-expressing, i.e. *crq* lineage-marked crystal cells, demonstrating for the first time that crystal cells may also differentiate from a cell pool of embryonic macrophage origin (Fig. 6A).

Upon induction of the cell mediated immune response, the lamellocytes differentiate. It is known that after immune induction, the lymph gland is decomposed (Crozatier et al., 2004), giving rise to lamellocytes. Our present results confirm this and extend it with the finding that a subset of plasmatocytes also originates from the *Dot* lineage, showing that plasmatocytes may originate from the lymph gland too. Additionally, a novel cell type is released from the lymph gland. These cells were first detected as small, round cells 3-4 μm in diameter, expressing *hdc>lacZ* (Márkus et al., 2009). These cells carry neither plasmatocyte nor lamellocyte markers, and do not take up bacteria, though they express the pan-hemocyte antigen, Hemese (Márkus et al., 2009). Our knowledge regarding these cells is fragmentary, and further studies are required to explore their possible function in metamorphosis and in adult life. It was earlier shown that lamellocytes originate from the sessile hematopoietic compartment (Márkus et al., 2009), and also from the lymph gland (Sorrentino et al., 2002). The lineage tracing experiments revealed that both the *crq* and the *Dot* lineages are involved in making up the lamellocyte population, possibly serving as a precursor pool in the sessile tissue and in the lymph gland (Fig. 6B). The origin of the lamellocytes therefore could be traced back to the embryonic macrophages and to the embryonic lymph gland, demonstrating the dual embryonic origin of this cell type. The *Dot*-marked cells fell into three morphological categories: lamellocytes, plasmatocytes, and a so far undefined cell type. As *crq* lineage-marked cells appear in all hemocyte populations, the data underscore the importance of the embryonic macrophage lineage in the production of all effector cell types: the plasmatocytes, the lamellocytes and the crystal cells. The results also confirm that the sessile tissue serves as a pool of lamellocyte precursors (Márkus et al., 2009).

The marker and functional analyses revealed that the *crq*, originally phagocytic (embryonic macrophage) lineage gives rise to non-phagocytic L6^+ lamellocytes, showing that phagocytic cells (embryonic macrophages or larval plasmatocytes) may transform to non-phagocytic lamellocytes (Figs. 6B and 7). The lamellocyte markers are expressed sequentially in time following immune stimulation, with the L1 and L4 molecules appearing early, and L2 and L6 late after immune stimulation, the latter two marking lamellocytes committed to terminal differentiation. The expression of lamellocyte markers partially overlaps with phagocytosis and with the expression of the plasmatocyte-specific marker NimC1, again indicating that phagocytic cells, e.g. plasmatocytes, may transform into lamellocytes. This transformation is confirmed by plasmatocyte-specific cell lineage tracing with *Pxn-GAL4* and *eater-GAL4*.

Overall, our findings reveal the multilineage origin of the major immune-cell types of *Drosophila melanogaster*. Moreover they highlight the fact that the formation of the immune system and the onset of the cell mediated immune response require considerable plasticity and the concerted action of the *Drosophila* hematopoietic compartments.

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REFERENCES

- Brehélin, M., 1982. Comparative study of structure and function of blood cells from two *Drosophila* species. Cell. Tissue Res. 221, 607-615.
- Crozatier, M., Ubeda, J.M., Vincent, A., Meister, M., 2004. Cellular immune response to parasitization in *Drosophila* requires the EBF orthologue collier. PLoS Biol. 2, E196.
- Goto, A., Kadowaki, T., Kitagawa, Y., 2003. *Drosophila hemolectin* gene is expressed in embryonic and larval hemocytes and its knock down causes bleeding defects. Dev. Biol. 264, 582-591.
- Hedengren, M., Asling, B., Dushay, M.S., Andó, I., Ekengren, S., Wihlborg, M., Hultmark, D., 1999. Relish, a central factor in the control of humoral but not cellular immunity in *Drosophila*. Mol. Cell 4, 827-837.
- Holz, A., Bossinger, B., Strasser, T., Janning, W., Klapper, R., 2003. The two origins of hemocytes in *Drosophila*. Development 130, 4955-4962.
- Honti, V., Kurucz, É., Csordás, G., Laurinyecz, B., Márkus, R., Andó, I., 2009. *In vivo* detection of lamellocytes in *Drosophila melanogaster*. Immunol. Lett. 126, 83-84.

