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“Host immune suppression by *Toxoneuron nigriceps* polydnavirus: use of *Drosophila melanogaster* as an experimental model system”

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to the soul of my beloved father

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Abstract

Host regulation strategies adopted by parasitic Hymenoptera are increasingly attracting the scientific interest, since several data support the idea that these strategies include targeting of evolutionarily conserved pathways.

This is in particular emerging from molecular and functional analyses of several parasitoid-associated polydnviruses (PDVs), whose gene products, expressed in the tissues of parasitized hosts, interact with key signaling molecules (Pennacchio and Strand. 2006; Espagne et al. 2004; Falabella et al. 2003; Falabella et al. 2007; Provost et al. 2004; Thoetkiattikul et al. 2005).

T. nigriceps bracovirus (TnBV) is the PDV associated with *Toxoneuron nigriceps* (Hymenoptera, Braconidae), the endophagous larval parasitoid of the tobacco budworm *Heliothis virescens* (Lepidoptera, Noctuide).

We recently identified a host gene, named 102, which is targeted by a small *TnBV* transcript. Several data indicate that this gene is involved in the immune response, but its mode of action is still unclear.

Since the 102 gene is highly conserved throughout evolution, to investigate the function of the protein encoded by the 102 gene, we decided to use *Drosophila melanogaster* as a model system, since it offers a wide range of molecular genetic tools not available in other systems: its genome is fully sequenced; detailed knowledge about the molecular pathways regulating cellular immune responses is available; several sophisticated genetic and molecular genetic techniques have been developed and can be used as powerful tools

to dissect the functional mechanisms underlying a wide range of biological processes.

In *Drosophila melanogaster* two putative protein sequences, showing 38% and 34% identity, respectively, with the translation product of the 102 gene, were found by Blast analyses. One of these is highly expressed in larval hemocytes, like its *Heliothis virescens* counterpart. Moreover, as reported in the FlyAtlas database (<http://www.flyatlas.org/>), the gene is strongly expressed also in the fat body.

I took advantage of the well-established RNAi strategies based on the GAL4/UAS binary system to interfere with the expression of this gene in immuno-competent tissues (i.e. hemocytes and fat body) of *Drosophila melanogaster*. First of all, I evaluated the potential impact of this tissue-specific RNA interference on viability and found a dramatic lethal effect approaching 100%. Lethal phase measurements indicated that mortality was prominent in larval and pupal stages. Morphological analyses of the larvae in which the 102 gene had been targeted by RNA interference showed the presence of huge melanotic masses freely floating in the hemocoel. This tumorous-like phenotype is usually associated with altered and/or excessive performance of the immune system. Accordingly, hemocyte counts indicated that hemocyte overproliferation occurred in larvae, and increased numbers of both plasmatocytes and lamellocytes were found in their hemolymph. Furthermore, in these larvae, the sessile hemocyte population, was also affected. Finally, morphological analyses of melanotic masses clearly identified their association with hemocytes, forming a cellular capsule around them.

Introduction

By the second half of the last century the need of a responsible exploit of planet's natural resources to protect the environment for future generations, had become a priority.

In this contest, a major issue is the indiscriminate use of pesticides to control food production, which must be drastically reduced. A valid alternative to chemical pesticides is represented by the bio-insecticides, natural molecules deriving from plants, bacteria, viruses and animals.

Bio-insecticides are environmentally safe, biodegradable and have a higher specificity and selectivity in comparison with chemical pesticides, which have a wide spectrum of negative effects on all organisms, including humans.

These considerations stimulated in the last decades a huge increase of studies aimed at the identification, isolation, characterization and production of molecules that could be used as bio-insecticides. In particular, strong efforts have been directed towards investigations focused on control strategies used by insect's natural enemies.

Among these enemies, parasitoids are attracting special interest. Parasitoids belong to diverse insect orders (Diptera, Coleoptera, Hymenoptera, Lepidoptera, Trichoptera, Neuroptera, Strepsiptera), and have developed a huge variety of strategies to colonize their hosts. Adult females lay their eggs in or on host bodies resulting in the death of the host. An astonishing number of Hymenoptera are parasitoids of other insect species and adopt a variety of host regulation strategies in order to create favourable conditions for the development of their progeny. Since parasitoid reproduction results in killing hosts, they can be viewed as a promising source of bioactive molecules that can be used to control insect pests attacking a wide variety of crops. Indeed, several data strongly

support the idea that host regulation exerted by the parasitoids targets host genes/proteins that play key roles in host physiology and development.

The study of different host-parasitoid associations, is already providing new insights in the molecular mechanisms underlying host regulation and this will lead to the characterization of the different pathways affecting host physiology and will eventually allow to develop new effective bio-insecticides.

1.1 Parasitoids and host regulation

Insect parasitoids have developed a huge variety of strategies to colonize their hosts through specialized mechanisms generated by long adaptive processes occurred within host-parasitoid interactions. (Vinson S.B. and Scott J.R. 1974; Vinson S.B. and Iwantsch G.F. 1980; Godfray H.C.J. 1994; Quicke D.L.J. 1997).

Parasitoids belong to several insect orders (Coleoptera, Diptera, Lepidoptera, Neuroptera, Strepsiptera, Trichoptera), but are particularly abundant in the order Hymenoptera. They can be classified in several ways, based on specific features: number of eggs laid, nutritional mode, behavior. According to the number of eggs laid, successfully developing in/on a single host, they can be classified as solitary or gregarious. With respect to the nutritional mode, parasitoids can be divided in ectoparasitoids, which feed outside the host body, and endoparasitoids, which feed inside the host body (Godfray H.C.J. 1994). Regarding their behavior, they can be classified in koinobionts and idiobionts; the latter, block host development by female injection of specific secretions which are able to preserve host tissue and/or facilitate digestion. Koinobionts, instead, allow host growth till their own maturation is complete.

Koinobionts include the so called *conformers*, endoparasitoids that conform their own development to host physiology, and the *regulators*, endoparasitoids able to alter host physiology to create an environment suitable for successful egg development. Generally, *regulators* parasitize early host stages and modulate host physiology, morphology and development, redirecting host metabolism for their own advantage. Therefore, a close anatomo-physiological interaction is established between the host and the parasitoid, which generally shows a significant degree of morphological simplification combined with a high degree of specialization (Pennacchio F. and Strand M.R. 2006). The latter is converted in the association of the parasitoid species with only a given host species, or a wider but homogenous systematic group. This has led, in turn, to the evolution of fine regulatory mechanisms allowing the parasitoid to evade host immune defenses.

Host regulation is exerted by the action of both maternally-derived and embryonic factors. The latter are polyploid cells, named teratocytes, generated by the dissociation of the embryonic membrane at the egg hatching, that freely circulate within the host's hemolymph, where they grow in size without undergoing cell division (Pennacchio F. and Strand M.R. 2006). These cells influence host metabolic and endocrine balance, allowing parasitoid development.

Maternal factors consist of venom and ovarian fluid proteins. They are injected into the host at oviposition, and play a key role in the induction of the major alterations observed in parasitized hosts. In certain wasp groups the ovarian fluids also contain a symbiotic virus of the family Polydnviridae. This is a unique virus family whose members share peculiar genomic features (Espagne E. et al. 2004).

On the basis of their association with braconid or ichneumonid wasps, polydnaviruses (PDVs) are included in two genera, Bracoviruses and Ichnoviruses, respectively, with distinct evolutionary origins (Espagne E. et al. 2004). PDVs of both genera are stably integrated as proviruses in the genome of the wasp they are associated with and are vertically transmitted through the germline. Their replication occurs only in the wasp ovaries to generate viral particles containing circular double-stranded DNA molecules of different size (Pruijssers A. J. and M Strand. 2006). These viral particles, injected into the body of the insect host along with the parasitoid egg, enter host cells and express their genes. Viral gene products prevent the host's immune system from killing the parasitoid's offspring and cause alterations in host growth that facilitate parasitoid development (Pruijssers A. J. and M Strand. 2006).

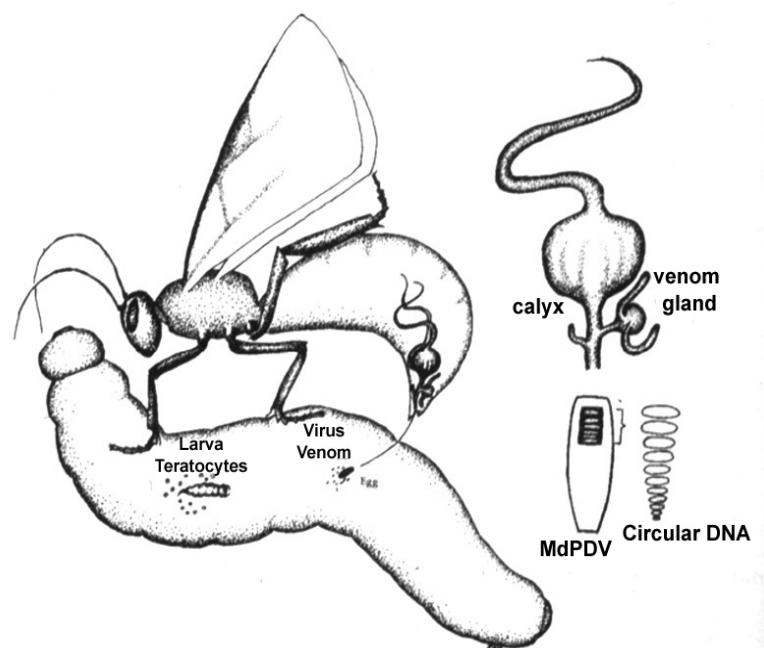


Fig.1 Maternally-derived and embryonic factors that play key roles in host regulation strategies.

1.2 The host-parasitoid association *H.virescens-T. nigriceps*

Toxoneuron nigriceps (Hymenoptera, Braconidae) is an endophagous parasitoid of the tobacco budworm *Heliothis virescens* (Lepidoptera, Noctuidae) larvae, associated with a symbiotic bracovirus (*TnBV*) that plays a major role in host regulation. In the last decades it has become a model system for investigations on host-parasitoid interactions.



Fig.2: *H.virescens* larva parasitized by a female *Toxoneuron nigriceps* wasp

Sequencing of the *TnBV* genome by the research group I work with led to the identification of 37 putative genes; some of these genes show similarity only with genes found in other polydnaviruses whose function is still unknown, while others code for proteins having conserved domains. In several cases these conserved domains are indicative of specific enzymatic activities, such as aspartic proteases and tyrosine phosphatases (Malva C et al, 2004).

Conserved domains are also found in the *TnBV* *ank1* gene which encodes for a protein that contain ankyrin repeats and resemble I κ B

family members, which act as negative regulators of NF- κ B transcription factors. However, the *TnBVank* proteins lack the sequence motifs necessary for signal-induced degradation. This strongly suggests that it may irreversibly bind host NF- κ B related molecules, disrupting the signal transduction pathways they belong to (Falabella P et al. 2007; Thoetkiattikul H. et al. 2005).

In a recent work, it has been reported that the expression of the *TnBVank1* gene in the *Drosophila* germline affects the distribution of several mRNAs whose gene products are involved in embryonic development. This is due to defects in the polarization of the microtubule network that lead to altered intracellular, microtubule-based, trafficking routes. These findings suggest that, by interfering with cytoskeleton organization, the *ank* gene family may play novel functions. (Duchi S et al, 2010).

It has been demonstrated that the expression of PDV genes in the host tissues is involved in the regulation of a number of endogenous host genes (Barat-Houari M. et al. 2006). However, the molecular mechanisms underlying these effects are largely unknown and, in most cases, a link between altered host gene expression and pathological consequences of viral infection still needs to be established.

The screening of a cDNA library raised from hemocytes of parasitized host larvae, using as probes a set of *TnBV* genomic clones, led to the isolation of a 1804nt long cDNA, named cDNA 102, potentially encoding a protein of 364aa with a predicted signal peptide of 31aa, showing 85% sequence identity with a protein present in the venom of *Lonomia obliqua* (Lepidoptera, Saturniidae) larvae (accession number: [AY829819.1](#)), which cause the hemorrhagic syndrome in humans by skin contact (Carrijo-Carvalho L.C. 2007). This protein

belongs to the XendoU protein family, whose members characterized so far display endoribonuclease activity.

It turned out that the 102 gene is a host gene expressed in *H. virescens* hemocytes that, in parasitized individuals, is targeted by a small *TnBV* transcript, which is virtually identical in sequence to the antisense strand of the 5' UTR region of the 102 gene itself.

This evidence, coupled with the finding that in host hemocytes there is a high expression of the 102 gene, strongly supports the idea that the protein (named P102) encoded by this gene, plays an important role in the immune response (Falabella et al, 2011. unpublished data), but its mode of action is still unclear.

Blast analyses, using as query the sequence of the putative protein encoded by the 102 gene, showed that it displays an extended region of similarity with proteins present all along the evolutionary tree. (see Table A p.62). In particular, in *Drosophila melanogaster*, two putative protein sequences (CG2145 and CG3303), showing 38% and 34% identity, respectively, with the translation product of the 102 gene, were found. Preliminary RT-PCR experiments performed in the lab indicated that the gene coding for one of these putative proteins (the one showing the highest similarity rate, tentatively named Dm-102), is highly expressed in hemocytes, like its *Heliothis virescens* counterpart.

Therefore, we decided to use *Drosophila melanogaster* as a model system to investigate the function of the protein encoded by the 102 gene. This choice was primarily based on the observation that a strong evolutionary conservation of many basic physiological processes exists between insects.

Moreover, research work on the very effective immune reactions in this Dipteran insect have already given insights on the immune

system of other insects that have harmful effects on humans as agricultural pests or as disease vectors. Finally, *Drosophila melanogaster's* fully sequenced genome (completed in 2000) strongly supported the development of new postgenomic technologies, such as microarrays, proteomics and RNA interference (RNAi), whose application greatly expanded the ability to analyze the immune system in this model organism (Lamaitre B. and Hoffmann J. 2007)

1.3 *Drosophila* immune system: the humoral response

In the last two decades *Drosophila melanogaster* has become a powerful animal system for investigations on the molecular pathways that regulate innate immunity, leading to the identification of many factors that play key roles in immune responses in both vertebrates and invertebrates (Lemaitre C and Hoffmann J. 2007).

The innate immune system of *Drosophila*, and more generally of insects, can be divided into two major components: the humoral system and the cellular system, both activated upon immune challenge. This distinction is somewhat arbitrary, for there is a constant overlap between humoral and cellular defenses. Indeed, hemocytes are an important source of many humoral molecules, as well as many humoral factors affect hemocyte functions.

Humoral defenses comprise the production of antimicrobial peptides (AMPs) (Williams M.J. 2007); the enzymatic cascades that lead to coagulation and/or melanization of the hemolymph (Muta T. and Iwanaga S. 1996; Gillespie et al. 1997); and the production of

reactive intermediates of oxygen (ROS) and nitrogen (Bogdan C. et al. 2000; Vass E. and Nappi A.J. 2001). The AMPs are produced and secreted in the circulating hemolymph by several tissues, including hemocytes, epidermis, and the fat body (an organ thought to be the equivalent of the liver in insects). Seven classes of AMPs have been described in *Drosophila*. Based on their main microbial target, these classes can be divided into three groups: diptericins, drosocins, cecropins and attacins are active against Gram-negative bacteria; defensins against Gram-positive bacteria; drosomicins and metchnikowins against fungi (Leclerc V. Reichhart JM. 2004). The expression of the different types of AMPs involves several pathways. In a very schematic and simplified way, Gram-negative bacteria induce the expression of *diptericin* and other genes through the IMD pathway, while fungi and Gram-positive bacteria induce the expression of *drosomylin* and other genes through the Toll pathway. In the recognition of microorganisms, a key role is exerted by pattern recognition receptors (PRRs), immune proteins that recognize general microbial components. In *Drosophila* two major families of PRRs have been identified: the Gram-negative binding proteins (GNBPs) and the peptidoglycan recognition proteins (PRPs). The recognition of a microorganism by a PRRs leads to the activation of the Toll or the Immune-deficiency (IMD) pathway. Both pathways culminate in the activation of a nuclear factor- κ B (NF- κ B)/reticuloendotheliosis (Rel) family transcription factor. (Fig1) (Leclerc V. and Reichhart J.M. 2004).

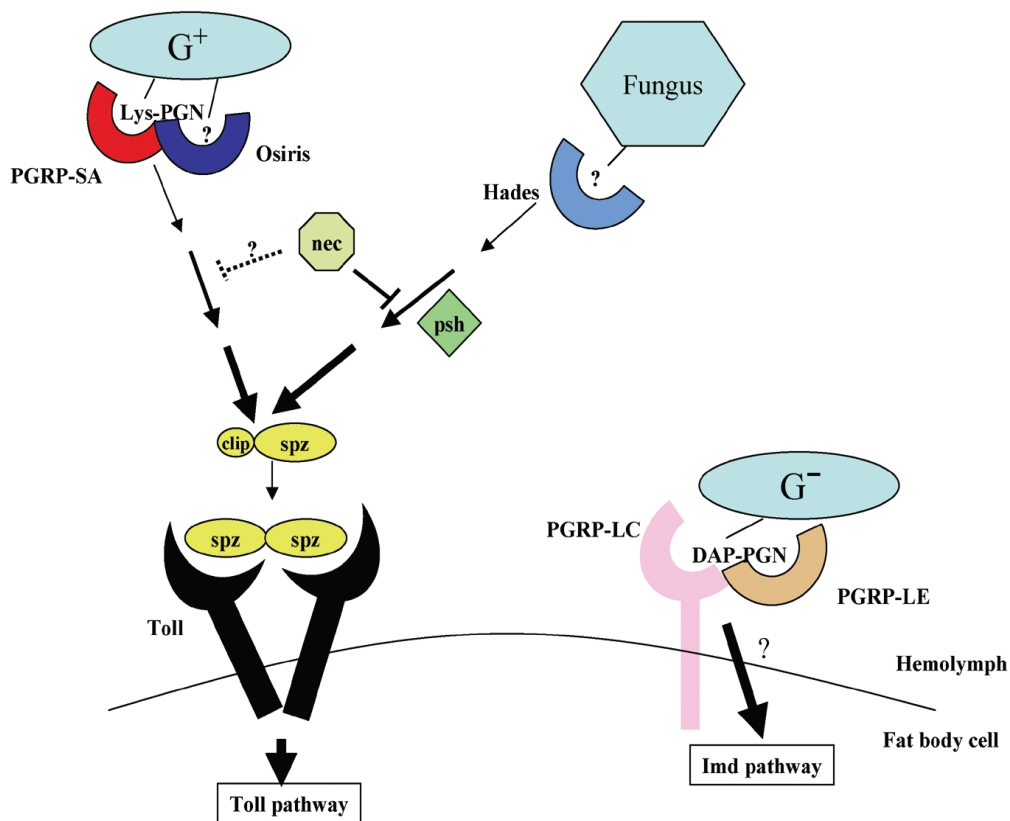


Fig. 3 Recognition of microorganisms in *Drosophila* hemolymph. The lysine-type peptidoglycan of Gram-positive bacteria (G^+) is recognized by a complex consisting of PGRP-SA and Osiris. An unknown fungal molecule is recognized by Hades. In both cases, a protease cascade is activated, resulting in the cleavage of the clip domain of spz and the subsequent activation of the Toll pathway. The diaminopimelic acid (DAP)-type peptidoglycan of Gram-negative bacteria (G^-) is recognized by PGRP-LC and PGRP-LE, possibly acting as a complex and activating the immune deficiency (IMD) pathway. (Adapted from Leclerc V. and Reichhart J.M. 2004)

1.4 Melanization

The primary humoral immune response of insects is melanization that produces black pigments resulting from the activation of a biochemical pathway leading to the conversion of tyrosine to melanin (Meister M. and Lagueux M. 2003). Melanin production is controlled by a serine proteases cascade that leads to the cleavage of the zymogen prophenoloxidase (PPO) to its active form (PO). Phenoloxidase, in turn, catalyses the oxydation of phenols to quinones, which polymerize non-enzymatically forming melanin. The production of the active form of PO is triggered by the cascade of serine proteases, activated by the recognition of a non self molecular pattern (i.e. peptidoglycan or LPS present on bacterial surface), with the final activation of the proPo-activating enzyme (PPAE) which, in turn, cleaves the PPO to its active form. This cascade is highly controlled to prevent PO activation when it is not necessary: enzymes involved in the cascade are synthesized as zymogens and activated only after proteolytic cleavage by a serine protease, and, moreover, inhibitors are involved in the regulation process (Fig. 4) (Cerenius L. and Söderhäll. 2004). In *Drosophila*, the different elements of the cascade have not yet been identified, but recently a key control serine protease inhibitor protein, that restricts phenoloxidase activity to the site of injury or infection has been identified (De Gregorio E. et al. 2002). This serpin (Serpín-27A) regulates the melanization cascade through the specific inhibition of the terminal protease prophenoloxidase-activating enzyme (PPAE), preventing the insect from excessive melanization.

