

**The role of a novel blood cell type,  
the Multinucleated Giant Hemocyte in the  
cell-mediated immune response of *Drosophila***

Ph.D. thesis

**Zita Lerner**

Supervisors: Dr. István Andó and Dr. Viktor Honti



Immunology Unit, Institute of Genetics  
Biological Research Centre, Szeged



Ph. D. School in Biology  
Faculty of Science and Informatics, University of Szeged

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# Introduction

The immune system of living organisms serves as the first line of defense against viruses, microorganisms and multicellular parasites. Insects have effective innate immunity, which consists of the cell-mediated immune response, driven by specialized blood cells and the humoral immune response, based on soluble factors. The fruit fly (*Drosophila melanogaster*) is an excellent model organism for studying the processes of innate immunity, since the regulatory factors and conserved defense mechanisms of the immune system show similarities to the immune processes found in vertebrates. Three hemocyte types are known in *Drosophila* larvae: the phagocytic plasmatocytes, the crystal cells, which are important in initiating melanization processes, and the capsule-forming lamellocytes that differentiate after immune induction and are involved in the formation of a multilayer capsule around foreign particles of non-phagocytic size. The effector hemocytes are located in three hematopoietic compartments: the circulation, the sessile hematopoietic tissue, and the lymph gland. It is known that after immune induction, for example by a parasitoid wasp infection, all three hematopoietic compartments contribute to the differentiation of effector hemocytes.

The innate immune response of *Drosophila melanogaster* has been studied in detail, however, little is known about it in other *Drosophila* species. The originally subtropical species of the *ananassae* subgroup, studied in my dissertation have become cosmopolitan, so

they are popular model organisms for evolutionary and population genetic studies. The possible immunological processes behind their success have not been known so far, therefore, in my Ph.D. work, I studied what alternative immune strategies may be responsible for the successful adaptation of different *Drosophila* species.

# Aims

Detailed investigation of the cell-mediated immune processes of innate immunity in insects may lead to a better understanding of the conserved immune mechanisms. Behind the success of the immune response of *Drosophila* species with different geographic distribution might stand alternative hematopoietic and differentiating processes, so our aims were:

1. To investigate the immune response of different *Drosophila* species upon parasitic wasp infection, furthermore to characterize the larval hemocytes, especially in *Drosophila ananassae* and *Drosophila bipectinata* species of the *ananassae* subgroup.
2. The structural characterization of the hemocytes with electron microscopy.
3. Production of hemocyte-specific monoclonal antibodies, to identify and investigate in detail the hemocyte-subsets of *D. ananassae*.
4. To develop *in vivo* genetic system by preparation of transgenic lines in *D. ananassae*.
5. To define the role of hemocytes in the cell-mediated immunity, and to follow up the differentiation in the hematopoietic compartments.
6. To explore the processes of the immune response against parasitic wasps.

# Methods

1. Preparation of hemocyte samples
2. Production of monoclonal antibodies
3. Generation of transgenic lines in *Drosophila ananassae*
4. Indirect immunofluorescence
5. Western blot assay
6. Maintenance of different parasitic wasp species
7. Preparation of the lymph gland
8. Phagocytosis assay
9. BrdU labeling of the larvae and detection of cell fusion *ex vivo*
10. Host-parasite interaction assay, parasitization assay
11. Investigating the effect of nitric-oxide on the immune response, DAF-FM assay
12. Immune electron microscopy
13. Statistical analysis
14. Microscopic analysis: stereo microscopy, epifluorescence microscopy, video microscopy, confocal microscopy, electron microscopy

# Summary of the results

1. As we investigated the larval hemocytes of various *Drosophila* species from the *ananassae* subgroup, we found that after immune induction with *Leptopilina bouhardi* parasitoid wasp, a so far unknown multinucleated cell type with filopodia, differentiates in the circulation, that we termed as Multinucleated Giant Hemocyte (MGH). We observed that, the precursors of MGHs are already found in the circulation of the naive larvae in the *bipunctinata* complex, however they differentiate only after immune induction. in the species of the *ananassae* complex.

2. Examining the ultrastructure of multinucleated giant cells, we found that there is a dense, continuous network of actin filaments and microtubule around the nuclei, but no cell membrane is visible between the nuclei, so MGHs form a true multinucleated structure. As a result of colchicine treatment, the giant cells are unable to unfold, remaining compact in structure, suggesting that microtubule polymerization plays an important role in forming their final structure. Based on the electron microscopic sections, we observed that the dual cell membrane structure uniquely forms a network in the cytoplasm. This system has been described as an extracellular peripheral labyrinth.