Hultmark, D., 1994. Insect immunology. Ancient relationships. *Nature* 367, 116-117.

Ito, K., Awano, W., Suzuki, K., Hiromi, Y., Yamamoto, D., 1997. The *Drosophila* mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. *Development* 124, 761-771.

Jankovics, F., Brunner, D., 2006. Transiently reorganized microtubules are essential for zippering during dorsal closure in *Drosophila melanogaster*. *Dev. Cell* 11, 375-385.

Kimbrell, D.A., Hice, C., Bolduc, C., Kleinhesselink, K., Beckingham, K., 2002. The *Dorothy* enhancer has Tinman binding sites and drives hopscotch-induced tumor formation. *Genesis* 34, 23-28.

Krzemień, J., Dubois, L., Makki, R., Meister, M., Vincent, A., Crozatier, M., 2007. Control of blood cell homeostasis in *Drosophila* larvae by the posterior signalling centre. *Nature* 446, 325-328.

Kurucz, É., Márkus, R., Zsámboki, J., Folkl-Medzihradzsky, K., Darula, Z., Vilmos, P., Udvardy, A., Krausz, I., Lukacsovich, T., Gateff, E., Zettervall, C.J., Hultmark, D., Andó, I., 2007a. Nimrod, a putative phagocytosis receptor with EGF repeats in *Drosophila* plasmatocytes. *Curr. Biol.* 17, 649-654.

Kurucz, É., Váczi, B., Márkus, R., Laurinyecz, B., Vilmos, P., Zsámboki, J., Csorba, K., Gateff, E., Hultmark, D., Andó, I., 2007b. Definition of *Drosophila* hemocyte subsets by cell-type specific antigens. *Acta Biol. Hung.* 58 Suppl, 95-111.

Kurucz, É., Zettervall, C.J., Sinka, R., Vilmos, P., Pivarcsi, A., Ekengren, S., Hegedűs, Z., Andó, I., Hultmark, D. 2003. Hemese, a hemocyte-specific transmembrane protein, affects the cellular immune response in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 100, 2622-2627.

Lanot, R., Zachary, D., Holder, F., Meister, M., 2001. Postembryonic hematopoiesis in *Drosophila*. *Dev. Biol.* 230, 243-257.

Lebestky, T., Chang, T., Hartenstein, V., Banerjee, U., 2000. Specification of *Drosophila* hematopoietic lineage by conserved transcription factors. *Science* 288, 146–149.

Mandal, L., Martinez-Agosto, J.A., Evans, C.J., Hartenstein, V., Banerjee, U., 2007. A Hedgehog- and Antennapedia-dependent niche maintains *Drosophila* haematopoietic precursors. *Nature* 446, 320-324.

Márkus, R., Laurinyecz, B., Kurucz, É., Honti, V., Bajusz, I., Sipos, B., Somogyi, K., Kronhamn, J., Hultmark, D., Andó, I., 2009. Sessile hemocytes as a hematopoietic compartment in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U.S.A.* 106, 4805-4809.

Nappi, A.J., Vass, E., Frey, F., Carton, Y., 1995. Superoxide anion generation in *Drosophila* during melanotic encapsulation of parasites. [Eur. J. Cell Biol.](#) 68, 450-456.

Olofsson, B., Page, D.T., 2005. Condensation of the central nervous system in embryonic *Drosophila* is inhibited by blocking hemocyte migration or neural activity. *Dev. Biol.* 279, 233-243.

Rizki, M.T., Rizki, R.M., 1959. Functional significance of the crystal cells in the larva of *Drosophila melanogaster*. *J. Biophys. Biochem. Cytol.* 5, 235-240.

Rizki, T.M., Rizki, R.M., 1980. Properties of larval hemocytes of *Drosophila melanogaster*. *Experientia* 36, 1223-1226.

Russo, J., Dupas, S., Frey, F., Carton, Y., Brehélin, M., 1996. Insect immunity: early events in the encapsulation process of parasitoid (*Leptopilina boulardi*) eggs in resistant and susceptible strains of *Drosophila*. *Parasitology* 112, 135-142.

Shrestha, R., Gateff, E., 1982. Ultrastructure and cytochemistry of the cell types in the larval hematopoietic organs and hemolymph of *Drosophila melanogaster*. *Dev. Growth Differ.* 24, 65–82.