Melanogenesis, in addition to the production of melanin, generates intermediates (i.e. quinones, semiquinones, tryhydroxyindoles and

Reactive Oxygen Species) that represent a powerful cytotoxic potential against pathogens (Nappi A.J. And Christensen B.M. 2005).

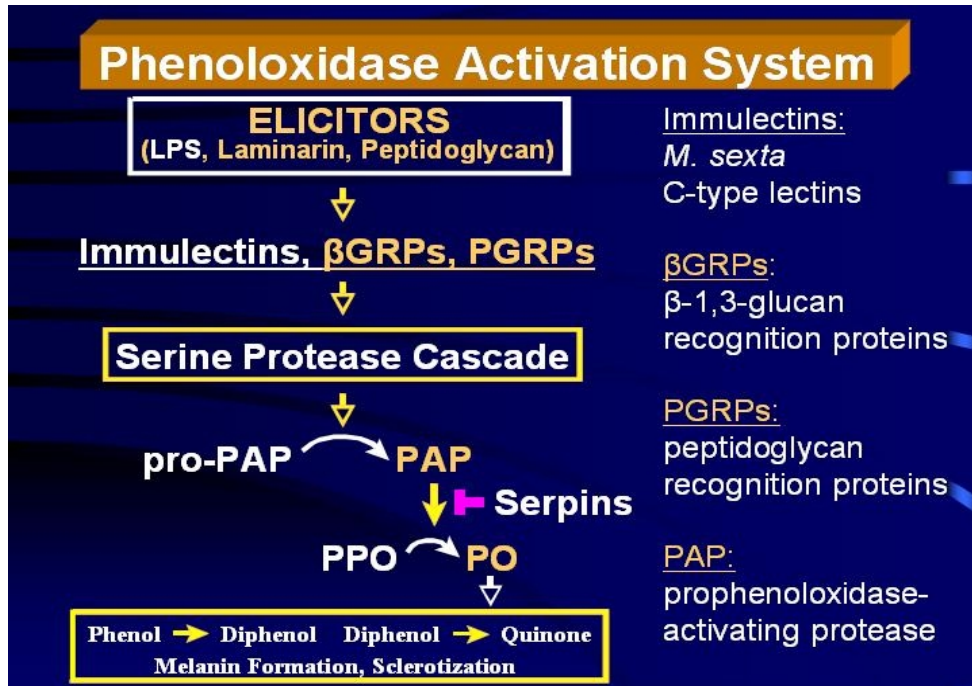


Fig. 4: Activating cascade of ProPhenolOxydase.

1.5 The cellular response

The cellular response is mediated by circulating and sessile immune surveillance cells (hemocytes) and include phagocytosis, coagulation, and encapsulation.

1.6 Hemocytes

Based on their morphological and functional characteristics, *Drosophila* hemocytes can be divided into three types.

The most abundant (90-95% in healthy larvae) are plasmatocytes, small cells involved in phagocytosis and encapsulation, which also produce AMPs. Plasmatocytes are similar to the mammalian monocyte/macrophage lineage (Williams M J. 2007), while the equivalents in Lepidopteran insects are named granular cells. Crystal cells are less abundant (5% of total hemocytes), and smaller than plasmatocytes and display many inclusions that contain large amounts of prophenoloxidase in crystallized forms. Upon immune challenge they disrupt and release this material in the hemolymph, thus giving rise to the prophenoloxidase cascade that leads to melanization .

The third type of hemocytes are the so called lamellocytes, equivalent to Lepidopteran plasmatocytes (Ribeiro C. and Brehélin M. 2006), large flattened cells never present, or at least present in a very low percentage (3%) in healthy larvae; lamellocytes are involved in encapsulation of intruders too big to be phagocytized by plasmatocytes (i.e. parasitoid eggs). Lamellocytes differentiate and proliferate soon after an immune challenge such as a parasitoid attack.

1.7 Hematopoiesis

In *Drosophila* two waves of hematopoiesis occur, distinct in space and time. During embryonic development hemocytes arise from the head mesoderm and then populate the whole embryo (Fig.5).

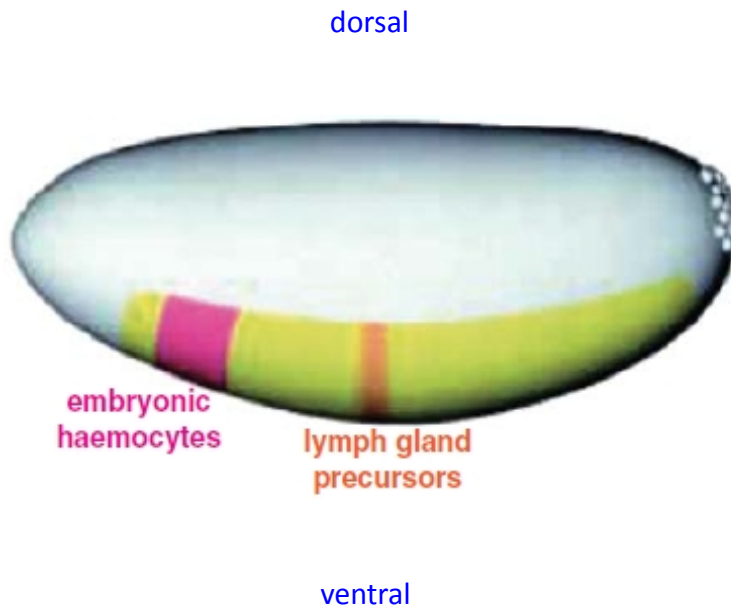


Fig.5 Embryonic hematopoiesis.

Hemocytes arise from the head mesoderm (magenta); in orange the area of the cardiogenic mesoderm containing lymph gland precursors. (Modified from Crozatier et al. 2007)

These hemocytes of embryonic origin, persist and replicate in the hemolymph of larval stages. In the larva, a second wave of hematopoiesis occurs in a specialized organ named lymph gland. The lymph glands originate in the cardiogenic mesoderm of the embryo (Fig 5) and grow by cellular proliferation during larval stages. In the late embryo lymph glands consist of a single pair of lobes with about 20 cells each, and are flanked by a cluster of pericardial cells, postulated to have nephrocyte functions. During the second larval stage, the primary lobes contain approximately 200 cells, but 2-3 new pairs of posterior lobes appear, that act as a pro-hemocytes reservoir (Jung S.H. 2005).

By the late third larval stage, lymph glands have increased 10- fold in size and are composed by 2 to 7 pairs of hemocyte-containing lobes flanking the dorsal vessel, the aorta/heart tube of the open circulatory system of *Drosophila*.

Hemocyte differentiation in the lymph gland first occurs in the primary lobes of early third instar larvae. Under normal conditions, very few hemocytes differentiate in the secondary lobes, but an immune challenge (i.e. wasp infestation) leads to the differentiation of all types of hemocytes in these lobes.

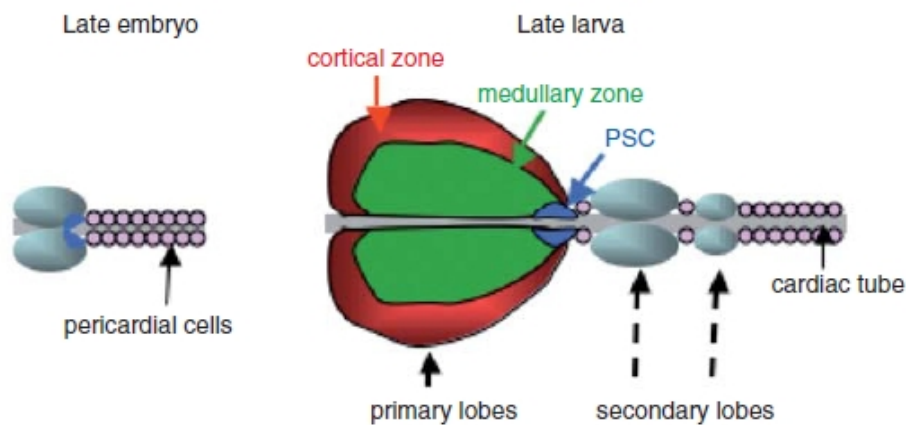


Fig.6 Lymph gland architecture.

Embryonic lymph glands are composed by a pair of lobes flanked by pericardial cells with putative nephrocyte functions. In late III larval stage lymph glands are composed by a pair of primary lobes and several secondary lobes. In the primary lobes a cortical zone (in red), a medullary zone (green) and a Posterior Signalling Centre (PSC, blue) can be observed. (Adapted from Croazier et al. 2007)

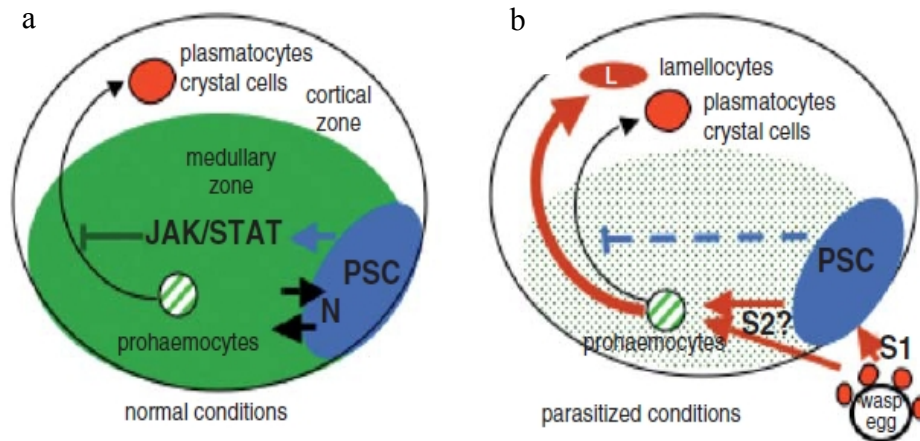


Fig.7 Larval hematopoiesis.

a. During normal development, PSC cells (blue) act to maintain JAK/STAT signalling in the medullary zone cells (green). JAK/STAT activity is needed to prevent premature differentiation of prohemocytes of the medullary zone in plasmatocytes and crystal cells that would migrate in the cortical zone (white). Notch (N) signalling, via *Ser* expression, is required to maintain high levels of *col* transcription.

b. Wasp infestation triggers a strong immune reaction and prohemocytes are reprogrammed. Plasmatocytes (in red) recognize the wasp egg and produce a yet unknown signal that reaches the prohemocytes, either directly (S2) or indirectly via the PSC, or both. *Col* activity in the PSC is required for lamellocyte differentiation. (Adapted from Crozatier et al. 2007)

At metamorphosis the lymph glands disintegrate and release their content in the hemolymph, while all pro-hemocytes differentiate into plasmatocytes. In the adults, lymph glands are absent and no hemocyte proliferation has been observed; thus hemocyte

population of pupae and adults consists of many thousands of mature plasmatocytes of both embryonic and larval origin (Crozatier M. et al. 2007).

Morphologically, in the lymph gland primary lobes of early third instar larvae, three areas can be distinguished: a cortical zone of loosely arranged cells; a medullary zone consisting of compactly arranged cells that can be identified by the expression of *domeless* (*dom*), which encodes the receptor for the JAK/STAT (Janus Kinase/signal transducer and activator of transcription) signalling pathway; and a posterior signaling center (PSC), that can be identified by a small group of cells expressing the transcription factor Collier (*Col*) and the Notch ligand Serrate (*Ser*). *Col* is necessary for PSC identity and for the differentiation of lamellocytes triggered by wasp infestation (Fig.4) (Crozatier M. et al. 2007). The PSC works as an organizing centre; the medullary zone contains hemocyte precursors, while the cortical zone contains differentiating hemocytes. (Fig.7).

In the embryo, the earliest marker of the haemocyte cell fate is *Serpent* (*Srp*), a GATA transcription factor homolog, involved in the differentiation of plasmatocytes and crystal cells; its expression is necessary for cell maintenance during development (Fosset N. et al. 2003). Initially all pro-hemocytes express the transcription factors glial cell missing (*gcm*) and *gcm2*. Later, *gcm/gcm2* transcription is inhibited and *lozenge* (*lz*), a RUNT transcription factor homolog, starts to be activated in the first row of pro-hemocytes, while in the other rows *gcm/gcm2* expression is maintained; the pro-hemocytes of these rows will differentiate into plasmatocytes. 60% of the *lz*⁺ expressing progenitors maintain *lz* expression via an autoactivation loop and will differentiate into crystal cells, while in the last 40%

the presence of residual Gcm interferes with *Iz* expression and promotes plasmatocyte differentiation (Fig. 8) (Crozatier M. et al. 2007).

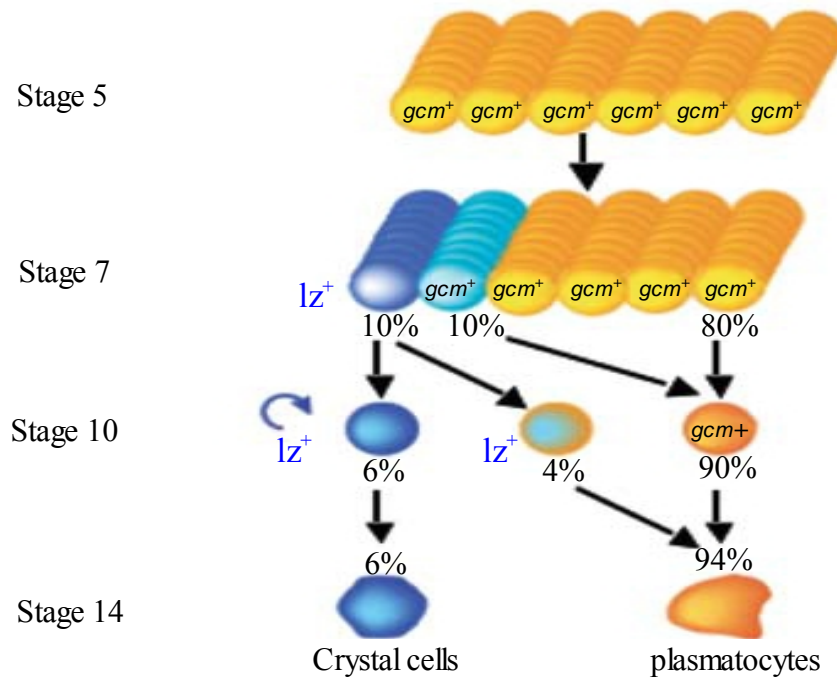


Fig.8 Hemopoiesis in embryo; see text for details. (Adapted from Williams 2007)

Lamellocytes are rarely seen in healthy larvae, but their differentiation is triggered rapidly by an immune challenge (i.e. parasitic attack). Several signalling pathways are involved in their differentiation, including Toll (Qiu P. et al. 1998.), JNK (Zettervall C.J.2004) and JAK/STAT pathways (Luo H. et al. 1995).

Hemese, which encodes a transmembrane protein, and *Yantar*,

encoding a protein with a putative RNA-processing role, inhibit the differentiation of lamellocytes (Kurucz et al. 2003; Sinenko 2004). Recent studies indicated the existence of an alternative pathway for lamellocyte differentiation (Stofanko M. 2010). Stofanko and colleagues demonstrated, by lineage tracing, that plasmatocytes differentiate into lamellocytes in response to wasp infection; moreover they showed that over-expression of the CoREST transcription factor *charlatans* in plasmatocytes induces lamellocyte differentiation in lymph glands and hemolymph. In these experiments the increase in lamellocyte number was coupled with the extinction of plasmatocyte markers, further supporting the idea that plasmatocytes transform into lamellocytes (Stofanko M. 2010).

1.8 Phagocytosis.

Phagocytosis is an evolutionarily conserved mechanism against microorganism infection (i.e. bacteria, fungi) and for the removal of cell debris, deriving from dead cells, and other foreign bodies. In *Drosophila*, plasmatocytes are the main actors in this process. The first step of phagocytosis is the attachment of the phagocyte (i.e. plasmatocyte) to the target particle, followed by cytoskeleton modifications that lead to the internalization and the destruction of the engulfed particles inside the phagosomes (Meister M. 2004). The first gene to be identified as a gene coding for a pattern recognition receptor that binds bacteria, is the scavenger receptor dSR-CI (Rämer M. et al. 2001). Other genes involved in the engulfment of bacteria are the peptoglycan recognition protein LC (PGRP-LC), which recognizes Gram-negative bacteria, and *eater*, encoding a EGF domain transmembrane receptor. RNAi knockdown of *eater* in S2

cells inhibits phagocytosis by 70%. *Eater* is expressed in larval plasmatocytes and several evidences point to a role of this receptor in the recognition of a broad range of microbial pathogens, including *Escherichia coli* (Gram-negative bacterium) and *Staphylococcus aureus* (Gram-positive bacterium) (Williams M J. 2007). More recently, the TEP (thioester-containing) proteins have been implicated in phagocytosis. In the *Drosophila* genome, six genes encodes TEP proteins (TEP1-TEP5 and Mcr, Macroglobulin-complement related). They are postulated to act as opsonins that induce phagocytosis. Several evidences point to a role for TEP2 in phagocytosis of *E.coli*; for TEP3 in phagocytosis of *S.aureus* and for Mcr in phagocytosis of the fungus *C.albicans*. The Ig superfamily receptor Down syndrome cell adhesion molecule (Dscam), is also required for proper phagocytosis by plasmatocytes (Watson F.L. Et al. 2005). Alternative splicing of Dscam can lead to the production of more than 30,000 isoforms with distinct extracellular domains. Therefore, it can be postulated that Dscam is able to supply a great number of pathogen recognition receptors (Williams M J. 2007), including opsonization factors (Lemaitre C. and Hoffmann J. 2007).