3. In order to investigate the cell-mediated processes of innate immunity in detail, *D. ananassae* and *D. bipunctinata* hemocyte-specific monoclonal antibodies were generated to define blood cell

subpopulations (plasmatocytes, crystal cells, and multinucleated giant hemocytes) in these species. We identified MGH-specific and plasmatocyte-specific immunological markers, and determined their molecular weights by Western blot analysis.

4. Further on we investigated the function of hemocytes in the immune response of *D. ananassae* upon immune induction. We found that plasmatocytes are phagocytic cells, however, the MGHs are unable to engulf bacteria. These cells, similarly to lamellocytes, are involved in the formation of the multilayer capsule around the parasitic wasp egg.

5. It is known that encapsulating hemocytes of *D. melanogaster* differentiate from phagocytic plasmatocytes. To analyze whether plasmatocytes are able to transform into terminally differentiated encapsulating MGHs in *D. ananassae* as well, we analyzed the changes of morphology and function of circulating hemocytes at different time points after wasp infection. We observed that 24h after infestation, small mononuclear cells with filopodia appeared in the circulation, carrying the marker molecule (7C5) for MGHs, these cells were capable of engulfing bacteria. However, 48 and 72h after immune induction the multinuclear, terminally differentiated giant cells did not take up bacteria. In conclusion, phagocytic hemocytes of *D. ananassae* are capable of converting into non-phagocytic, encapsulating cells upon immune induction, similarly to *D. melanogaster*.

6. In order to study the hemocytes and the processes of hemocyte differentiation *in vivo*, we developed a genetic system in *D. ananassae*. *Hml*-GFP and *Pxn*-GFP transgenic lines were generated and together with the immunological marker system we used them to investigate the compartmental origin of multinucleated giant cells. We found that MGHs differentiate in the circulation and in the sessile hematopoietic tissue, upon immune induction. The structure of the lymph gland - in contrast to the processes known in *D. melanogaster* - remained intact after immune induction, and only a small percentage of anti-phosphohistone H3 staining was observed, indicating cell division. We did not detect any sign of giant cell differentiation in this compartment.

7. In order to understand the function of the lymph, we investigated the expression pattern of Collier transcription factor in the lymph gland of *D. ananassae* before and after immune induction. Collier is responsible for maintaining the integrity of the lymph gland. Its expression is characteristic of all cells of the primary lobes in first instar larva and later it is restricted to cells of PSC (Posterior Signaling Centre). In *D. melanogaster*, it is known that after immune induction the primary lobe disintegrates and only Collier-positive PSC cells remain intact. Recent findings suggest that Collier is also responsible for maintaining the prohemocyte state of cells. Thus, the constant Collier expression observed in *D. ananassae*, which persists in all cells of the primary and posterior lobes, even after immune induction, suggests that the lymph gland is composed of prohemocyte



cells. Therefore, the primary lobe does not disintegrate and mature hemocytes do not originate from it.

**8.** By using video microscopy and a special system based on BrdU labeling, we studied the formation of MGHs, and found that they are formed by fusion of activated mononuclear plasmatocytes, similar to mammalian multinucleated giant cells involved in granuloma formation.

**9.** We investigated the immune response of *D. ananassae* against *Leptopilina* parasitoid wasp species with different host specificities. We found a very efficient immune response against the *L. boulardi* host-specialist species. In the majority of the cases the encapsulating MGHs differentiated in the circulation, while the parasitoid could not hatch from any of the pupae. The host immune response was less effective against the generalist *L. vicoriae* and *L. heterotoma* species. Following *L. heterotoma* infection, decreased giant cell differentiation was observed and this species was able to hatch from the host pupa in 1-2% of cases. *L. vicoriae* infection effectively inhibited giant cell differentiation and resulted in very low total cell counts. In some cases, both the host and the parasitoid died and in 4% the wasp was able to hatch from the host pupa.

**10.** Although the encapsulation in *D. ananassae* was not accompanied by visible melanization, it led to effective destruction of the parasitoid. It is known that in addition to toxic melanin, other reactive oxygen and nitrogen species can contribute to the killing of invaders. To test this, we investigated the role of the nitric oxide (NO)

synthesis pathway in the immune response, following various parasitoid wasp infections. The source of NO synthesis - the L-arginine amino acid - and the inhibitor of the key enzyme of the pathway (nitric oxide synthase-NOS) - the L-NAME - were added to the larval food. We found that L-NAME reduced the number of successful capsule formation against parasites and as a result of the treatment, the rate of the giant cell differentiation decreased. In the case of L-arginine treatment, more efficient capsule formation and an increased rate of giant cell differentiation were observed. The intensity of intracellular NO levels changed almost equally in multinucleated giant cells and plasmatocytes as a result of different treatments, suggesting that NO and the resulting free radicals may serve mainly as a differentiation signal.

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