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

Stramer, B., Wood, W., Galko, M.J., Redd, M.J., Jacinto, A., Parkhurst, S.M., Martin, P., 2005. Live imaging of wound inflammation in *Drosophila* embryos reveals key roles for small GTPases during in vivo cell migration. *J. Cell Biol.* 168, 567-573.

Tokusumi, T., Shoue, D.A., Tokusumi, Y., Stoller, J.R., Schulz, R.A., 2009. New hemocyte-specific enhancer-reporter transgenes for the analysis of hematopoiesis in *Drosophila*. *Genesis* 47, 771-774.

Zettervall, C.J., Anderl, I., Williams, M.J., Palmer, R., Kurucz, É., Andó, I., Hultmark, D., 2004. A directed screen for genes involved in *Drosophila* blood cell activation. *Proc. Natl. Acad. Sci. U.S.A.* 101, 14192-14197.

FIGURE LEGENDS

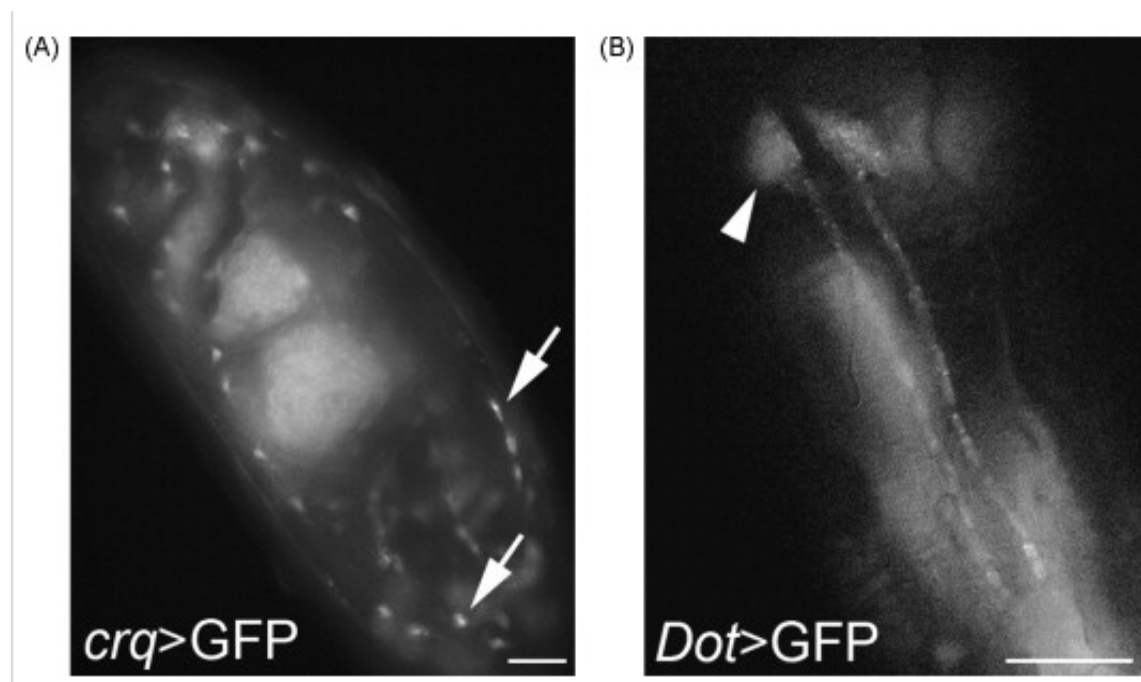


Fig. 1. Expression of *crq*>GFP and *Dot*>GFP in the *Drosophila* embryo. (A) *crq*>GFP expression in embryonic macrophages (arrows). (B) *Dot*>GFP expression in the embryonic lymph gland (arrowhead) (scale bars: 20 μ m).