1.9 Coagulation

In insects, coagulation of wound is a critical process to limit hemolymph loss and for the onset of the wound healing, and represent an important immune defense, forming a barrier to infection immobilizing and killing bacteria. In *Drosophila*, soon after an injury, a clot of fibers trapping hemocytes is formed at the site of the wound. This reaction is independent of melanization, since a primary clot is still formed in mutants defective of the

prophenoloxidase enzyme. However, it is clear that a second step in wound closure include melanization and epithelial movements. *Hemolectin* is a specific plasmatocyte gene encoding a protein that is a major component of the clotting fibers of *Drosophila*, and is required for proper clotting (Goto A. et al. 2003). The silencing of this gene in hemocytes result in bleeding defects. Bleeding defects are also observed in larvae in which the *fondue* gene has been silenced by RNAi. Fondue is an abundant hemolymph protein regulated by the Toll pathway, not involved in the formation of the primary clot fibers, but in the following cross linking of these fibers (Scherfer C. et al. 2006). However, wounding of larvae defective in these two genes do not determine an increase in mortality compared to wounded controls, but lead to the formation of larger scrabs.

1.10 Encapsulation.

Encapsulation occurs when foreign bodies, too big to be phagocytized by plasmatocytes, are detected. The recognition of the parasitoid egg is exerted by plasmatocytes, which in turn generate a yet unknown signal that alerts the PSC in the lymph glands. Then, the PSC transduces the signal, via a yet unclarified pathway, to the medullary zone. This elicits a strong cellular response that leads to the release of hemocytes from the lymph glands (Williams M J. 2007), as well as from a subepidermal population of sessile hemocytes, which forms peculiar clusters in the posterior-most segments of larval cuticle. Recent studies have indeed assigned to these sessile population of hemocytes a role as a second hematopoietic organ (Marcus R. et al. 2009). Once plasmatocytes

have recognized the intruder, cytoskeletal modifications have to occur to allow these cells to adopt adhesive features and undergo a complete spreading process around the foreign body. This process includes the formation of septate junctions that physically separate the intruder from the hemocoel (Williams M.J. et al. 2006). Plasmatocytes adherence to the surface of the foreign body is followed by lamellocyte recognition, that leads these cells, through a yet unknown mechanism, to attach to the plasmatocytes surrounding the surface of the foreign body. The process ends with the melanization of the capsule, triggered by the rupture of the crystal cells which release their intracellular molecules giving rise to the prophenoleoxidase cascade (see 1.4). A key role in this process must be exerted by genes controlling adhesion features and cell shape changes. Accumulating evidences point in this direction. Williams et al., demonstrated that Rac1 and Rac2 are required for proper encapsulation of the eggs layed into *Drosophila* larval body by the parasitoid wasp *Leptopilina boulardi*. Rac1 and Rac2 encode for proteins belonging to the Rho-GTPase family, known to be involved in cytoskeleton modifications. In Rac2 mutants, plasmatocytes and lamellocytes correctly recognize and attach to the parasitoid egg, but fail to form septate junctions and thus fail to form a melanized capsule (Williams M.J. et al. 2005; 2006). Rac1 and *basket* (JNK homolog) are also involved in the encapsulation process, by regulating the formation of actin and focal adhesion kinase (FAK)-rich placodes in hemocytes (Williams M.J 2006). FAK are large dynamic protein complexes through which the cytoskeleton of cells connect to the extracellular matrix. In several cell types integrins are known to signal upstream of Rho GTPases, and this likely occurs also in hemocytes. Actually, several evidences point to a role for

mysospheroid (*mys*) in proper encapsulation. *Mys* encodes an integrin subunit, and in *mys* mutant flies lamellocytes differentiate correctly, but are unable to attach to the wasp egg (Irwin P. et al. 2005). The molecular pathways involved in the encapsulation process still have to be clarified. A genome wide study based on microarray analysis identified several genes whose expression level is modulated by wasp infection: their function may be further investigated to achieve a detailed understanding of encapsulation mechanism. Interestingly, both TOLL and JAK/STAT pathways were found to be up-regulated after parasitization. These data fit with independent findings showing that Toll and *hopschotch* (encoding the JAK kinase) are necessary for proper encapsulation (Sorrentino R.P. et al. 2004).

1.11 Melanotic tumor mutants

Mutations leading to constitutive activation of *hopschotch*, as well as gain-of-function mutations in Toll or loss-of-function mutations in *cactus* (coding for a I κ B homologue), cause hyperproliferation of circulating and lymph gland hemocytes and exhibit a melanotic tumor phenotype, characterized by the presence of melanized bodies (melanotic masses) free floating in the hemocoel or attached to internal organs. These masses resemble melanotic capsules that form around a wasp egg, as both contain multi-layers of melanized lamellocytes (Lemaitre B. and Hoffmann J. 2007). Several studies have clearly demonstrated the involvement of the cellular immune response in the formation of melanotic masses in larvae (Rizki and Rizki 1983; Qiu et al. 1998; Harrison et al. 1995). Mutants showing melanotic masses can be subdivided into two classes (Watson K.L. et

al. 1994). Mutants belonging to Class 1 form melanotic masses due to an auto-immune reaction resulting from an altered immune response or to a supposedly normal immune response to an abnormal target tissue. Rizki and Rizki postulated that rupture of the basement membrane that surrounds tissues can induce hemocyte adhesion and capsule assembly leading to the formation of melanotic masses. Indeed these authors demonstrated that *Drosophila* larvae encapsulate transplanted self tissues when they are mechanically damaged; in contrast, tissue fragments with intact basement membrane are not encapsulated (Rizky and Rizki. 1980). These data support an intriguing hypothesis: hemocytes could recognize foreign bodies due to the absence of a yet unknown factor present on insect own basement membrane (Lemaitre B. and Hoffmann J. 2007). Mutants belonging to Class 2 show over-proliferation of hemocytes that leads to capsule formations. Mutations in several genes of the JAK/STAT and TOLL pathways (see above) belong to this class. An alternative classification of melanotic mutants, based on a morphological and immunohistochemical characterization of melanotic masses, was proposed by Minakhina and Steward. The authors subdivided melanotic masses in *melanotic nodules*, engaging the hemocyte-mediated encapsulation, and in *melanizations* not encapsulated by hemocytes. Melanotic nodules are found free floating in the hemocoel or in association with the lymph gland, while melanizations are found in the gut, in salivary glands, in the cuticle and in the tracheae (Minakhina S. and Steward R. 2006).

*Materials
and
Methods*

2.1 **Culture of *Drosophila melanogaster***

Drosophila stocks are maintained at 25°C or 18°C and are cultured in plastic vials, with hydrofobic cotton wool as stopper. The fly food is composed by agar, sugar, yeast, maize meal, Nipagin (methyl hydroxy benzoate).

For 2L of water:

- 15g of agar
- 200g of sugar
- 100g of yeast
- 180g of maize meal
- 5g of nipagin melted in ethanol 96%

2.2 **Proper staging of larvae**

0,05% of Bromophenolblue was added to the fly food to visualize the gut content of larvae. Early- and late-wandering third-instar larvae can be distinguished by the presence or absence of food in the gut, respectively (Zettersal C J et al., 2004). Therefore, early-wandering larvae are identified by the presence of blue coloured gut, while late-wandering larvae are characterized by a white gut.

2.3 **Agar-apple juice plates**

For 200ml of water:

- 9g of agar
- 100ml of apple juice
- 5ml of 10% nipagine-ethanol.

2.4 Fly stocks

Drosophila strain w¹¹¹⁸ was obtained from Bloomington Stock Centre. UAS-CG2145^{IR} strain number 14874 was obtained from Vienna *Drosophila* RNAi Centre (VDRC). Collagene type IV(cg)-GAL4 strain was kindly provided by Prof. Giuseppe Gargiulo (Dipartimento di Biologia Evoluzionistica e Sperimentale, Bologna University). Flies were kept on a standard corn molasses meal diet at between 21-25°C. GAL4/UAS lines were crossed at 29°C.

2.5 Antibodies

Lamellocyte specific mouse monoclonal antibody (L1) and plasmatocyte specific monoclonal mouse antibody (NimrodC1) (kindly provided by Dr. Istvan Andó, Biological Research Center–Szeged–Hungary) were used undiluted. TRITC coniugated phalloidine (Sigma-Aldrich, MO, USA) and anti-mouse Texas red (Mol. Probes) were used according to manufacturer instructions. All samples were analysed using Leica DM6000 microscope. Images were acquired using a CCD Leica DFC380 and processed using Leica Application Software-Application Fluorescence (LAS-AF).

2.6 GAL4/UAS – mediated RNA interference

The system allows the rapid generation of individual strains in which ectopic expression of the gene of interest (the target gene) or, as in this case, of a sequence coding for a specific dsRNA, can be directed to different tissues or cell types (Brand and Perrimon. 1993). The method separates the target gene from its transcriptional activator in two distinct transgenic lines. In one line the target gene remains silent in the absence of its activator, in the second line the activator protein is present but has no target gene to activate. This ensures that the parental lines are viable. Only when the two lines are crossed, the target gene is turned on in the progeny, and the phenotypic consequences of misexpression (including lethality) can be conveniently studied. More in details, the system uses the yeast transcriptional factor GAL4 and its target upstream activated sequences (UAS), to which GAL4 binds to activate transcription. GAL4 can be easily expressed in several tissues just putting it under the control of distinct tissue-specific promoters. Because the UAS sequences are not present naturally in *Drosophila melanogaster*, the transgene will be transcribed only when GAL4 will be transcribed.

I crossed a transgenic line bearing on the second chromosome the insertion UAS-CG2145dsRNA (*transformant ID: 14874 generated by the Vienna Drosophila RNAi Center*) to the driver line collagen type IV (cg)-GAL4, which specifically drives the expression of the UAS-bearing line in hemocytes and fat body.

2.7 RNA extraction from larval hemocytes

Larvae of the proper stage were collected, washed in ethanol 96%, rinsed in water and dried on a tissue paper.

Larvae were then collected in a basket with a nylon mesh at the bottom (mesh size 40 μ m) and squashed in 0,5ml of buffered saline solution (PBS 1X) using the tip of a eppendorf tube .

The basket was washed twice with 0,5ml of PBS 1X.

PBS containing hemolymph was collected in a eppendorf tube and centrifuged at 100g x 10' at 4°C, to separate larval tissue debris.

The supernatant, containing hemocytes, was collected in a fresh tube and hemocytes were pelleted by spin down at 300g x 10' at 4°C.

RNA was extracted from hemocytes using TRIreagent (Sigma-Aldrich, MO, USA) according to manufacturer instructions. Briefly, hemocytes were lysed for 5' at room temperature and RNA was extracted in 20% chloroform solution. RNA was precipitated using 0,7 volume isopropanol and the resulting pellet was washed in 70% ethanol, resuspended in DEPC treated water and quantified using nanodrop 1000 (Thermo Scientific, DE, USA).

2.8 DNase treatment

To confirm absence of DNA contamination in RNA preparation, RNA samples were subjected to DNase treatment using Turbo DNase (Ambion, Austin, TX, USA) according to manufacturer instructions where one enzyme unit was used to digest DNA from 2 µg RNA for 30' at 37°C. The enzyme was inactivated by adding EDTA to a final concentration of 15mM and heating at 75°C for 10'.

2.9 cDNA synthesis

2 µg RNA was used as template for cDNA synthesis using M-MLV reverse transcriptase (Ambion, Austin, TX, USA) according to manufacturer instructions. The reaction was placed at 42°C for 1 hour and the enzyme was destroyed for 10 minutes at 95°C. Synthesis of the first cDNA strand was performed using random primers.

2.10 Real time PCR

Real-time quantitative RT-PCR was performed on Applied Biosystems 7900HT fast real time Quantitative Thermal Block using SYBR green chemistry and real-time fluorescence measurements. Gene specific primers of Dm-102 gene were designed for real-time PCR to amplify the mid-open reading frame area of Dm-102 based on the instructions provided by the thermal cycler producer with forward (5' TCCATCGACAGCGCAGATGA 3') and reverse (5' CAAGATGCGGCTGCTGTTCA 3'). Real-time RT-PCR was performed in SYBR green PCR master mix (Applied Biosystems, Life Technologies, CA, USA). The reaction mixture (20 μ l) consisted of 1X Fast SYBR green Master mix, 200 nM each of RT primers, and 20 ng of cDNA. The reaction was performed under the following conditions; one cycle of 20 seconds at 95°C for activation of AmpliTaq Fast DNA Polymerase, 40 cycles under 94°C for 1 sec and 20 sec under 60°C to allow annealing of the primers and extension of PCR. The RP49 gene was also performed on each sample as an internal control for equivalence of template with forward (5' GAGCTGGAGGTCCTGCTCAT 3') and reverse (5' GCGCTCGACAATCTCCTTGC 3') primers. Fluorescence values were measured and amplification plots were generated in real time by the Exicycler program. Quantitative analysis of Dm-102 transcript expression was done using the comparative CT (Δ CT) method (Livak *et al.*, 2001).

2.11 Immunofluorescence on circulating hemocytes

Bled hemocytes from larvae in 50µl PBS 1X on polylysine glass slides

Wait 5' to allow the hemocytes to attach to the glass slide

Replace Mead with Grace's medium 30' at RT (in a humid chamber from now on)

Fix cells 10' with 100µl 3,7% paraformaldehyde-PBS 1X

Wash 3 x 5' in PBS 1X

Add 50µl Blocking solution* : 30' at RT with agitation

Wash cells twice in PBS 1X x 5'

Incubate with primary antibody in 50µl Blocking solution O/N at 4°

Wash 3 x 5' in PBS 1X 0,1% Triton

Incubate secondary antibody in Blocking solution 30' at RT

Wash 4 x 5' in PBS 1X 0,1% Triton

Muont cells in Prolong Gold antifade reagent with Dapi (Invitrogen, Eugene, Oregon, USA)

*Blocking solution (PBS 1X, 10% Normal Goat Serum, 1% BSA, 0,1% Triton)

2.12 Immunofluorescence on melanotic masses

Open larvae on polylysine glass slides

Remove carcass

Fix tissues and melanotic masses at -80°C for several days

Allow to cool at room temperature and fix in 3,7% paraformaldehyde-PBS 1X for 10' at room temperature (in a humid chamber from now on)

Wash 3 x 5' in PBS 1X

Add 50µl Blocking solution* : 30' at RT with agitation

Wash cells twice in PBS 1X x 5'

Incubate with primary antibody in 50µl Blocking solution O/N at 4°

Wash 3 x 5' in PBS 1X 0,1% Triton

Incubate secondary antibody in Blocking solution 30' at RT

Wash 4 x 5' in PBS 1X 0,1% Triton

Muont cells in Prolong Gold antifade reagent with Dapi (Invitrogen, Eugene, Oregon, USA)

*Blocking solution (PBS 1X, 10% Normal Goat Serum, 1% BSA, 0,1% Triton)

2.13 Visualization of GFP expressing hemocytes *in vivo*

Larvae were washed in water and anesthetized with ether.

Then were transferred in a drop of PBS 1X on an object slide and observed under a Leica DM6000 microscope. Images were acquired using a CCD Leica DFC380 and processed using Leica Application Software-Application Fluorescence (LAS-AF).

Results

3.1 Identification of a putative *Drosophila* homolog of the *H.virescens* 102 gene

Our lab is carrying out an extensive characterization of the virulence factors encoded by the polydnavirus (*TnBV*) associated with *Toxoneuron nigriceps* (Viereck) (Hymenoptera, Braconidae), an endophagous parasitoid of *Heliothis virescens* (Lepidoptera, Noctuide) larvae. A host gene, named 102, was identified in the frame of this work as the potential target of a small non-coding *TnBV* transcript. The 102 gene is highly expressed in hemocytes and is likely involved in the immune response (Falabella et al., unpublished data)

BLAST analyses, using as query the sequence of the putative translation product of the 102 gene, identified related proteins in both invertebrate and vertebrate species (see Table A, p.62). Two of them, sharing with the *Heliothis virescens* P102 protein 38% and 34% identity respectively, were *Drosophila melanogaster* proteins with unknown function (Table A). RT-PCR experiments demonstrated that the gene (CG2145) coding for the *Drosophila* protein most closely related to P102 was highly expressed in hemocytes (Fig. 9), like its *Heliothis virescens* counterpart.

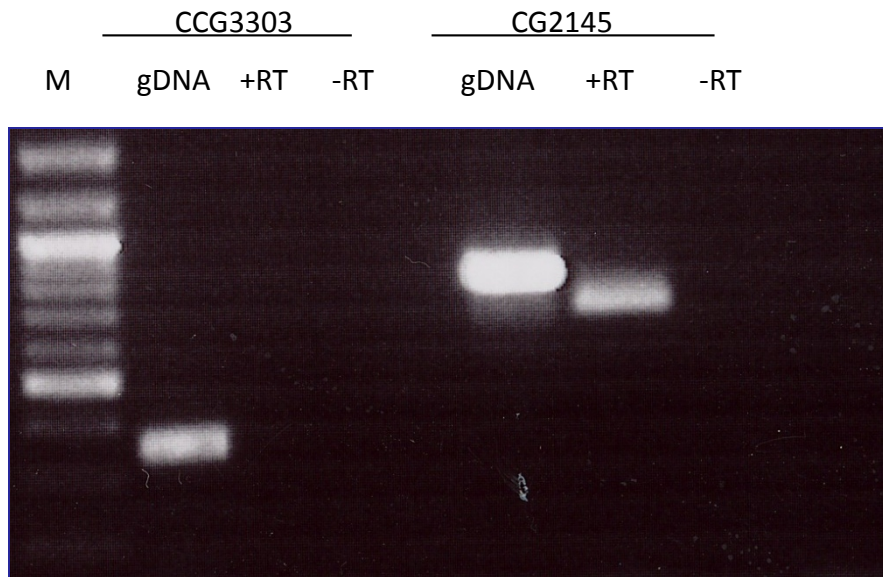


Fig.9: RT-PCR. Expression of CG2145 and CG3303 genes in *Drosophila* larval hemocytes. CG2145 is highly expressed in hemocytes. (gDNA: positive control using as template *Drosophila* genomic DNA; +RT: reverse transcribed RNA; -RT: negative control, containing all components of the reverse transcriptase reaction except the enzyme).

Moreover, as reported in the Flyatlas database, (<http://www.flyatlas.org/>), the gene is strongly expressed also in the larval fat body (Table B, p.67).

3.2 RNA interference on Dm-102 gene

Drosophila melanogaster is a powerful model organism for studying innate immunity and offers a wide range of molecular genetic tools not available in other model systems. In addition, it is recognized that basic physiological processes are evolutionarily conserved between insects. We therefore decided to use *Drosophila melanogaster* as an experimental model system and started a

functional analysis of the CG2145 gene, re-named Dm-102, mainly focusing on its potential role in the immune response. I took advantage of the well established Gal4/UAS binary system to silence the expression of this gene in immuno-competent tissues (i.e. hemocytes and fat body) of *Drosophila melanogaster* (Fig. 10).

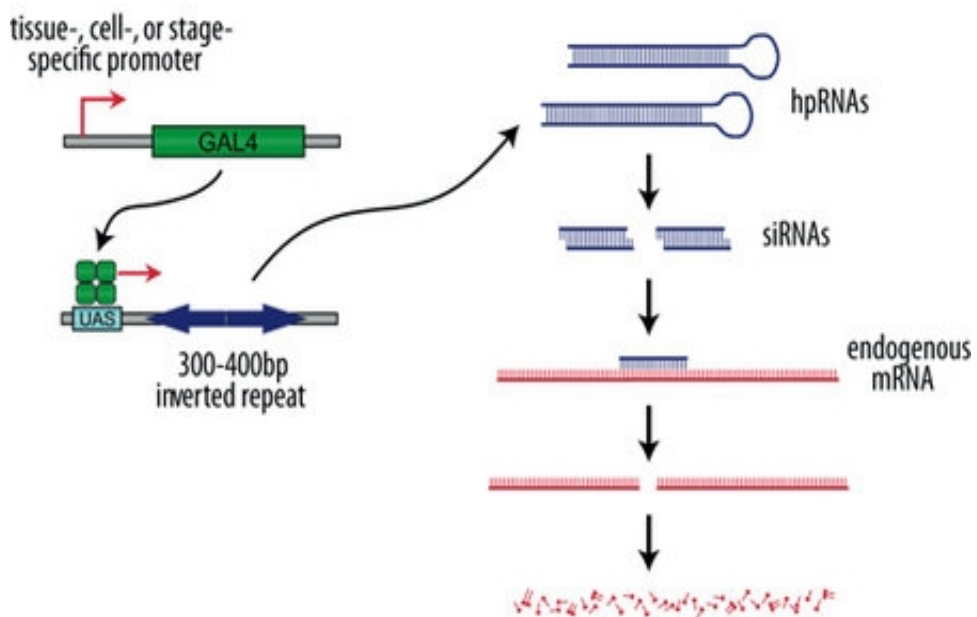


Fig. 10: The GAL4/UAS system can be used to drive the expression of hairpin RNA molecules (hpRNAs). These double-stranded RNAs are processed into siRNAs which direct sequence-specific degradation of the target mRNA.

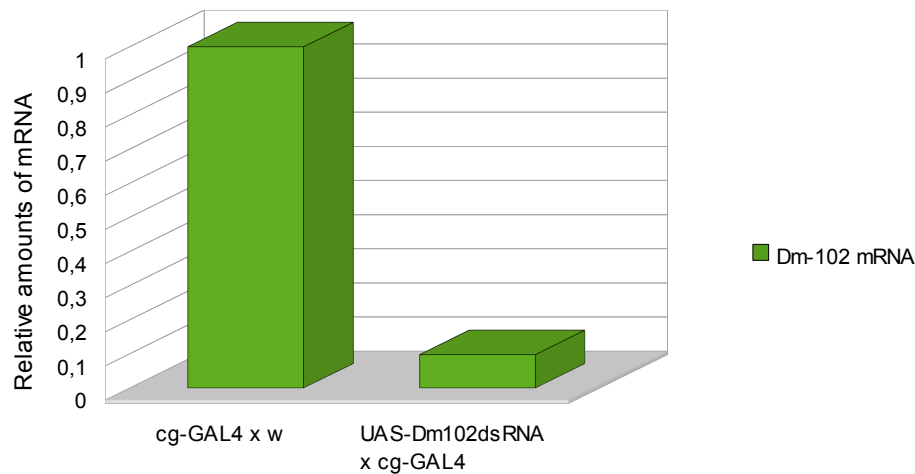


Table 1: Relative quantification of Dm-102 mRNA using qRT-PCR. RNA samples were extracted from hemocytes of early third instar wandering larvae deriving from the indicated crosses. Real Time PCR was performed using SYBR green technology. Data analysis was performed using ΔC_t method (Livak et al., 2001).

To silence the Dm-102 gene specifically in hemocytes and fat-body, females of a “target” line bearing a synthetic construct directing the transcription, under the control of the yeast UAS sequence, of a hairpin RNA targeting the Dm-102 transcript, were crossed with males of a “driver” line expressing the yeast GAL4 protein under the control of the hemocyte/fat-body specific collagene-type IV promoter (cg-Gal4). The standard rearing temperature was 29°C, since the optimal expression of the GAL4-UAS system is at this temperature. Relative quantification of Dm-102 mRNA using qRT-PCR confirmed the reduced expression of the gene in hemocytes extracted from larvae deriving from this cross (Table 1). These

individuals were analyzed phenotypically.

3.3 Viability test and lethal phase identification

First of all I evaluated the potential impact of the tissue-specific RNA interference directed against the Dm-102 gene in fat body and hemocytes on viability and found a dramatic lethal effect, since virtually no adult flies were obtained from the test cross (data not shown). I therefore set up an experimental procedure to identify the developmental stage at which lethality occurred.

30 females of the target line (UAS-Dm102dsRNA) were crossed, at 29°C, with 20 males of the driver line (cg-GAL4). The eggs laid in a 4 hours time interval were collected on agar-apple juice plates, counted and allowed to develop at 29°C. The larvae emerged from hatched eggs were in turn counted, transferred in vials supplemented with fly food and left at 29°C to complete their development. Appropriate controls, in which either the cg-GAL4 or the UAS-Dm102dsRNA line were crossed with the w^{1118} strain, were treated in the same experimental conditions. This revealed that mortality was prominent in larvae and pupae. As shown in Table 2, embryos viability in the test cross (hereafter indicated as Dm-102i) had slightly reduced viability in comparison with embryos generated in control crosses (62% of Dm-102i embryos developed into larvae compared with 78% in cg-GAL4 x w^{1118} and 89% in UAS-Dm102dsRNA x w^{1118}). The negative effect on viability became more dramatic during larval development (8% survival in Dm.102i, compared with 93% in cg-GAL4 x w^{1118} and 79% in UAS-Dm102dsRNA x w^{1118}). Moreover, surviving larvae died during the pupal stage, with very few escapers emerging as adults (less than 1% in Dm-102i, compared with 87% in cg-GAL4 x w^{1118} and 86% in UAS-Dm102dsRNA x w^{1118}).

The obtained results were validated using the Pearson's chi-squared test; in Table 2 the statistically significant ($p < 0,0001$) differences are marked with an asterisk (contingency table: embryos $X^2=5,02$; $df=2$; $p=0,0813$; larvae $X^2=171,6$; $df=2$; $p < 0,0001$; pupae $X^2=189,5$; $df=2$; $p < 0,0001$).

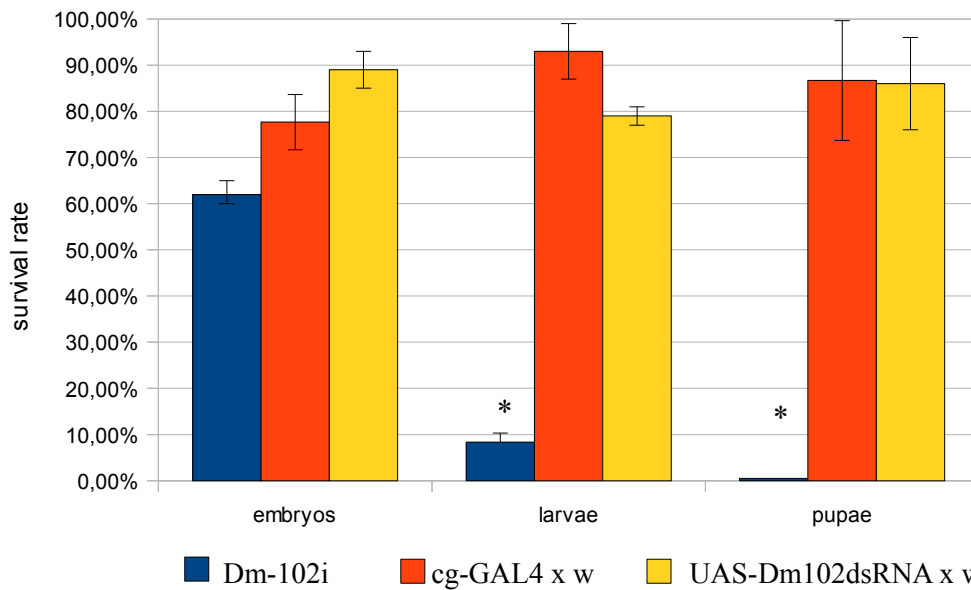


Table 2: Progeny development at 29°C (* = significant difference)

It should be noticed that, in the cg-GAL4 driver line, GAL4 is expressed in the embryonic hemocytes only starting from stage 13 and does not seem to be present in the developing fat body (Asha et al. 2002). Therefore, the slight effect observed on the viability of Dm-102i embryos, even if not statistically significant, may be a hint that the Dm-102 gene is required for embryonic development. Additional experiments will be needed for addressing this issue. However, the strong effect produced on viability by Dm-102

interference during larval and pupal development, indicates that Dm-102 gene plays an important function in immune competent tissues during these stages. This notion was supported by morphological analyses that revealed the presence of aberrant, dark masses in the hemocoel of Dm-102i larvae, which also appeared to be delayed in their development compared with controls (Fig. 11).

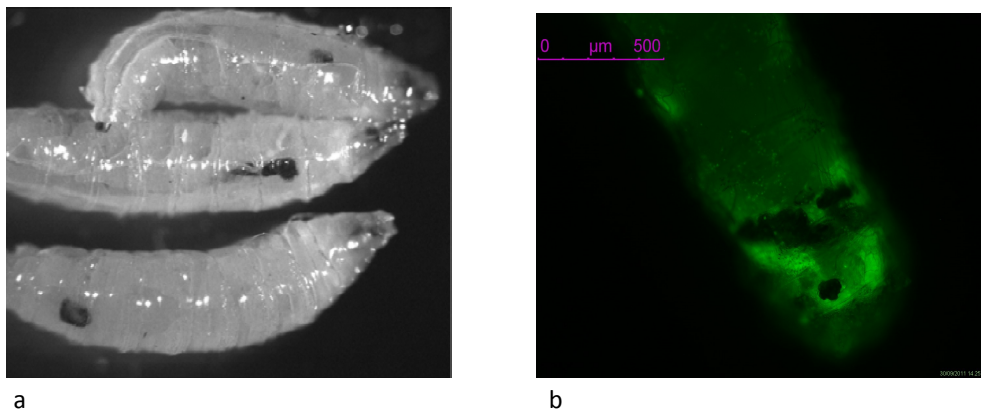


Fig. 11: Black melanotic masses formed in UAS-Dm-102dsRNAi/cg-GAL4; UAS-GFP larvae. a) Bright field. b) GFP filter.

3.4 Melanotic masses identification

Dark masses started to form in Dm-102i larvae during the mid-third larval instar and increased, both in number and size, over time. On a total number of 160 Dm-102 larvae analysed 8 days after egg-laying, 71% showed the presence of dark masses in the hemocoel. These masses were found free floating in the hemocoel and were located at different positions, with a preference for the posterior body region. They closely resembled melanotic cell clusters found in many tumorous-like mutants, which are in turn very similar to the

cellular capsules formed around non-self objects entering the hemocoel, like parasitoid eggs (Watson et al., 1991).

3.5 Hemocyte proliferation

Formation of melanotic masses is usually triggered by abnormal activation of the cellular immune response, but this is not always the case (Minakhina and Steward, 2006). To test whether the melanotic masses observed in Dm-102i larvae were associated with cellular immunity, I counted the number of circulating plasmatocytes and traced the presence of lamellocytes in the larval hemolymph. Since the number of circulating hemocytes increases during larval development, I paid special attention to proper larval staging. Early- and late-wandering third-instar larvae can be distinguished by the presence or absence of food in the gut, respectively (Zetterval C J et al., 2004). I therefore added a suitable stain to the fly food to allow visualization of the gut content and selected early third-instar larvae for hemocyte counts.

Dm-102i larvae showed a 3-fold increase in total plasmatocyte counts and the appearance of several lamellocytes, which were absent or very rare in control larvae (Fig. 12, 13, 14, 15). The observed increase of plasmatocyte number, as well as the occurrence of lamellocytes differentiation, resembled the cellular immune response of *Drosophila* larvae to parasitization by the hymenopteran parasitoid wasp *Leptopilina boulardi* (Figitidae) (Labrosse C. et al. 2004), supporting that melanotic masses formation in DM-102i larvae may be due to an autoimmune response.

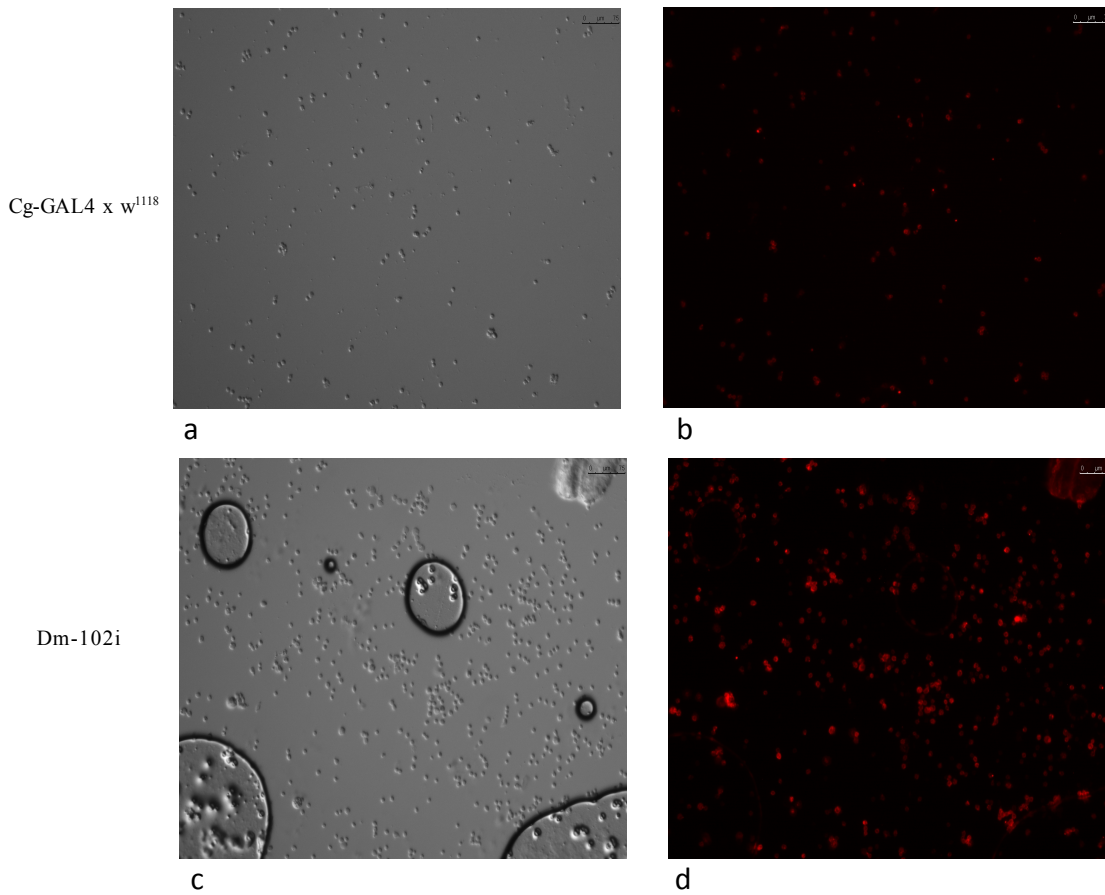


Fig. 12: Circulating hemocytes isolated from a control larva (generated by the *cg-GAL4 x w¹¹¹⁸* cross) and from a *Dm-102i* larva and stained with anti-NimC1 (red) to visualize plasmatocytes. a-c) Bright field. b-d) anti-NimC1. 10X magnification.

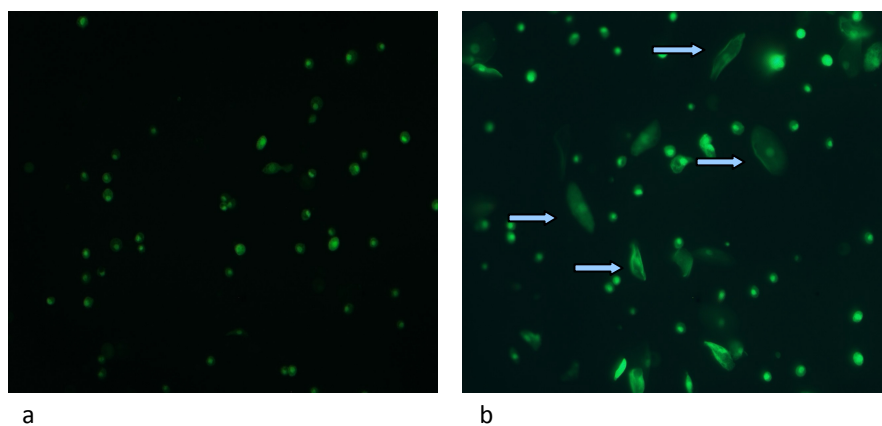


Fig. 13 : GFP expressing hemocytes isolated from early-wandering third instar larva. a) *cg-GAL4; UAS-GFP*. b) *UAS-Dm102dsRNA/cg-GAL4;UAS-GFP*. Note the presence of several lamellocytes (arrows) in b. 20X magnification.