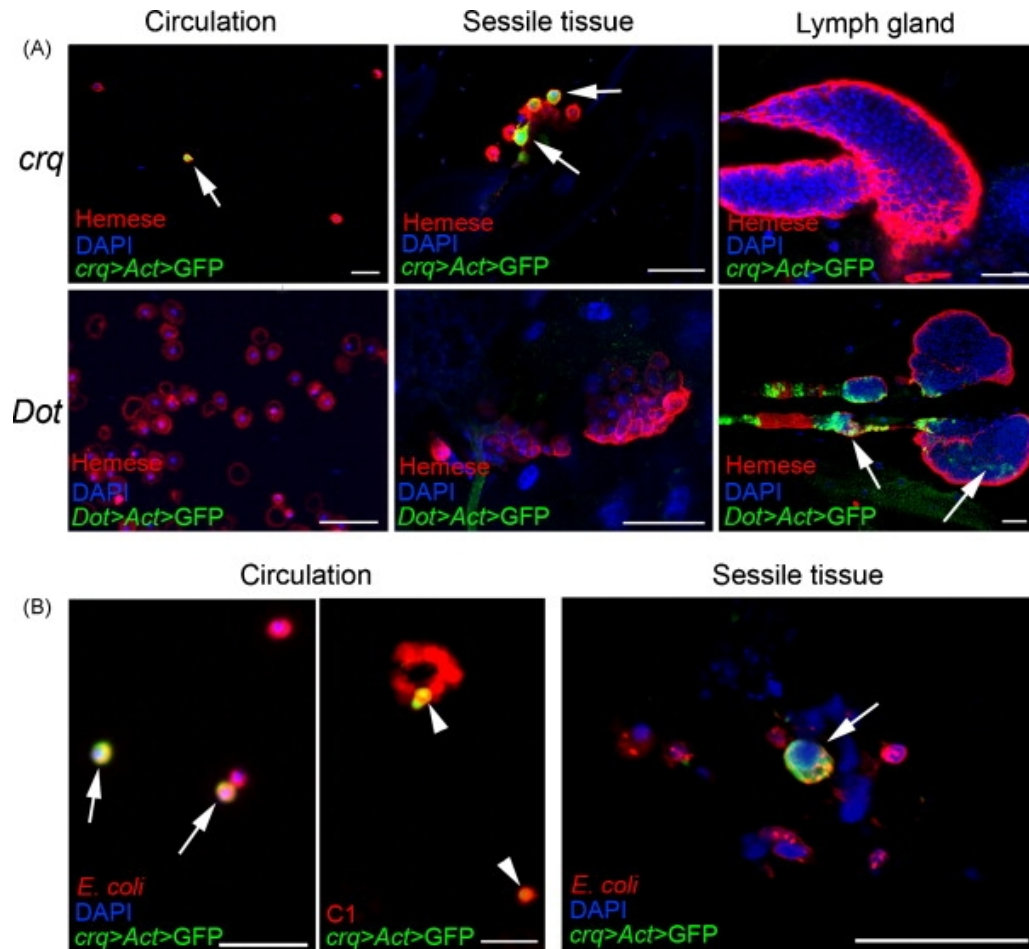


Fig. 2. Localization and function of hemocytes derived from the embryonic *crq* and *Dot* lineages in the different larval hemocyte compartments. (A) *crq* and *Dot* lineage tracing in the circulation, in the sessile tissue and in the lymph gland (arrows indicate lineage-traced cells). (B) Hemocyte types of the *crq* lineage in the circulation and in the sessile tissue. The arrows indicate phagocytic cells; the arrowheads point to crystal cells. Bacteria and hemocyte-specific markers were visualized by red fluorescence (scale bars: 20 μ m).