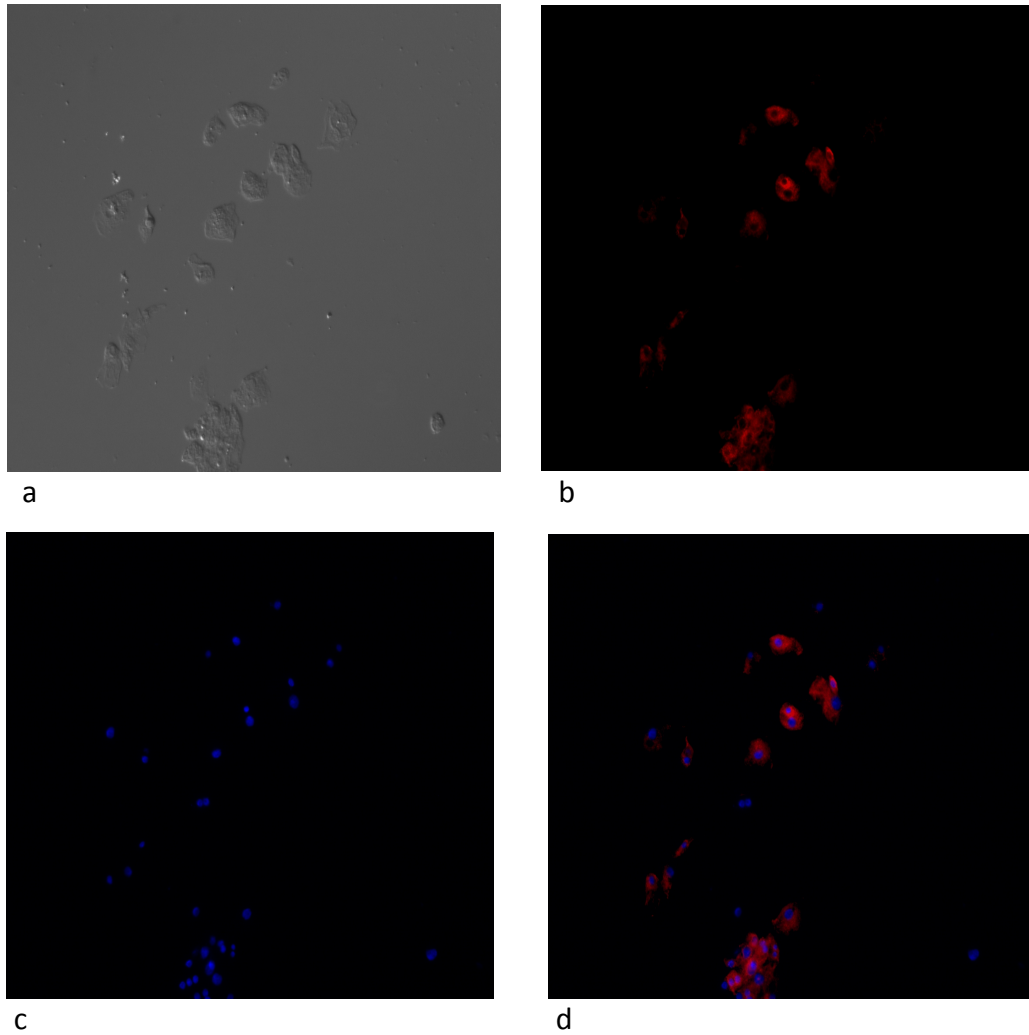


Fig. 14: Hemocytes isolated from a Dm-102i wandering third instar larva and stained with an antibody that specifically recognizes lamellocytes (anti-L1, red) and with Dapi to visualize nuclei (blue). a) Bright field. b) anti-L1. c) Dapi. d) b,c merge. 20X magnification.

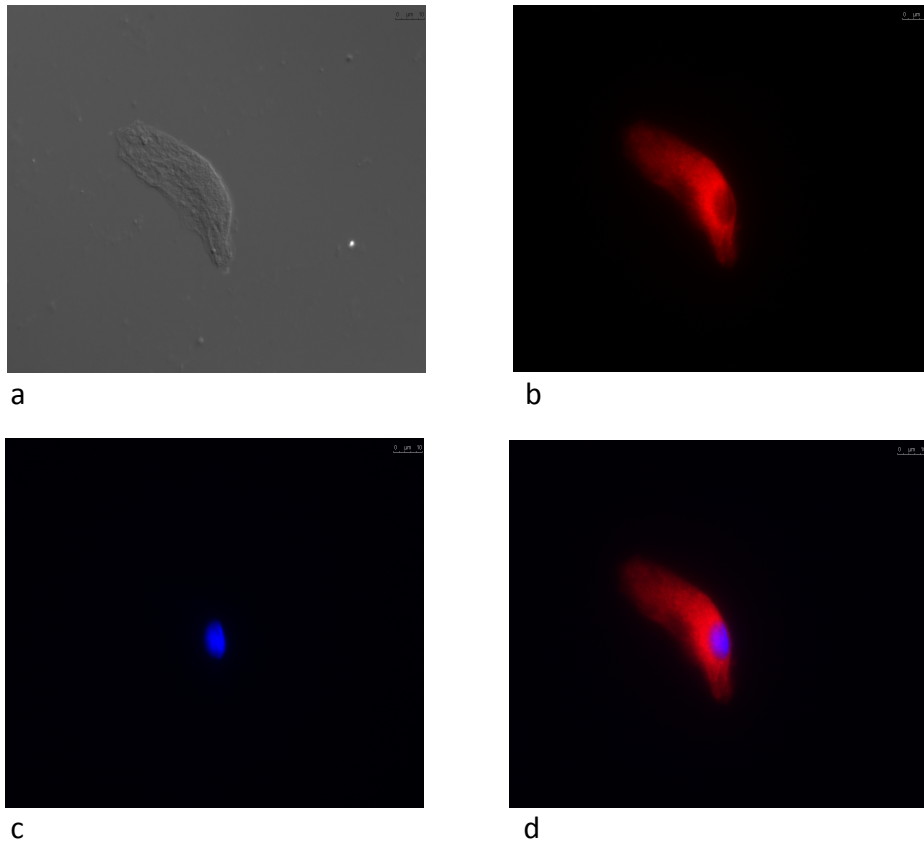


Fig. 15: Lamellocyte isolated from a Dm-102i third instar larva stained with the anti-L1 antibody, which specifically recognizes lamellocytes (red) and Dapi (blue) to visualize nuclei. a) Bright field. b) Anti-L1. c) Dapi. d) b,c merge. 63X magnification.

Immunostaining experiments on lymph glands extracted from early third instar larvae using the anti-NimC1, antibody that specifically recognizes plasmacytes, provided further support to this hypothesis, since, in Dm-102i larvae, NimC1⁺ positive cells were present not only in the lymph gland primary lobes, but also in all the secondary lobes (Fig. 16d,h), resembling hemocyte differentiation in lymph glands of immune challenged larvae (Crozatier M. et al. 2007). On the contrary, in control early third instar larvae, plasmacyte differentiation only occurred in the lymph gland primary lobes, with very few plasmacytes observed in the secondary lobes (Fig. 16c,g). Interestingly, several pericardial cells separating the lymph gland lobe appeared to be NimC1⁺ positive in Dm-102i larvae (16h, arrows). Further investigations are needed to shed light on the functional meaning of this observation.

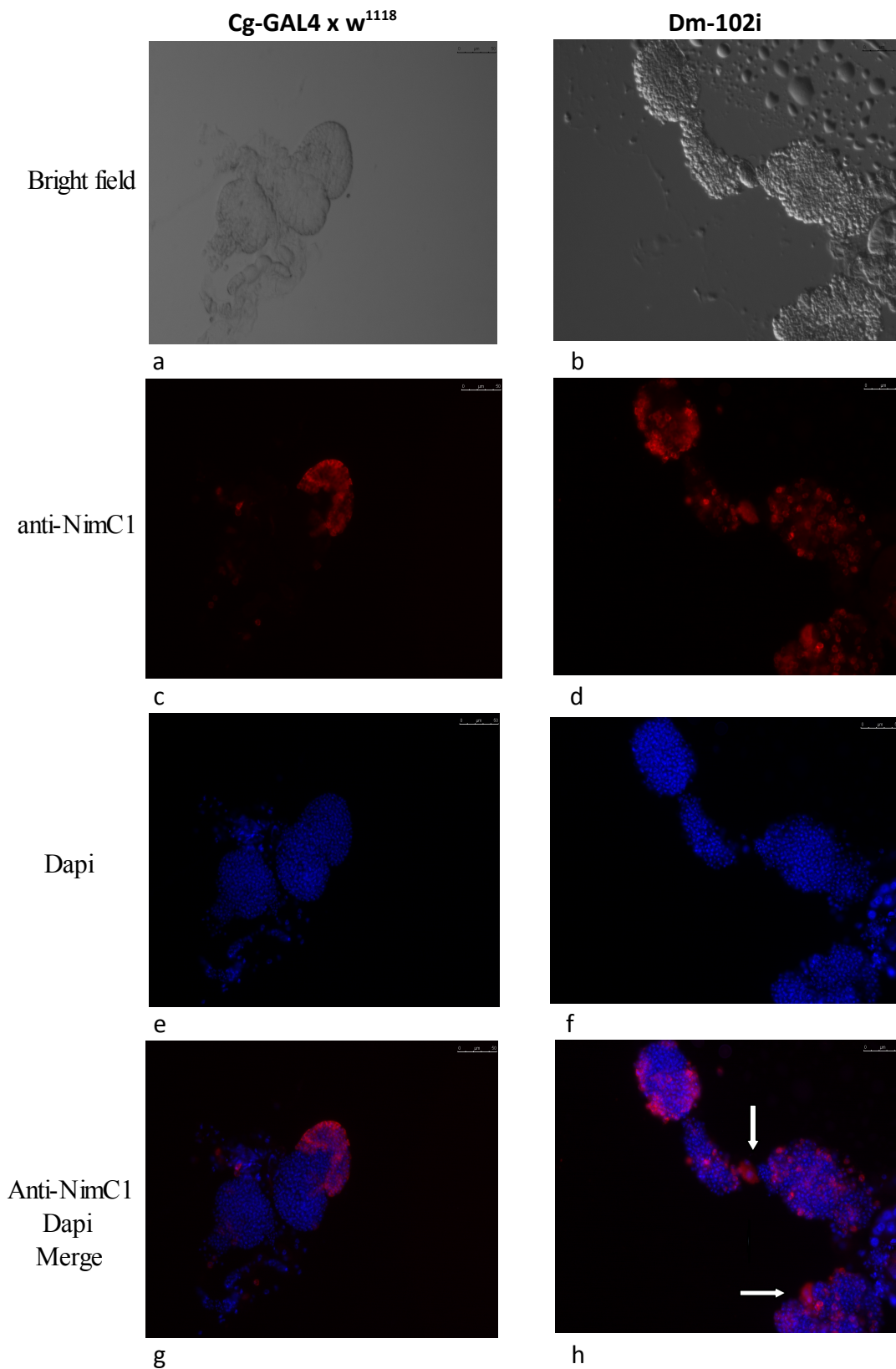


Fig. 16: Lymph gland extracted from early third instar larvae and stained with the anti-NimC1 antibody (red) to visualize plasmatocytes and with Dapi (blue) to highlight nuclei. (a,c,e,g) control larva (generated by the *cg-GAL4 x w¹¹¹⁸* cross); (b,d,f,h) *Dm-102i* larva. Arrows in h point to NimC1⁺ pericardial cells. 20X magnification.

3.6 Melanotic masses morphology

A classification of melanotic masses, based on a morphological and immunohistochemical characterization, was proposed by Minakhina and Steward (2006). The authors subdivided melanotic masses in *melanotic nodules*, engaging the hemocyte-mediated encapsulation, and in *melanizations* not encapsulated by hemocytes. Melanotic nodules are found free floating in the hemocoel or in association with the lymph gland, while melanizations are found in the gut, in salivary glands, in the cuticle and in the tracheae. Since the melanotic masses observed in Dm-102i larvae were found free floating in the hemocoel, they should be classified as melanotic nodules. They were highly variable in number, size, melanization level, and shape not only in different larvae but even within each single larva. To validate their classification as melanotic nodules I analyzed them for the presence of hemocytes on their surface. Therefore, I performed immunostaining experiments using antibodies that specifically recognize lamellocytes (anti-L1) or plasmatocytes (anti-NimC1). I arbitrarily divided the melanotic masses isolated into two groups depending on the extent of the melanized area. When single melanotic spots were present in a scattered pattern, both lamellocytes (Fig.17) and plasmatocytes (Fig. 18) were found on the surface of the dissected melanotic mass. On the contrary, when a complete melanized capsule was present around the melanotic mass, plasmatocytes were no more visible (Fig. 19). This was probably due to the masking effect exerted by thick and continuous layer of melanin on the fluorescent signal emanating from underlying cell layers. However, anti-L1 staining was still visible (Fig. 20). These findings indicate that the melanotic capsules found in Dm-102i larvae have the typical structure of the

immune capsules, where the external cell layers are formed by lamellocytes, while plasmatocytes constitute the innermost cell layer, in direct contact with the encapsulated target.

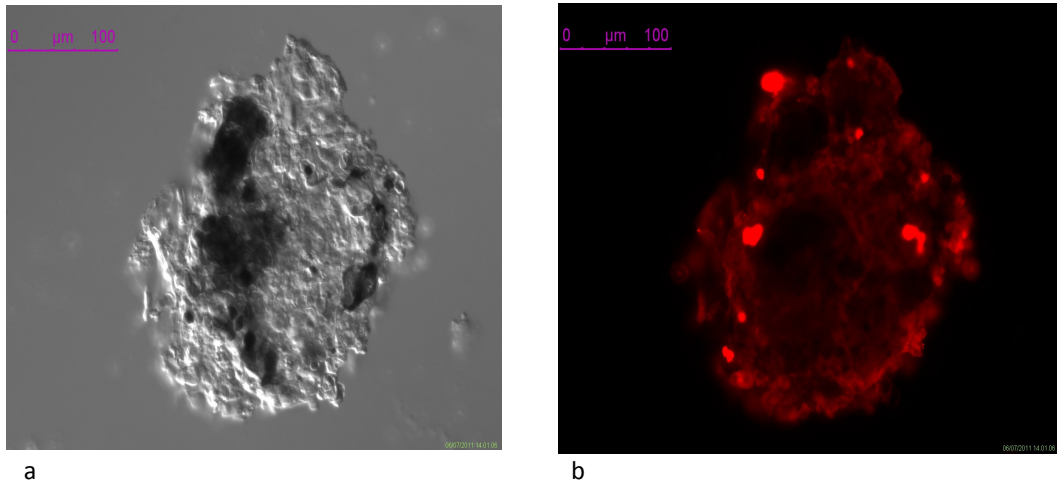


Fig. 17: Melanotic nodule with melanized spots isolated from a Dm-102i larva and stained with the anti-L1 antibody to visualize lamellocytes (red). a) Bright field. b) anti-L1.

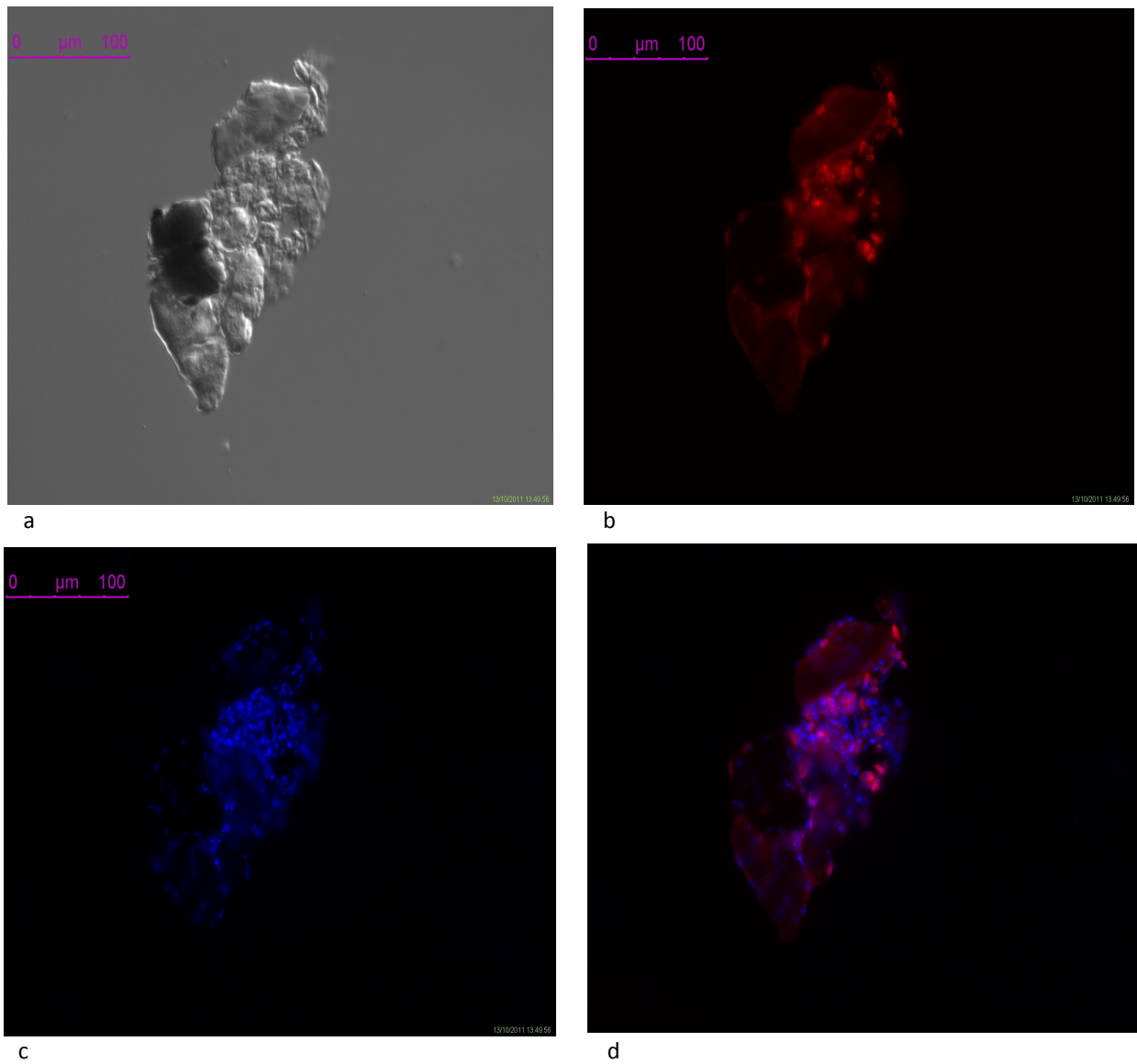


Fig. 18: Melanotic nodule with melanized spots isolated from a Dm-102i larva and stained with the anti-NimC1 antibody to visualize plasmatoytes (red) and with Dapi to highlight nuclei. a)Bright field. b) anti.NimC1. c) Dapi. d) b,c merge.

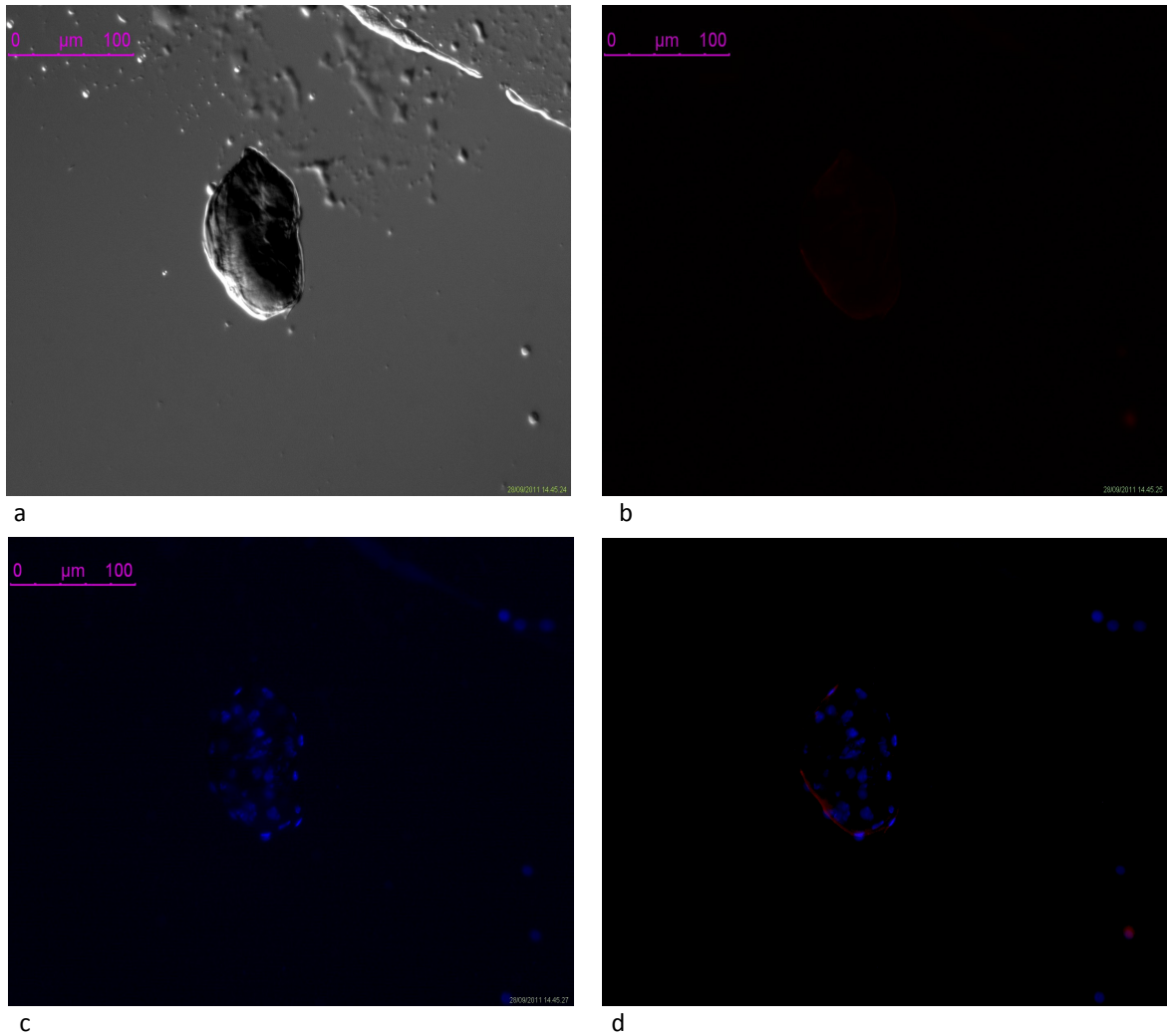


Fig. 19: Melanotic nodule showing a complete melanized capsule, isolated from a Dm-102i larva stained with anti-NimC1 to visualize plasmatocytes (red) and with Dapi to visualize nuclei (blue). a) Bright field. b) anti-NimC1. c) Dapi. d) b,c merge. No anti-NimC1 staining is detected in b.

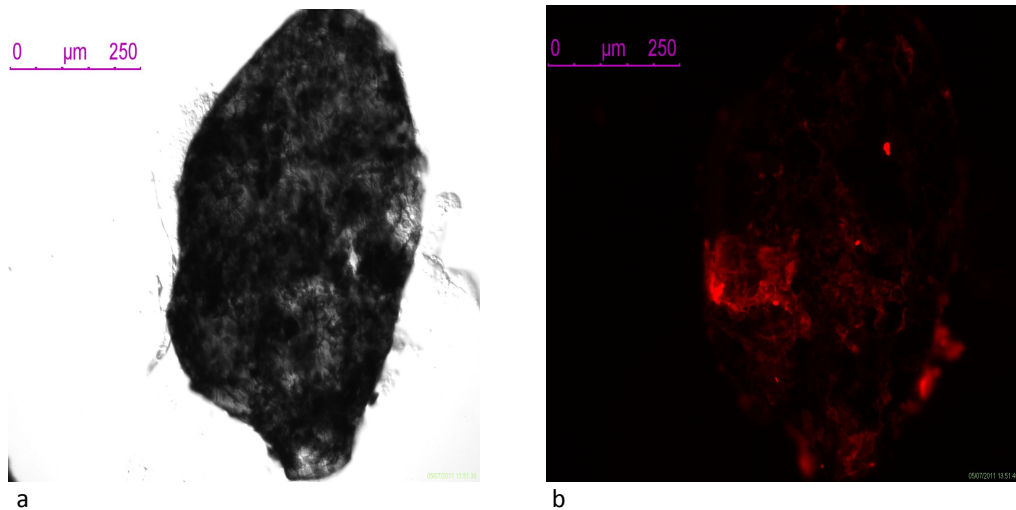


Fig. 20: Melanotic nodule showing a complete capsule isolated from a Dm-102i larva, stained with the anti-L1 antibody to visualize lamellocytes (red). a) Bright field. b) anti-L1.

3.7 Circulating hemocytes morphology

Immunofluorescence analyses performed on preparations of fixed circulating hemocytes from Dm-102i larvae, revealed the occasional presence of several large clusters of lamellocytes with different size and shape (Fig. 21). These clusters were easily lost during the immunostaining procedure, but were readily identified when the hemolymph was extracted from the larva in buffered saline solution and quickly observed in phase contrast microscopy (Fig.22). Interestingly, among the cells forming the clusters, I occasionally observed melanized hemocytes (arrows in Fig. 22).

The lamellocytes clusters observed in Dm-102i larvae resembled lamellocytes clumps described in blood cell samples from larvae of

melanotic tumor (tu) mutant strains (Rizki and Rizki. 1980; Rizki and Rizki. 1984; Rizki and Rizki. 1990; Lemaitre B. et al. 1995). Their presence indicated that lamellocytes have strong adhesive properties in Dm-102i larvae.

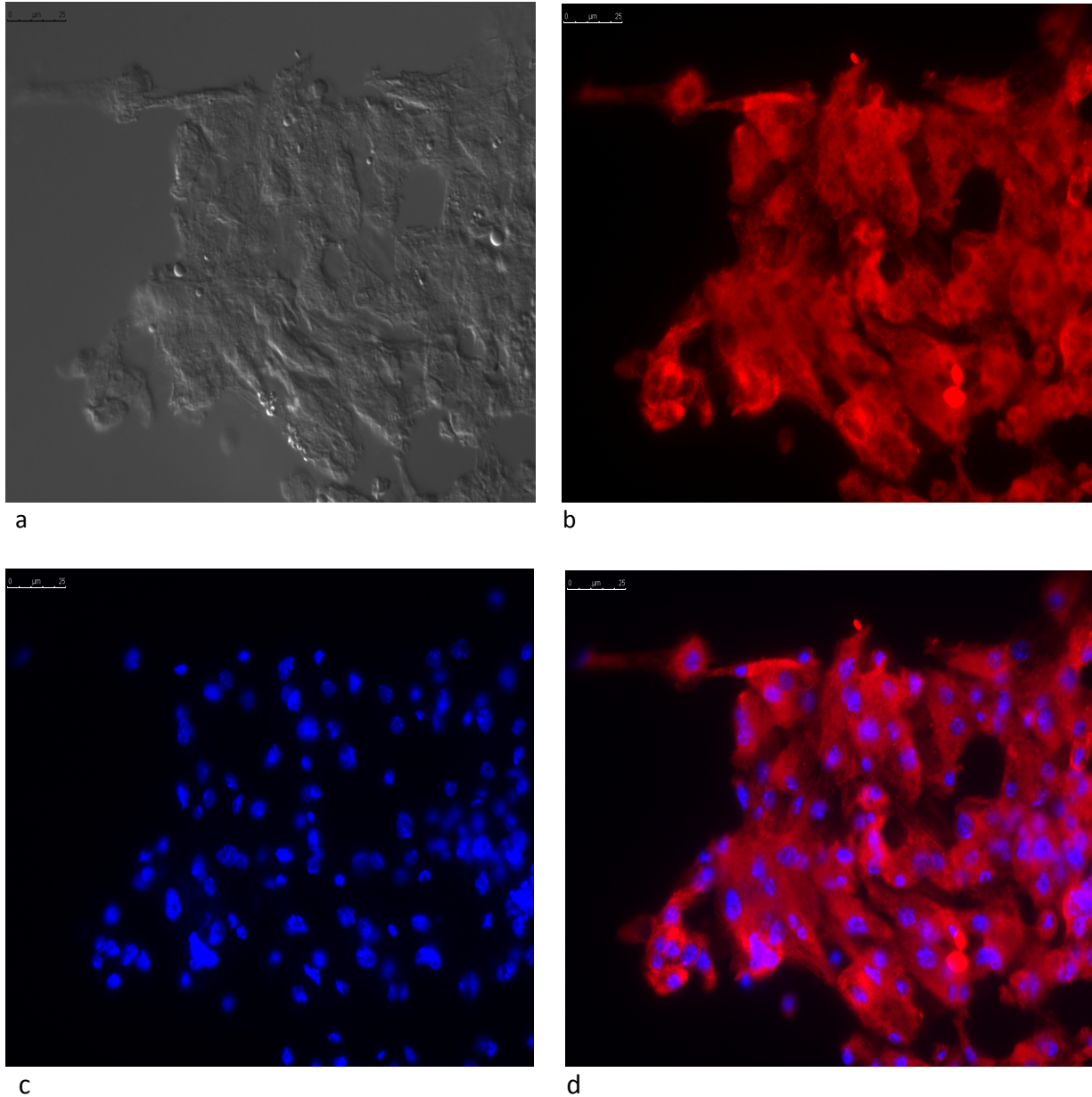
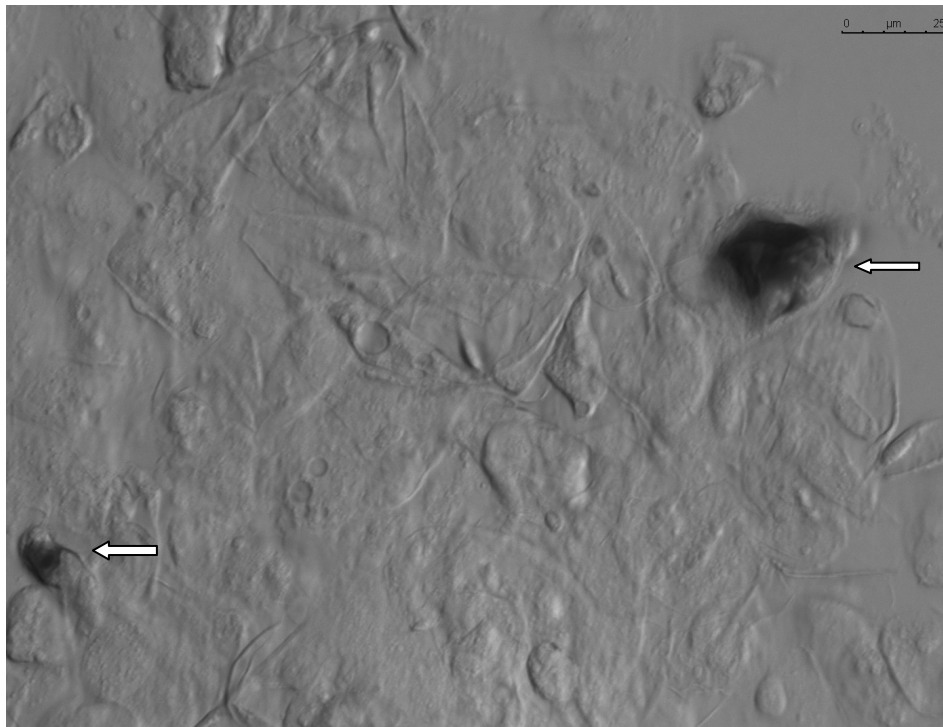
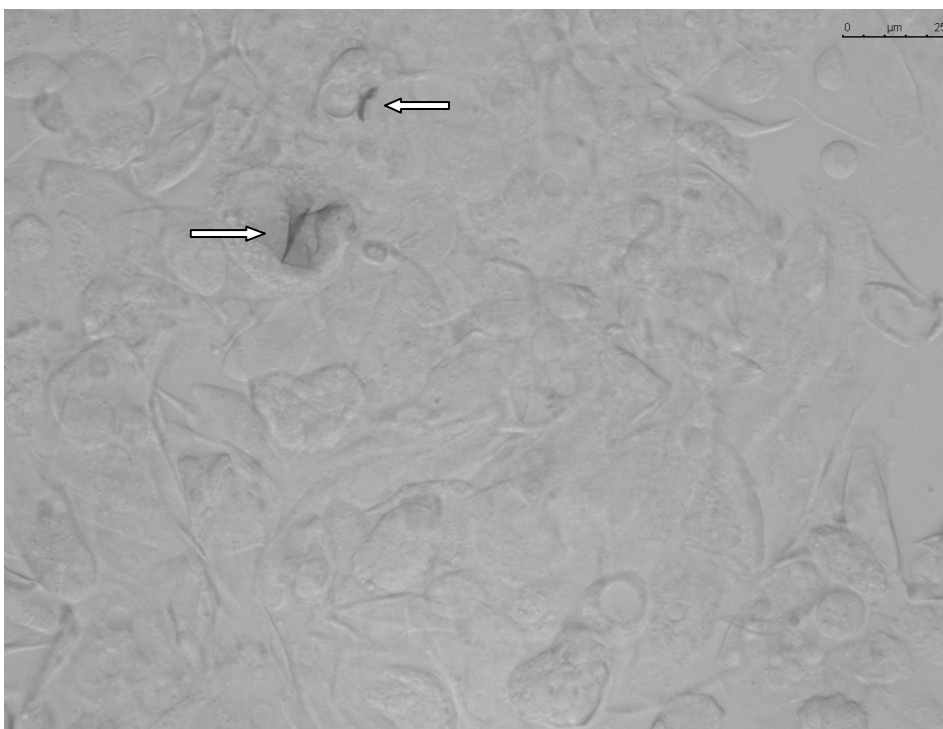


Fig. 21: Lamellocyte cluster isolated from a Dm-102i larva and stained with the anti-L1 antibody (red) to visualize lamellocytes and Dapi (blue) to visualize nuclei. a) Bright field. b) anti-L1. c) Dapi. d) b,c merge. 40X magnification



a



b

Fig. 22: a) and b) Lamellocyte clusters identified in unfixed cell preparations from Dm-102i larval hemolymph. Arrows point to melanizations. 40X magnification.

However, in Dm-102i larvae I also observed several lamellocytes with altered morphology. In particular, instead of showing the typical discoidal shape they displayed an elongated bipolar form (Fig. 23, 24). This closely resembled the phenomena observed in larvae parasitized by virulent wasps, where lamellocytes are targeted by virulent factors which alter their morphological and functional features (Rizki and Rizki. 1984; Rizki and Rizki. 1990).

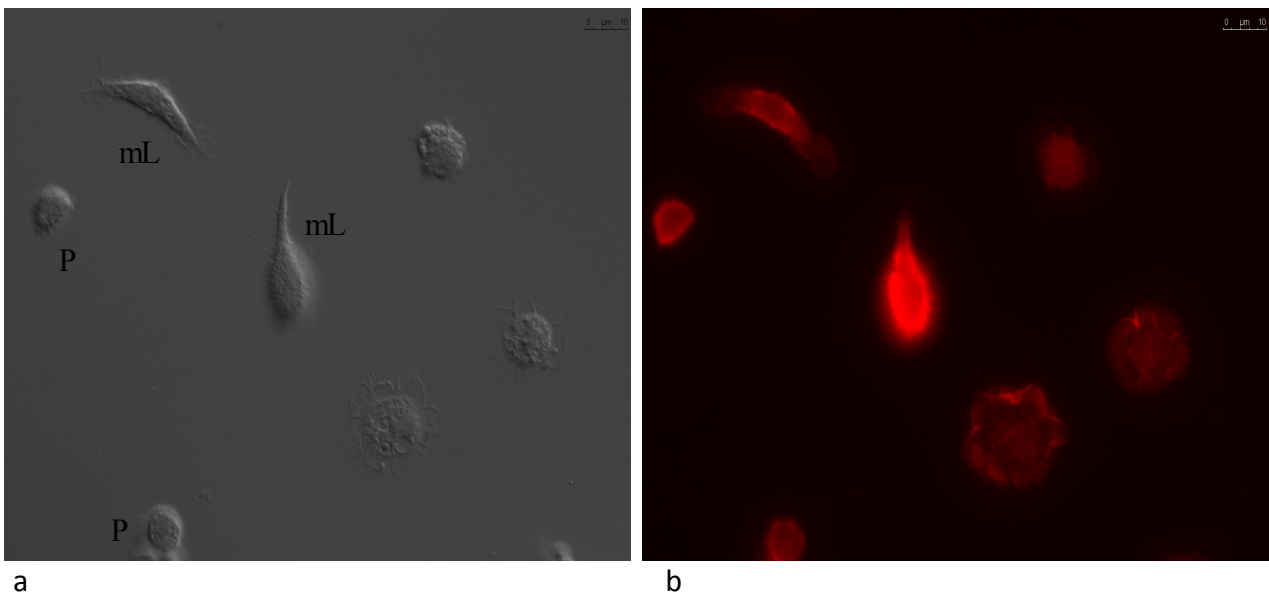


Fig. 23: Phalloidin staining to reveal the actin cytoskeleton (red) on circulating hemocytes from a third instar Dm- 102i larva.
a) Bright field. b)phalloidin staining. mL: modified lamellocyte;
P: plasmatocyte. 63X magnification.