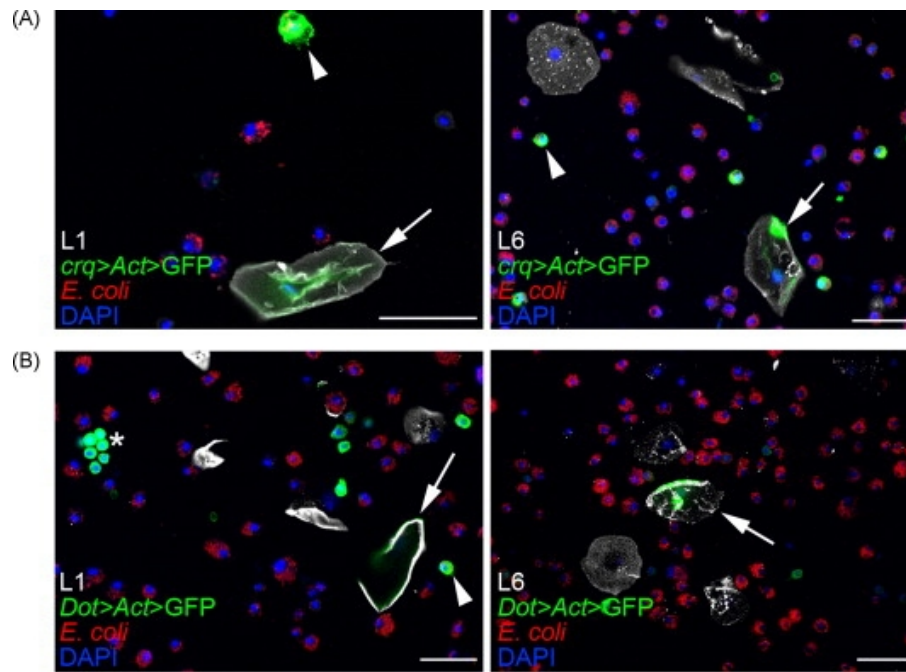


Fig. 3. Contributions of the *crq* and *Dot* lineages to effector hemocyte production following immune-induction. Expression of the lamellocyte-specific L1 and L6 antigens (far red - white) and phagocytosis of bacteria (red) in *crq* (A) and *Dot* (B) lineage-traced hemocytes (green) upon immune induction. The arrows indicate lamellocytes from the *crq* and *Dot* lineages; the arrowheads point to phagocytic cells; the asterisk marks a small cluster of non-phagocytic GFP-expressing cells (scale bars: 20 μ m).