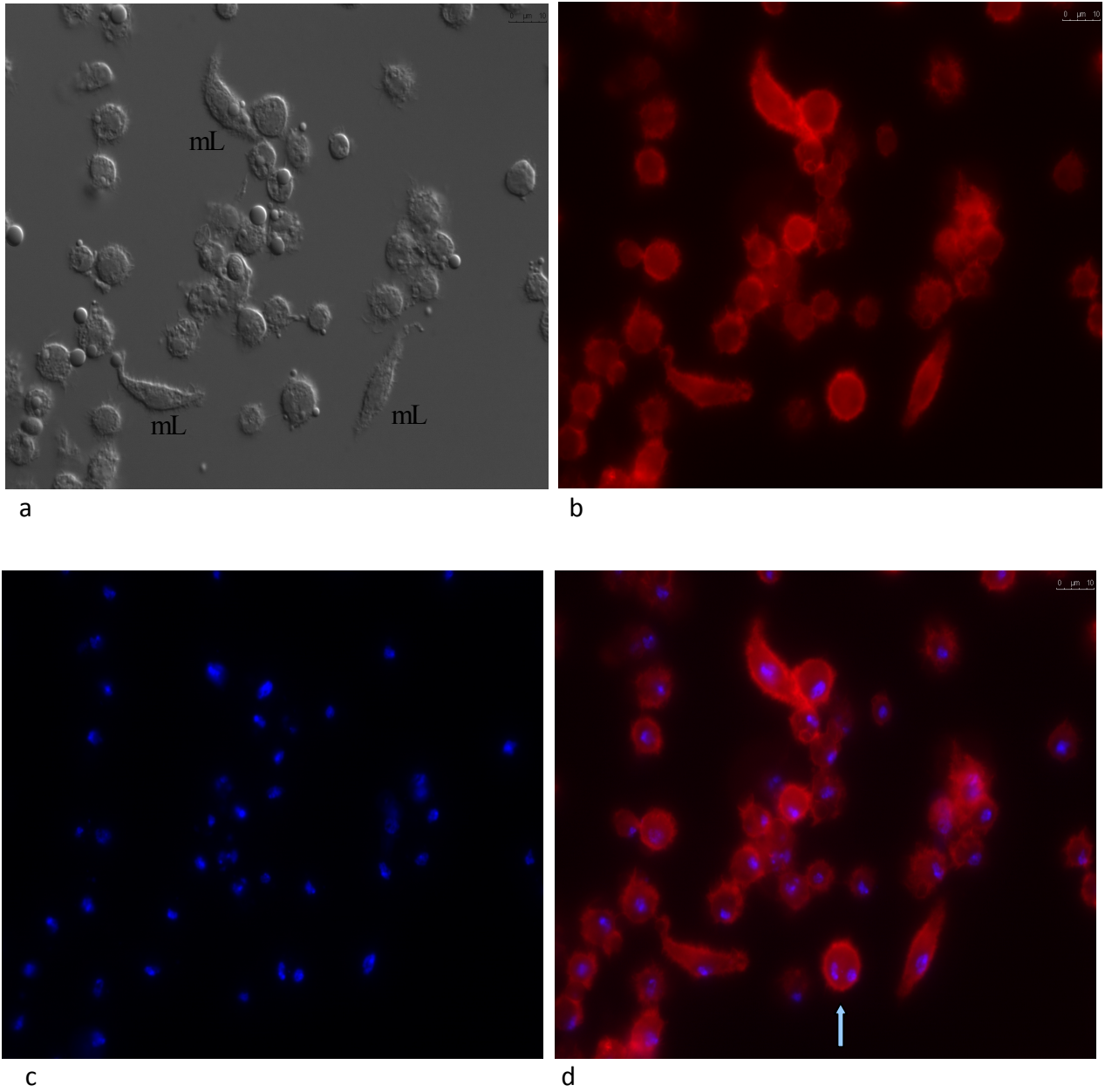


Fig. 24: Phalloidin staining on circulating hemocytes of a third instar Dm-102i larva. a) Bright field. b) Phalloidin staining. c) Dapi staining. d) b,c merge. mL: modified lamellocyte. Arrow point to a binucleate hemocyte. This abnormal feature is frequently observed in Dm-102i larvae. 63X magnification.

Immunofluorescence analyses on circulating hemocytes using the anti-NimC1 antibody that specifically recognizes plasmatocytes, identified large NimC1⁺ positive cells containing two nuclei, which may result from a defect in cytokinesis (Fig. 25, arrow. See also Fig. 24d, arrow)

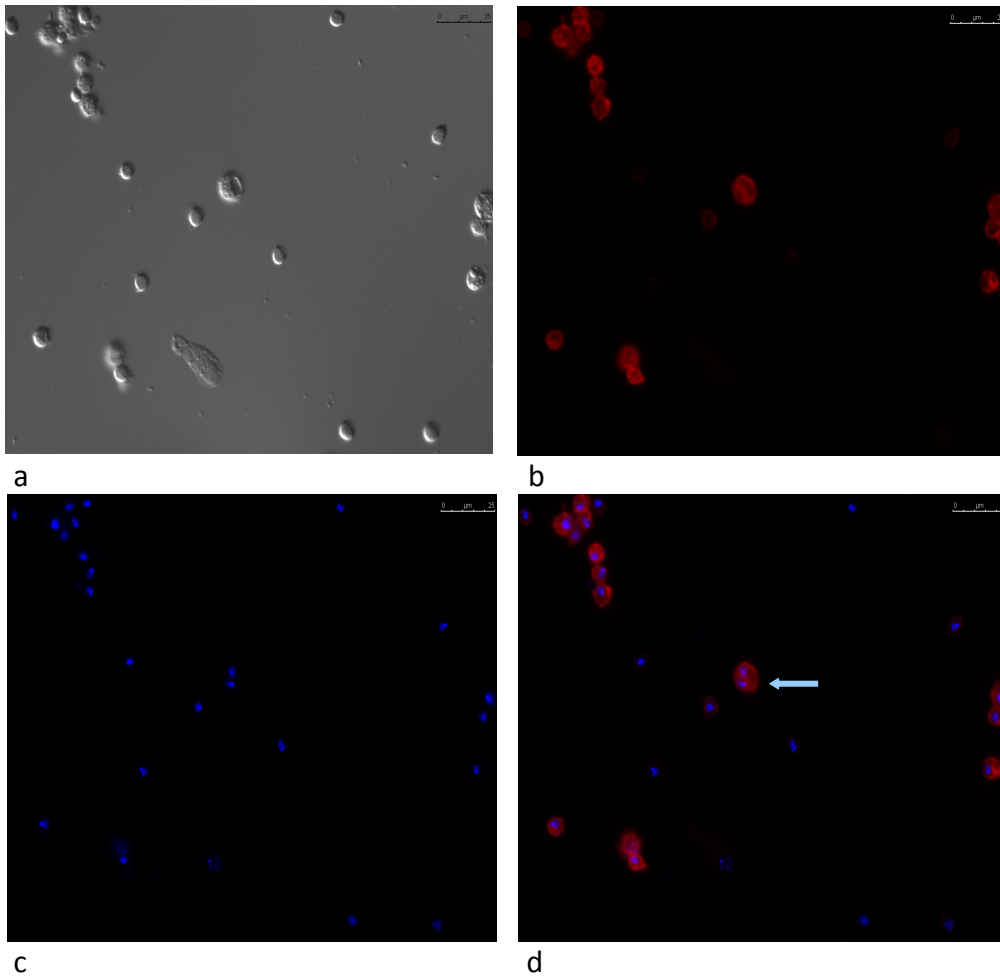


Fig. 25: Circulating hemocytes isolated from a third instar Dm-102i larva stained with the anti-NimC1 antibody (red) to visualize plasmatocytes and with Dapi to visualize nuclei. a) Bright field. b) anti-NimC1. c) Dapi. d) b,c merge. Arrow point to a binucleate plasmatocyte. 40X magnification.

3.8 Sessile hemocytes pattern

The results obtained so far have shown that Dm-102i larvae display a mutant phenotype that is usually associated with fly autoimmune disorders: formation of melanotic nodules freely floating in the hemolymph, hemocyte-mediated encapsulation, overproliferation of plasmatocytes and differentiation of lamellocytes. Generally lamellocytes are rarely seen in healthy larvae. As mentioned above, they are specialized for the encapsulation of invading pathogens that are too large to be engulfed by plasmatocytes, as well as for the encapsulation of damaged tissues (Rizki R.M. and Rizki T.M. 1980). The differentiation of lamellocytes correlates with the disappearance of a subepidermal population of sessile blood cells, which form a recently identified second hematopoietic compartment, in addition to lymph glands. These sessile hemocytes are arranged in two denser organ-like clusters in the posterior end of the larva (Kurucz E. et al. 2007; Marcus R. et al. 2009). In healthy larvae these clusters exhibit a well defined pattern (Fig. 26a), but an immune challenge, such as a parasitoid wasp attack, leads to the release of hemocytes and the clusters are no more visible. In the same manner, the posterior hemocyte clusters are disrupted in many melanotic mutants that cause overproliferation of hemocytes and differentiation of lamellocytes (Zetterval C.J. Et al. 2004). To test if also in Dm-102i larvae the posterior clusters were affected, I took advantage of a GFP reporter transgene that allowed me to visualize the hemocytes *in vivo*. As shown in Fig. 26b, in Dm-102i larvae the pattern of these clusters is disrupted and the posterior sessile hemocytes are no longer visible.

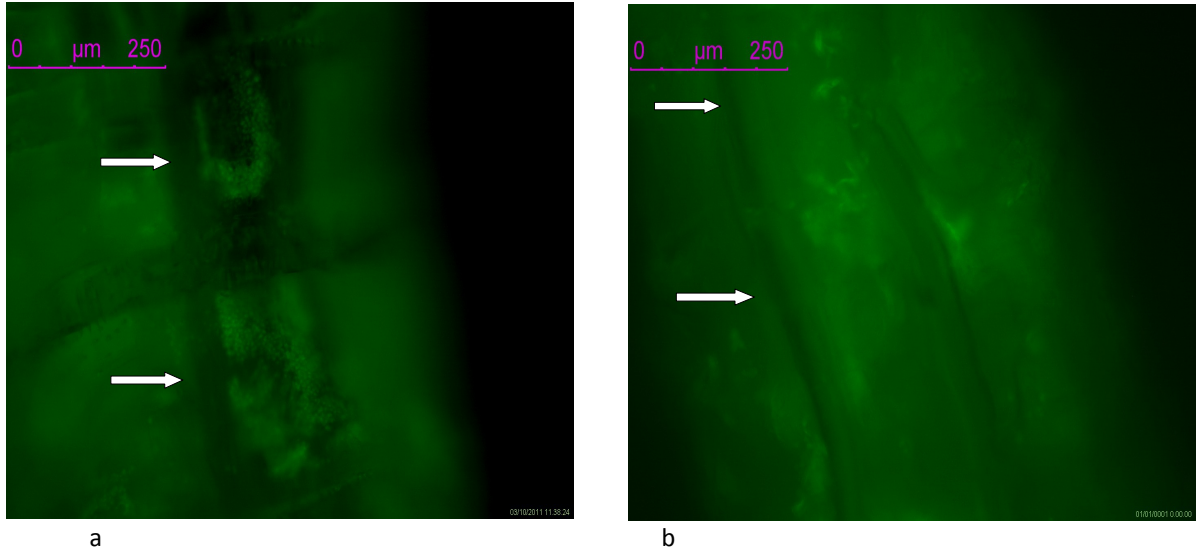


Fig. 26: Sessile hemocytes pattern in the larval body wall. a) Two posterior clusters of sessile hemocytes are clearly visible in a *cg-GAL4/+;UAS-GFP* larva (white arrows). b) The typical pattern of the posterior hemocyte clusters are disrupted in a *cg-GAL4/UAS-Dm102dsRNAi; UAS-GFP* larva; white arrows point to the area where posterior clusters should be.

Table A

Query: P102 *Heliotis virescence*

EHJ69917.1	putative serine protease-like protein 2 [Danaus plexippus]	649	649	82%	0.0	86%
AAV91433.1	putative serine protease-like protein 2 [Lonomia obliqua]	520	520	65%	0.0	86%
EHJ66341.1	hypothetical protein KGM_08584 [Danaus plexippus]	215	215	63%	1e-61	43%
EFZ16938.1	hypothetical protein SINV_06492 [Solenopsis invicta]	202	202	69%	5e-58	38%
EFA05915.1	hypothetical protein TcasGA2_TC008731 [Tribolium castaneum]	197	197	66%	2e-57	39%
XP_968069.1	PREDICTED: similar to CG2145 CG2145-PA [Tribolium castaneum]	195	195	66%	6e-56	39%
EFN80023.1	Placental protein 11 [Harpegnathos saltator]	197	197	63%	2e-55	40%
EFN80024.1	Placental protein 11 [Harpegnathos saltator]	199	199	69%	2e-54	39%
XP_001963598.1	GF20477 [Drosophila ananassae] >gb EDV44674.1 GF20477 [Drosophila ananassae]	194	194	62%	1e-53	39%
ADD19839.1	hypothetical conserved protein [Glossina morsitans morsitans]	192	192	62%	2e-53	38%
XP_003398251.1	PREDICTED: hypothetical protein LOC100644025 [Bombus terrestris]	194	194	58%	2e-53	41%
XP_001603664.1	PREDICTED: poly(U)-specific endoribonuclease homolog [Nasonia vitripennis]	187	187	62%	3e-53	40%
XP_002025808.1	GL18244 [Drosophila persimilis] >gb EDW32704.1 GL18244 [Drosophila persimilis]	191	191	62%	1e-52	37%
XP_003398082.1	PREDICTED: hypothetical protein LOC100642339 [Bombus terrestris]	193	193	63%	2e-52	41%
XP_001606738.1	PREDICTED: hypothetical protein LOC100123127 isoform 1 [Nasonia vitripennis]	192	192	58%	2e-52	40%
XP_003425281.1	PREDICTED: hypothetical protein LOC100123127 isoform 2 [Nasonia vitripennis]	192	192	63%	2e-52	38%
XP_003486024.1	PREDICTED: hypothetical protein LOC100746004 [Bombus impatiens]	191	191	58%	4e-52	41%
XP_002106640.1	GD16994 [Drosophila simulans] >gb EDX17598.1 GD16994 [Drosophila simulans]	190	190	62%	6e-52	39%
XP_003489126.1	PREDICTED: hypothetical protein LOC100746337 [Bombus impatiens]	192	192	62%	6e-52	41%
XP_001354551.2	GA15266 [Drosophila pseudoobscura pseudoobscura] >gb EAL31604.2 GA15266 [Drosophila pseudoobscura pseudoobscura]	191	191	62%	7e-52	37%
EFN64513.1	Placental protein 11 [Camponotus floridanus]	188	188	62%	1e-51	39%
XP_002010982.1	GI16258 [Drosophila mojavensis] >gb EDW05824.1 GI16258 [Drosophila mojavensis]	190	190	62%	2e-51	39%
XP_002056809.1	GJ16723 [Drosophila virilis] >gb EDW62295.1 GJ16723 [Drosophila virilis]	189	189	62%	2e-51	38%
XP_001977186.1	GG18381 [Drosophila erecta] >gb EDV46113.1 GG18381 [Drosophila erecta]	188	188	62%	3e-51	39%
EFX76267.1	Endoribonuclease-like protein [Daphnia pulex]	179	179	64%	1e-50	39%
NP_572668.1	CG2145 [Drosophila melanogaster] >sp Q9VZ49.1 ENDOU_DROME RecName: Full=Poly(U)-specific endoribonuclease homolog; AltName: Full=Protein endoU; AltName: Full=Uridylate-specific	187	187	62%	1e-50	38%

	endorigonuclease homolog; Flags: Precursor >gb AAAF47979.1 CG2145 [Drosophila melanogaster]						
XP_625112.2	PREDICTED: hypothetical protein LOC552734 [Apis mellifera]						
XP_003396146.1	PREDICTED: poly(U)-specific endorigonuclease homolog [Bombus terrestris]	177	177	63%	2e-49	34%	
XP_003251941.1	PREDICTED: poly(U)-specific endorigonuclease homolog [Apis mellifera]	175	175	62%	3e-49	36%	
XP_001991480.1	GH12680 [Drosophila grimshawi] >gb EDW00105.1 GH12680 [Drosophila grimshawi]	183	183	62%	8e-49	37%	
ACJ64345.1	endorigonuclease XendoU [Culex tarsalis]	173	173	60%	9e-49	39%	
XP_001607440.2	PREDICTED: hypothetical protein LOC100123738 [Nasonia vitripennis]	182	182	63%	1e-48	37%	
XP_002041919.1	GM11444 [Drosophila sechellia] >gb EDW45767.1 GM11444 [Drosophila sechellia]	179	179	62%	8e-48	38%	
XP_002100929.1	GE15899 [Drosophila yakuba] >gb EDX02037.1 GE15899 [Drosophila yakuba]	179	179	57%	8e-48	38%	
XP_311978.5	AGAP002925-PA [Anopheles gambiae str. PEST] >ref XP_003436311.1 AGAP002925-PB [Anopheles gambiae str. PEST] >ref XP_003436312.1 AGAP002925-PC [Anopheles gambiae str. PEST] >gb EAA07623.5 AGAP002925-PA [Anopheles gambiae str. PEST] >gb EGK96701.1 AGAP002925-PB [Anopheles gambiae str. PEST] >gb EGK96702.1 AGAP002925-PC [Anopheles gambiae str. PEST]	177	177	62%	1e-47	38%	
XP_001864562.1	endoU protein [Culex quinquefasciatus] >gb EDS40407.1 endoU protein [Culex quinquefasciatus]	174	174	63%	2e-46	39%	
XP_002017040.1	GL22078 [Drosophila persimilis] >ref XP_002137707.1 GA27370 [Drosophila pseudoobscura pseudoobscura] >gb EDW34140.1 GL22078 [Drosophila persimilis] >gb EDY68265.1 GA27370 [Drosophila pseudoobscura pseudoobscura]	168	168	62%	5e-46	36%	
XP_001660282.1	endoU protein, putative [Aedes aegypti] >gb EAT38484.1 endoU protein, putative [Aedes aegypti]	171	171	63%	4e-45	38%	
EFR21399.1	hypothetical protein AND_17100 [Anopheles darlingi]	171	171	58%	6e-45	39%	
XP_002070081.1	GK11216 [Drosophila willistoni] >gb EDW81067.1 GK11216 [Drosophila willistoni]	165	165	62%	7e-45	34%	
XP_002031000.1	GM24268 [Drosophila sechellia] >gb EDW41986.1 GM24268 [Drosophila sechellia]	164	164	62%	1e-44	34%	
XP_001980118.1	GG16962 [Drosophila erecta] >gb EDV49076.1 GG16962 [Drosophila erecta]	164	164	62%	1e-44	35%	
NP_650508.1	CG3303 [Drosophila melanogaster] >gb AAK93334.1 LD39912p [Drosophila melanogaster] >gb AAF55248.2 CG3303 [Drosophila melanogaster] >gb AAN71399.1 RE41114p [Drosophila melanogaster] >gb ACL85673.1 CG3303-PA [synthetic construct] >gb ACL90515.1 CG3303-PA [synthetic construct]	164	164	62%	2e-44	34%	
XP_001999656.1	GI22951 [Drosophila mojavensis] >gb EDW15117.1 GI22951 [Drosophila mojavensis]	161	161	62%	3e-43	34%	
XP_001954992.1	GF18549 [Drosophila ananassae] >gb EDV43553.1 GF18549 [Drosophila ananassae]	160	160	62%	6e-43	35%	
XP_002097677.1	GE24349 [Drosophila yakuba] >gb EDW97389.1 GE24349 [Drosophila yakuba]	155	155	62%	4e-41	33%	