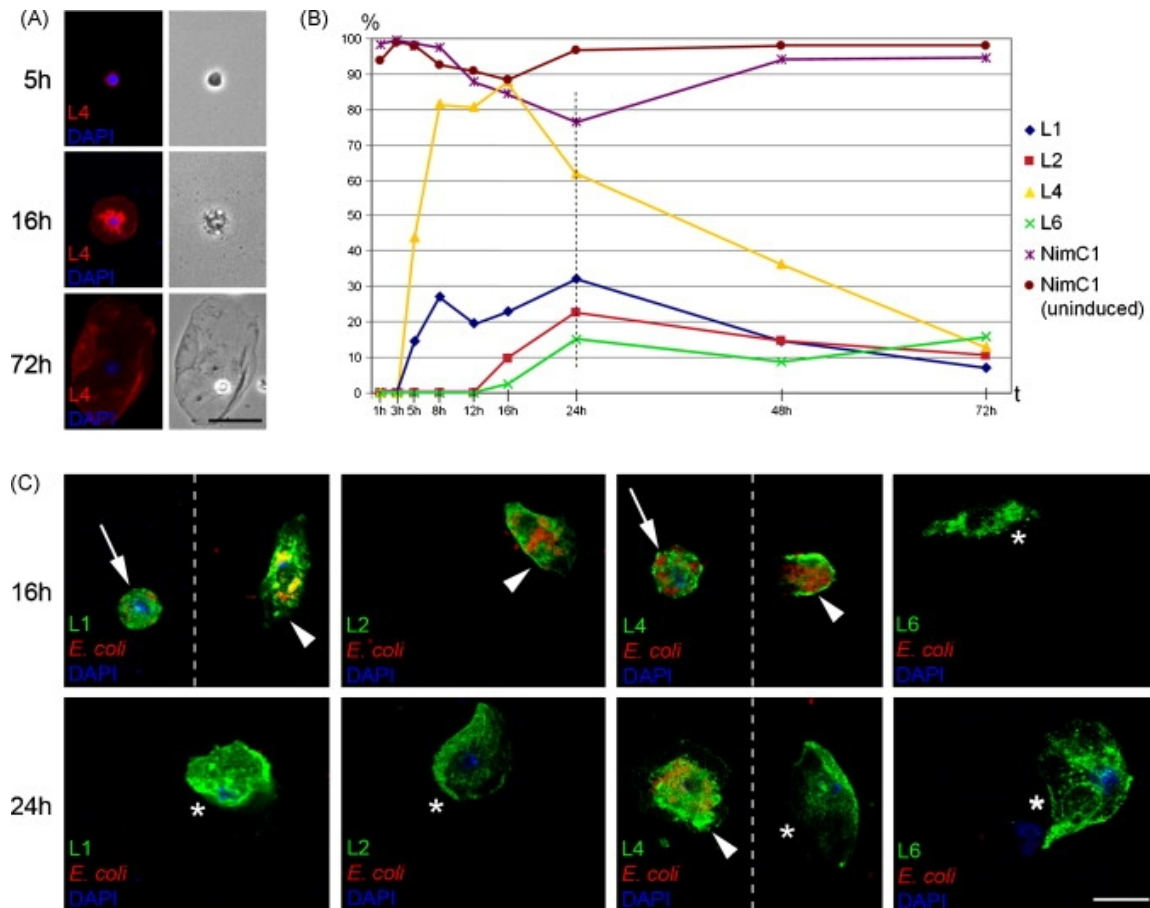


Fig. 4. Morphological and functional transformation of circulating hemocytes following immune induction. (A) Typical morphology of hemocytes expressing the L4 antigen (red) 5, 16 and 72 h after immune induction. (B) Kinetics of hemocyte populations expressing the lamellocyte-specific L1, L2, L4 and L6 antigens and the plasmatocyte-specific NimC1 antigen after infestation. The NimC1-expressing hemocyte population in uninduced larvae is indicated as control. (C) Phagocytosis of bacteria (red) by hemocytes expressing lamellocyte-specific antigens (green). The arrows indicate round cells; the arrowheads point to discoidal cells; the asterisks mark elongated lamellocytes (scale bars: 20 μ m).

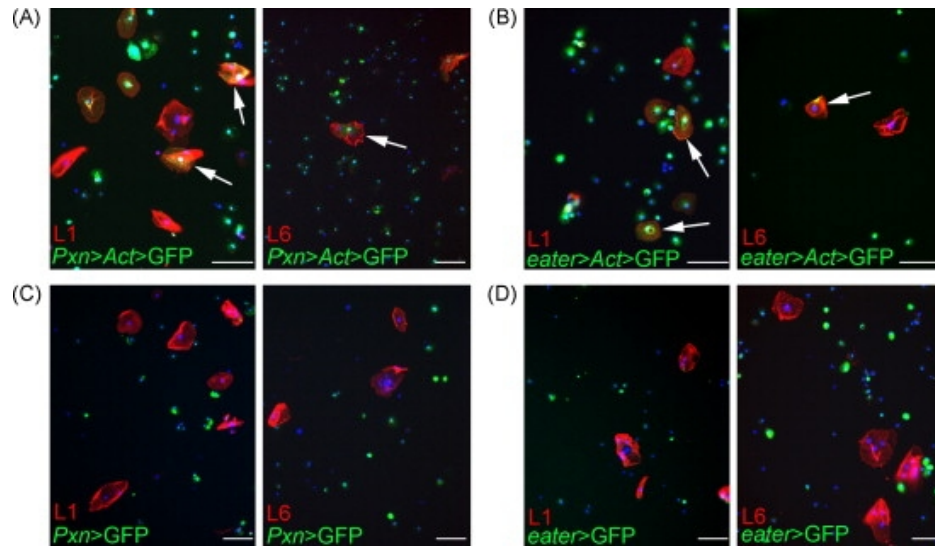


Fig. 5. Lineage tracing of plasmatocyte-specific *Pxn-GAL4* and *eater-GAL4*-expressing hemocytes following immune-induction. Expression of GFP (green) in *Pxn*- (A) and *eater*- (B) lineage-traced hemocytes. Expression of *Pxn-GAL4* (C) and *eater-GAL4* (D) drivers. GFP⁺ lamellocytes (red) are indicated by arrows (scale bars: 20 μm).

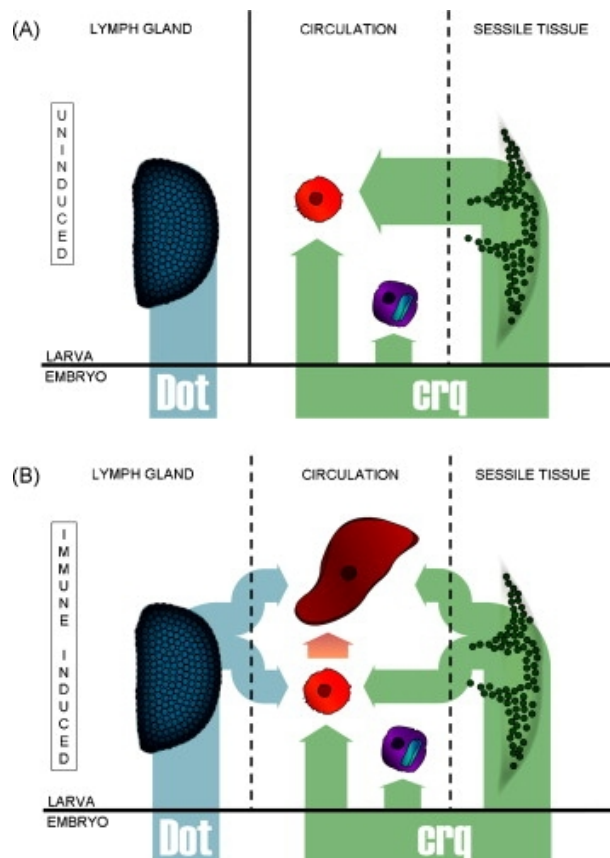


Fig. 6. Embryonic cell lineages contributing to the formation of larval compartments and the production of lamellocytes upon immune-induction. (A) The cells of the embryonic *Dot* lineage (blue) take part in the formation of the larval lymph gland, while hemocytes derived

from the embryonic *crq* lineage (green) integrate into the sessile tissue and produce circulating plasmatocytes (orange) and crystal cells (purple) of the larvae. The cells of the lymph gland do not enter the circulation in uninduced larvae. (B) Upon immune induction, both sessile hemocytes and cells from the lymph gland enter the circulation, and take part in lamellocyte differentiation. A fraction of circulating plasmatocytes also contribute to the lamellocyte pool (red arrow) upon immune induction.