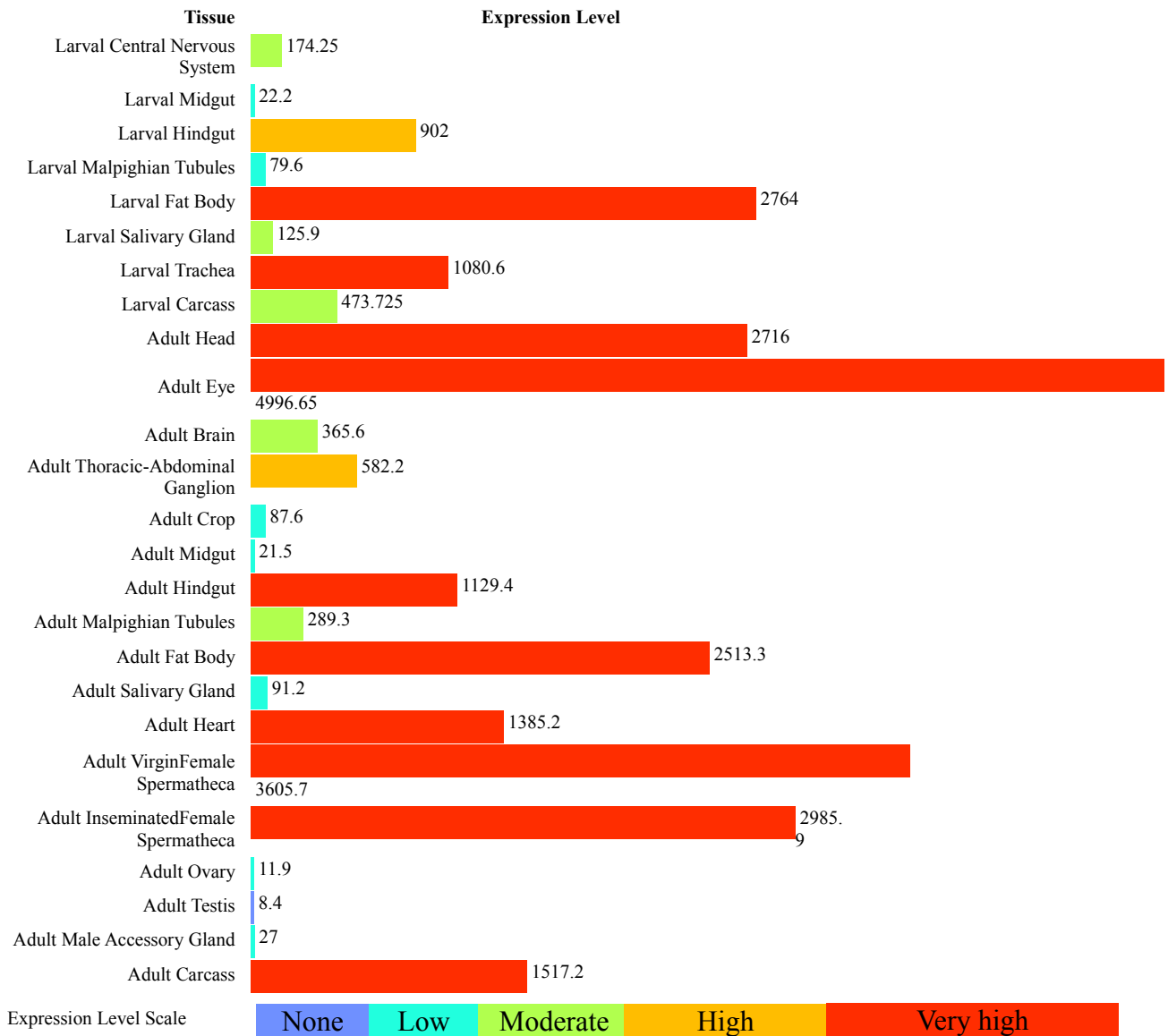
XP_001994204.1	GH23462 [Drosophila grimshawi] >gb EDV94940.1 GH23462 [Drosophila grimshawi]	155	155	62%	5e-41	34%
EFN67408.1	Placental protein 11 [Camponotus floridanus]	153	153	56%	2e-38	37%
EFX74375.1	Endoribonuclease-like protein [Daphnia pulex]	146	146	63%	4e-38	33%
EFX68974.1	Endoribonuclease-like protein [Daphnia pulex]	145	145	63%	1e-37	35%
EFN88107.1	Placental protein 11 [Harpegnathos saltator]	151	151	63%	1e-37	34%
EFX74601.1	Endoribonuclease-like protein [Daphnia pulex]	144	144	63%	3e-37	36%
EFX74564.1	Endoribonuclease-like protein [Daphnia pulex]	144	144	63%	2e-36	36%
XP_003424318.1	PREDICTED: hypothetical protein LOC100677901 [Nasonia vitripennis]	144	144	63%	6e-35	31%
XP_002124182.1	PREDICTED: similar to placental protein 11 related [Ciona intestinalis]	134	134	57%	7e-34	35%
EFZ20078.1	hypothetical protein SINV_15633 [Solenopsis invicta]	132	132	44%	2e-31	39%
XP_003213826.1	PREDICTED: poly(U)-specific endoribonuclease-like, partial [Meleagris gallopavo]	127	127	60%	2e-30	33%
EGI59688.1	Placental protein 11 [Acromyrmex echinatior]	131	131	75%	2e-30	27%
XP_002712715.1	PREDICTED: placental protein 11 [Oryctolagus cuniculus]	127	127	67%	3e-30	31%
XP_625106.3	PREDICTED: hypothetical protein LOC552728 [Apis mellifera]	130	130	63%	4e-30	28%
XP_002601869.1	hypothetical protein BRAFLDRAFT_215427 [Branchiostoma floridae] >gb EEN57881.1 hypothetical protein BRAFLDRAFT_215427 [Branchiostoma floridae]	125	125	63%	6e-30	33%
EFN67407.1	Placental protein 11 [Camponotus floridanus]	130	130	69%	6e-30	29%
XP_003485986.1	PREDICTED: hypothetical protein LOC100741570 [Bombus impatiens]	129	129	63%	8e-30	28%
XP_002740967.1	PREDICTED: placental protein 11-like [Saccoglossus kowalevskii]	122	122	54%	1e-29	32%
EFX61611.1	hypothetical protein DAPPUDRAFT_69104 [Daphnia pulex]	120	120	49%	4e-29	34%
XP_424494.2	PREDICTED: similar to Rap guanine nucleotide exchange factor (GEF) 3 [Gallus gallus]	127	127	60%	5e-29	33%
XP_003398098.1	PREDICTED: hypothetical protein LOC100644333 [Bombus terrestris]	127	127	63%	7e-29	28%
XP_001376122.2	PREDICTED: poly(U)-specific endoribonuclease-like [Monodelphis domestica]	124	124	61%	9e-29	33%
XP_002124119.1	PREDICTED: similar to Placental protein 11 precursor (PP11) [Ciona intestinalis]	124	124	63%	2e-28	31%
XP_002933735.1	PREDICTED: poly(U)-specific endoribonuclease-D-like [Xenopus (Silurana) tropicalis]	120	120	61%	3e-28	32%
XP_003224518.1	PREDICTED: poly(U)-specific endoribonuclease-like [Anolis carolinensis]	121	121	61%	3e-28	32%
NP_001095560.1	poly(U)-specific endoribonuclease precursor [Bos taurus] >sp A6QLQ8.1 ENDOU_BOVIN RecName: Full=Poly(U)-specific endoribonuclease; AltName: Full=Protein endoU; AltName: Full=Uridylate-specific endoribonuclease; Flags: Precursor >gb AAI48053.1 P11 protein [Bos taurus] >gb DAA29867.1 endonuclease, polyU-specific [Bos taurus]	122	122	63%	4e-28	32%
EFN88106.1	Placental protein 11 [Harpegnathos saltator]	123	123	63%	1e-27	29%

XP_001635145.1	predicted protein [Nematostella vectensis] >gb EDO43082.1 predicted protein [Nematostella vectensis]	117	117	60%	2e-27	31%
ADY47090.1	Poly(U)-specific endoribonuclease [Ascaris suum]	118	118	64%	2e-27	30%
XP_003515414.1	PREDICTED: poly(U)-specific endoribonuclease-like, partial [Cricetulus griseus]	115	115	63%	1e-26	30%
XP_002192417.1	PREDICTED: hypothetical protein, partial [Taeniopygia guttata]	114	114	56%	1e-26	31%
NP_001074167.1	poly(U)-specific endoribonuclease precursor [Danio rerio] >sp A1L237.1 ENDUA_DANRE RecName: Full=Poly(U)-specific endoribonuclease-A; AltName: Full=Protein endoU-A; AltName: Full=Uridylate-specific endoribonuclease-A; Flags: Precursor >gb AAI29333.1 Zgc:158628 [Danio rerio]	115	115	62%	1e-26	31%
EFZ20057.1	hypothetical protein SINV_00725 [Solenopsis invicta]	120	120	65%	2e-26	29%
XP_002593543.1	hypothetical protein BRAFLDRAFT_88522 [Branchiostoma floridae] >gb EEN49554.1 hypothetical protein BRAFLDRAFT_88522 [Branchiostoma floridae]	116	116	62%	2e-26	31%
AAI53520.1	Zgc:158628 protein [Danio rerio]	114	114	62%	3e-26	31%
XP_001622792.1	hypothetical protein NEMVEDRAFT_v1g248296 [Nematostella vectensis] >gb EDO30692.1 predicted protein [Nematostella vectensis]	114	114	64%	5e-26	30%
A8E624.1	RecName: Full=Poly(U)-specific endoribonuclease-C; AltName: Full=Placental protein 11 homolog; AltName: Full=Protein endoU-C; AltName: Full=Uridylate-specific endoribonuclease-C; AltName: Full=XendoU-C; Flags: Precursor >gb AAI53812.1 Unknown (protein for MGC:181775) [Xenopus laevis]	114	114	61%	5e-26	32%
XP_851796.2	PREDICTED: poly(U)-specific endoribonuclease [Canis lupus familiaris]	116	116	63%	6e-26	30%
XP_003376047.1	putative placental protein 11 [Trichinella spiralis] >gb EFV58708.1 putative placental protein 11 [Trichinella spiralis]	114	114	63%	7e-26	29%
NP_001177998.1	poly(U)-specific endoribonuclease precursor [Rattus norvegicus] >ref XP_002727017.1 PREDICTED: placental protein 11 related [Rattus norvegicus]	115	115	63%	8e-26	31%
XP_002601870.1	hypothetical protein BRAFLDRAFT_75913 [Branchiostoma floridae] >gb EEN57882.1 hypothetical protein BRAFLDRAFT_75913 [Branchiostoma floridae]	115	115	61%	9e-26	33%
XP_001692654.1	predicted protein [Chlamydomonas reinhardtii] >gb EDP03673.1 predicted protein [Chlamydomonas reinhardtii]	113	113	62%	9e-26	30%
XP_002935698.1	PREDICTED: poly(U)-specific endoribonuclease-like [Xenopus (Silurana) tropicalis]	115	115	70%	1e-25	29%
XP_002752133.1	PREDICTED: placental protein 11 isoform 3 [Callithrix jacchus]	114	114	60%	1e-25	31%
NP_001165911.1	poly(U)-specific endoribonuclease isoform 3 precursor [Homo sapiens] >dbj BAH13227.1 unnamed protein product [Homo sapiens]	112	112	63%	3e-25	30%
XP_001162696.1	PREDICTED: poly(U)-specific endoribonuclease isoform 1 [Pan troglodytes]	112	112	63%	3e-25	30%

XP_002752132.1	PREDICTED: placental protein 11 isoform 2 [Callithrix jacchus]	113	113	60%	3e-25	31%
NP_001018398.1	poly(U)-specific endoribonuclease-B [Danio rerio] >sp Q503V9.1 ENDUB_DANRE RecName: Full=Poly(U)-specific endoribonuclease-B; AltName: Full=Protein endoU-B; AltName: Full=Uridylate- specific endoribonuclease-B >gb AAH95160.1 Zgc:110053 [Danio rerio] >gb AAI64551.1 Zgc:110053 protein [Danio rerio]	111	111	62%	3e-25	31%

Table A : Blast sequence protein alignment. Query: P102 of Heliothis virescence

TABLE B



Guide to FlyAtlas expression level colors:

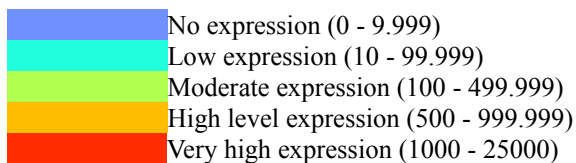


Table B: Flyatlas tissue expression of CG2145.

Discussion

The *H. virescens* 102 gene was isolated in our lab in the frame of a research project aimed at investigating the molecular bases of host-parasitoid interactions. This gene is targeted by the immunosuppressive activity of the polydnavirus associated with the parasitoid wasp *Toxoneuron nigriceps* and is involved in host immune response, in particular, in the encapsulation process of foreign bodies (Falabella et al. 2011, unpublished data).

To elucidate the role(s) that the 102 gene plays in immunity, I decided to use a well-established model organism, which could offer me a variety of experimental tools not available in *H. virescens*.

Considering that many basic physiological processes are evolutionarily conserved between insects, *Drosophila melanogaster* appeared to be the best choice, not only because very useful molecular genetic techniques have been developed in this insect, but also because of the huge body of information available on *Drosophila* immune system. In fact, in the last decades, *Drosophila* has turned to be an excellent model for studying immune responses, as evidenced by the recent Nobel Prize in Medicine assigned to Jules A. Hoffmann and colleagues for their discoveries in this field (Lemaitre B. et al. 1996).

The functional analysis were conducted on the *Drosophila melanogaster* CG2145 gene, which, based on sequence homology as well as its expression pattern in hemocytes, was the putative orthologue of the 102 gene of *Heliothis virescens*.

By using a RNAi approach, I demonstrated that the function of the CG2145 gene, renamed Dm-102, was essential in immune competent tissues for larval and pupal viability. In addition, since in these experiments the larval stages were considerably extended in

time, the Dm-102 gene might be involved in larval growth and/or development.

The presence of melanotic masses in the larval hemocoel indicated that the gene plays a regulatory role in the immune response. In agreement with this hypothesis, melanotic mass formation was associated with increase in the number of circulating hemocytes, differentiation of functional lamellocytes, release of hemocytes from the sessile hematopoietic compartment and with the presence of hemocytes in primary and secondary lobes of the lymph gland.

On the basis of the classification reported by Minakhina and Steward (2006) the melanotic masses observed in Dm-102i larvae can be described as melanotic nodules, since they were freely floating in the hemocoel and surrounded by hemocytes, in particular lamellocytes. In Dm-102 larvae, the latter cell type was also engaged in the formation of large clumps that could be readily isolated from blood samples of third instar larvae. These structures resembled the lamellocyte clusters observed in previously described melanotic tumor (*tu*) mutant strains (Rizki and Rizki. 1980; Rizki and Rizki. 1984; Rizki and Rizki. 1990; Lemaitre B. et al. 1995). Their presence was a clear demonstration of the strong adhesive properties of lamellocytes in Dm-102i larvae.

Stimulation of the immune system in the absence of an immune challenge occurs in autoimmune disorders.

As suggested by Watson and colleagues (1991), these disorders may be due to alterations of the immune system itself or may involve the reaction of a normal immune system to the presence of abnormal tissues, which are recognized as non-self. The Dm-102 gene is highly expressed in both the fat body and the hemocytes. Thus, in principle, either of the above mentioned alternatives might explain

the “melanotic tumor” phenotype observed in Dm-102i larvae. However, preliminary data indicate that the fat body of third instar Dm-102i larvae is disgregated (data not shown). Therefore, the structural integrity of the fat body might be affected by the silencing of the Dm-102 gene and this could in turn trigger an immune response directed against this tissue, thus leading to melanotic nodules formation. As postulated by Rizki and Rizki (1980), the rupture of the basal membrane that surrounds all tissues can induce hemocyte adhesion and formation of melanotic masses. In this study, the authors demonstrated that *Drosophila* larvae encapsulated transplanted self-tissues, if mechanically damaged; in contrast, tissues with intact basement membrane were not encapsulated.

Moreover, it was reported that mutations causing alterations of the structure of the basal membrane induced the formation of “melanotic tumors” in larvae.

This is, for instance, the case of the *tumor^w* (*tu^w*) mutant strain, where the basal membrane of the caudal fat body was disrupted, determining hemocyte-mediated encapsulation of the tissue (Rizki and Rizki. 1974). Analogously, the *tu-Sz^{ts}* temperature-sensitive mutation caused the development of abnormal adipose cells and basal membrane ruptures leading to the formation of melanotic masses (Rizki and Rizki. 1979).

Interestingly, the components of the basement membrane are synthesized by hemocytes and fat body, the two tissue types where Dm-102 function was targeted in our RNAi experiments.

The experimental evidences obtained so far in *H.virescens* indicated that the P102 protein forms a fibrillar scaffold, which is interspersed among the hemocyte layers of the immune capsule (Falabella et al.,

unpublished). Fibrillar structures were previously described in immune capsules formed by different insect species (Akai and Sato, 1973; Beaulaton, 1968; Sass et al., 1994; Wigglesworth, 1973). In these studies it was shown that insect hemocytes release consistent amounts of fibrillar material, which also accumulates in the basement membrane lining the hemocel and all internal organs. In *Drosophila*, ultrastructural studies of the capsules formed in the *tu^w* melanotic mutant demonstrated that the plasma membranes of apposing lamellocytes are in close contact in some regions, whereas other regions show a space between the membranes of the two cells. This space is traversed by electron dense fibrillar material, apparently originating from secreted vesicles and it was suggested that these vesicles and the material contained therein might function in the adhesion between lamellocytes during their aggregation (Rizki and Rizki, 1979).

Based on our results we can hypothesize that the *Drosophila* Dm-102 protein may be involved in the formation of the fibrillar structures found in both basement membrane and immune capsules. Reduced Dm-102 protein levels (likely occurring in our Dm-102i larvae) might lead to breakage of the structural integrity of the basement membrane in several tissues and mainly in the fat body, which undergoes massive growth during larval stages. This would in turn trigger the autoimmune response that we observed. Interestingly, melanotic masses appeared quite late during larval development, indicating that encapsulation of self-tissues in Dm-102i larvae is a slow process, if compared with encapsulation, in wild type larvae, of a foreign object such as a parasitoid egg, which is completed in 48 hours. The finding that reduced levels of the Dm-102 protein slow down capsule formation is compatible with the

idea that this protein is involved in the formation of the fibrillar structures found in the immune capsules and that these fibrillar structures mediate hemocyte adhesion. Moreover, this scenario is in agreement with the data obtained in *H. virescens*. As stated above, the *H. virescens* P102 protein is localized in the fibrillar material that is found in a scattered pattern in the immune capsules surrounding foreign intruders. When the assembly/function of this fibrillar material was affected by the usage of antibodies directed against the P102 protein, the encapsulation of non-self objects was impaired (Falabella et al., unpublished).

In conclusion, based on a detailed morphological characterization of the mutant phenotype generated by a RNAi approach, the CG2145 *Drosophila* gene appears to be the *Drosophila* orthologue of the *H. virescens* 102 gene. Further functional studies will allow elucidating the molecular pathways underlying its role in cellular immunity. Due to the significant evolutionary conservation of this gene, it can be anticipated that the information gathered in *Drosophila* will shed light on molecular machines/mechanisms shared by many insects and possibly also other organisms. Therefore, in perspective, these studies may well be relevant to applied research, ranging from biotechnology for pest insect control to pharmacology.

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