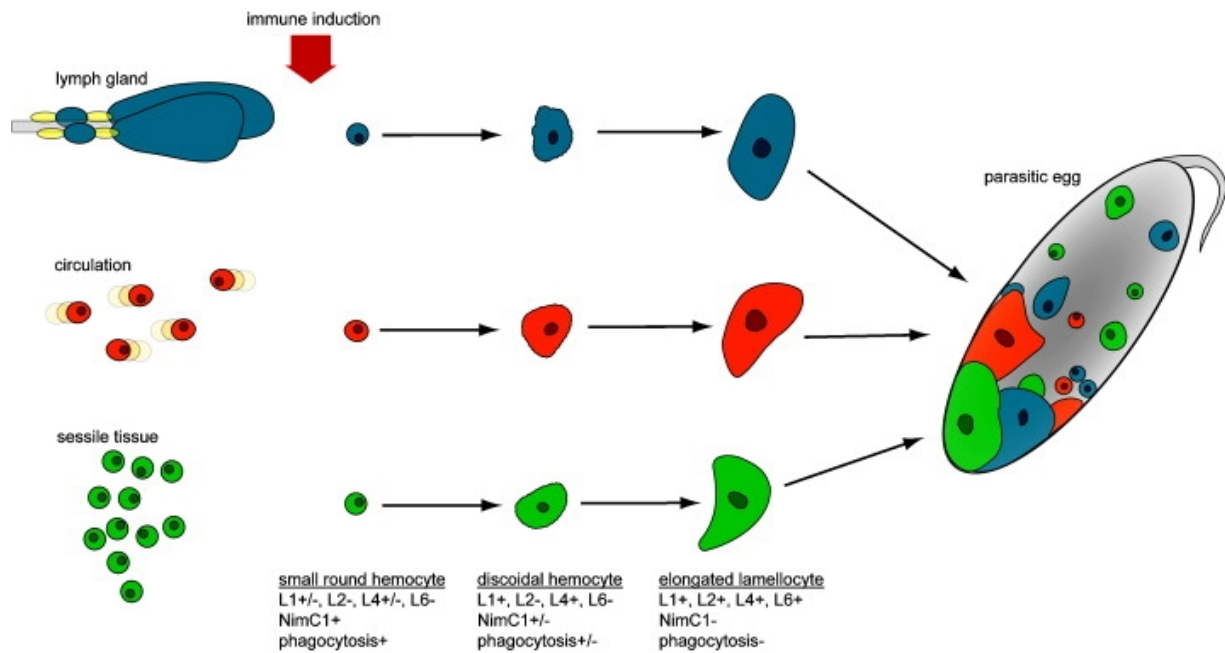


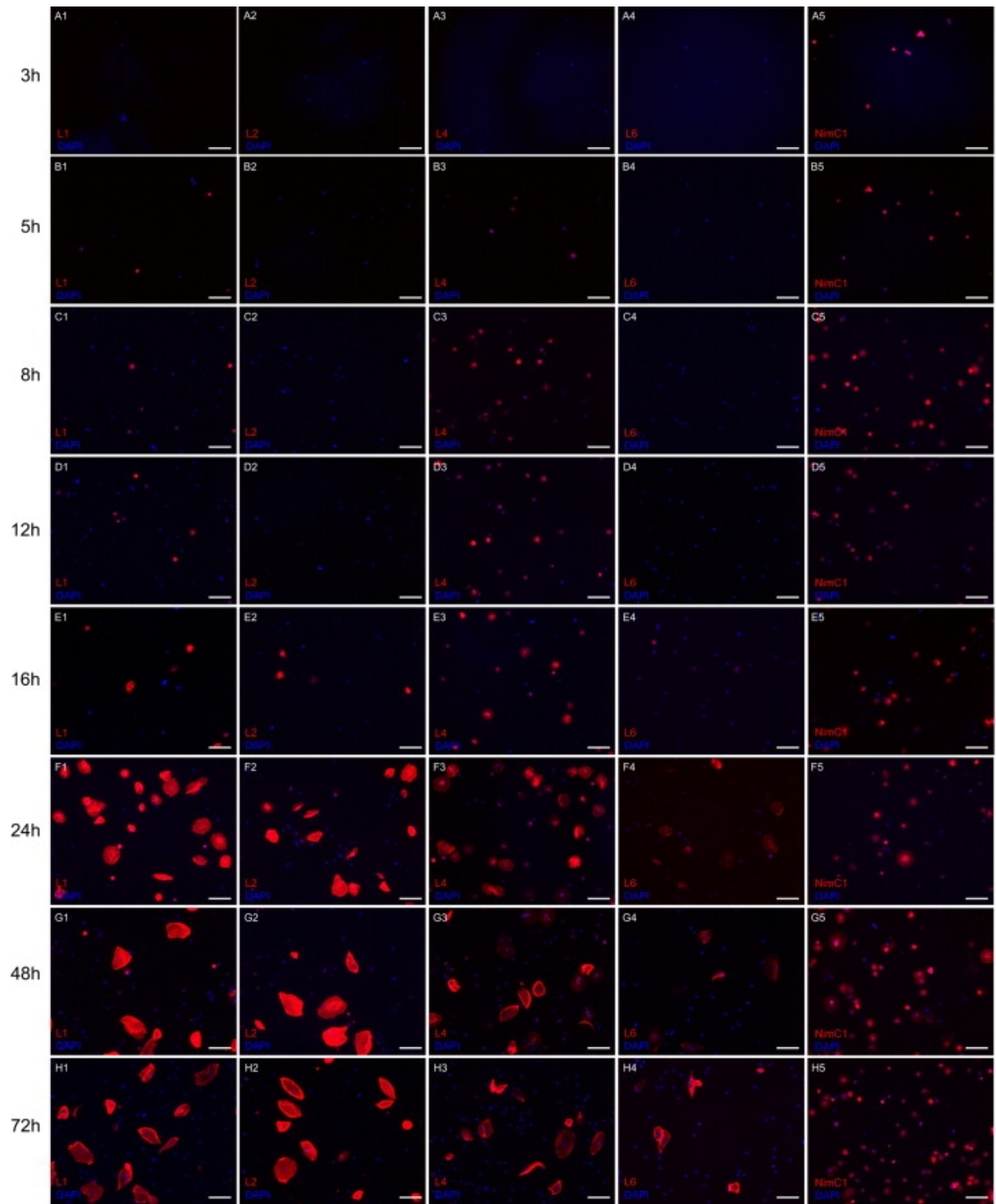
Fig. 7. The proposed model of lamellocyte differentiation and the encapsulation reaction.

<Movie1>

Supplementary Fig. S1. Time dependent visualization of *crq-GAL4*-expressing hemocytes during embryonic development (scale bar: 50 μ m).

<Movie2>

Supplementary Fig. S2. Time dependent visualization of *Dot-GAL4*-expressing hemocytes during embryonic development. The embryonic lymph gland is indicated by the arrow (scale bar: 50 μ m).



Supplementary Fig. S3. Time-dependent expression of lamellocyte and plasmatocyte-specific antigens. Hemocytes were isolated and adhered at the times indicated after infestation and stained for L1, L2, L4, L6 and NimC1 antigens (red) (scale bar: 50 μ m).