Project closing report-K 128762
Title: Linking a highly efficient immune response mounted by multinucleated giant hemocytes with a complex genomic analysis of invasive *Drosophila* species

In the framework of the research project conducted with the aid of grant K-128762 we gained insight into the fundamental molecular events of a highly efficient anti parasite immunity. The studies involved the identification of novel molecules, regulatory networks and structural changes underlying the discovered unorthodox immune processes. Furthermore, we discovered and characterized novel integrated genetic elements, which drive evolution of innate immunity in eukaryotes via the domestication of phage and bacterial toxins.

Identification and molecular characterization of cell type specific molecules
In the framework of the K 128762 grant we developed discriminative monoclonal antibodies to characterize the hemocyte subpopulations in *Zaprionus indianus* larvae and adults. Based on indirect immunofluorescence assays carried out in both naïve and *Leptopilina victoriae* parasitoid wasp induced animals, 30 monoclonal antibodies were identified, which divided the cellular elements of the hemolymph to three main classes as, the giant hemocytes, the plasmatocytes and the crystal cells. These antibodies reacted specifically with *Z. indianus* hemocytes and did not crossreact with blood cells of other insects. However, one antibody, recognizing the phagocytic plasmatocytes, showed a crossreaction with representatives of phylogenetically distant species, the Lepidoptera and the Hymenoptera families. As the antibody marks the phagocytic subpopulation of hemocytes, the granular cells (granulocytes) of the representative species (doi: 10.1016/j.dci.2020.103701, and unpublished observation) further studies will elucidate its possible involvement in the function of the corresponding blood cell subset in *Drosophilidae*, in the silkworm and in the honey bee.

Characterization of the blood cells and hematopoietic compartments in *Z. indianus*
We characterized three main hemocyte types: the plasmatocytes, the giant hemocytes and the crystal cells (https://doi.org/10.1159/000502646). The giant hemocytes includes multinucleated giant hemocytes (MGH) carrying more than one nucleus, nematocytes which are elongated cells carrying a single nucleus, and anucleated structures originated from the previous two cell types. Immune induction resulted in changes in the composition and morphological features of the circulating blood cell populations: the proportion of giant hemocytes and crystal cells expanded; the plasmatocytes, the multinucleated giant hemocytes and the crystal cells enlarged; moreover, the nuclei of the giant hemocytes also increased in size.

Furthermore, we determined the function of the respective blood cell populations in cellular immunity using phagocytosis assay and also parasitoid wasp encapsulation reaction. We observed that FITC labelled Gram positive and Gram negative bacteria were taken up by the plasmatocytes, however, the giant hemocytes never engulfed microorganisms. Using immunostaining with discriminative antibodies, in the analysis of the encapsulation of *L. victoriae* parasitoid larvae we observed that the plasmatocytes, were found in the same quantity on the wasp egg at each tested time point (24, 48 and 72 h) after infection, while the giant hemocytes were present in low numbers on the parasite at 24 h, and their presence gradually increased and peaked at 72 h. Using the crystal cell specific (anti prophenoloxidase-2) 12F6 antibody in immunofluorescence assay, we showed that the crystal cells and the prophenoloxydase ortholog is also involved in the encapsulation reaction and thus, the elimination of the parasite.

Studies in the involvement of the hematopoietic tissues in differentiation of the *Z. indianus* giant hemocytes revealed that both the lymph gland and the sessile tissue served as a source of precursor cells for the differentiating giant hemocytes. Moreover, we observed that the sessile hemocytes showed a characteristic spatial distribution in the form of aggregates, segmentally attached to the wall of the hemocoel and to the posterior part of the dorsal vessel, in naïve and also in infected larvae.

Using the mitotic marker, anti phospho-histone H3 (H3P) antibody in combination with the giant hemocyte specific 4G7 monoclonal antibody, we asked the question, whether nuclear division contributed to the formation of multinucleated cells: over 430,000 cells were tested for H3P expression and the signal appeared in the plasmatocytes, but never came up in the giant cells. To analyze the possible role of the cell fusion in differentiation of the multinucleated giant cells, immune induced *Z. indianus* larvae were grown on bromodeoxyuridine (BrdU) supplemented standard fly food, which hence incorporated into the DNA of dividing cells. Blood cells, isolated from BrdU treated and untreated immune induced larvae, were mixed and co-incubated *ex vivo*. After 45 min incubation both, BrdU labelled and unlabeled nuclei were detected in the same MGHs, showing, that cell fusion cold contribute to the formation of this cell type.

Analysis of the hemocyte ultrastructure by electron microscopy

Larval hemocytes were subjected to a detailed structural analysis, which also included immuno-transmission electron microscopic analysis, to reveal the specific ultrastructural characteristics of this unique cell type. We revealed that the nuclei of the multinucleated giant hemocytes are not separated by a plasma membrane and, contrary to the plasmatocytes, their cytoplasm has a distinctly sponge-like overall appearance, with an elaborate system of canals and sinuses (https://doi.org/10.1159/000502646). These canals and sinuses communicate with the hemolymph through openings on the outer surface of the cell. The canal system is abundantly equipped with protruding thin microvilli and thin lamellae. The membranes of the intracellular organelles, like the Golgi apparatus, the endoplasmic reticulum and mitochondrial membranes appear much thinner than the plasma membrane. We also observed multiform dense bodies with variable shape and size in the cytoplasm. The irregular shape of the cytoplasmic islands, the thin microvilli and lamellae, generated a large surface for the plasma membrane, especially in the spongy region. When analyzing the ultrastructure of the giant cell localized on the parasitoid wasp, we observed that the canal system was wider at the periphery, while it became narrower when approaching the surface of the embedded parasite. We found that plasmatocytes lack caverns, channels and sinuses, hence these structures were characteristic for the giant hemocytes.

We compared the ultrastructure of *Z. indianus* giant hemocytes and *Drosophila ananassae* MGHs, both representing the encapsulating cell type of the respective species, and identified similarities but also differences in their features. Both cell types carried large number of lipid droplets, which may serve as a reservoir for cholesterol and acyl-glycerols, required for the formation and maintenance of the outstretched membrane system (https://doi.org/10.1021/bi400862q). Formation of a special multiform dense body system is characteristic for both cell types, the giant cells of *Z. indianus* and also the MGHs of *D. ananassae*. Multiform dense bodies were more abundant in the basal region of the encapsulating cells, which is closer to the engulfed parasitoid, and they form an electron-dense layer firmly attached to the invader, likely to be involved in the efficient killing process. Hemocyte treatment with LysoTracker dye revealed that the multiform dense bodies and the electron-dense layer were found to be acidic (https://doi.org/10.1159/000520110). While the

canalicular system protruded the whole cytoplasm of the giant cells in *Z. indianus* (https://doi.org/10.1021/bi400862q), we found this network localized close to the cell periphery in the MGHs of *D. ananassae* (https://doi.org/10.1159/000520110). Furthermore, we observed that the giant hemocytes generated and released anucleated structures and giant cell exosomes into the hemolymph. We have identified several clotting factors and also, the accumulation of the anucleated structures around the wounding sites, a sign for their involvement in blood clotting and wound healing (manuscript in preparation). It is known that cells from bacteria to vertebrates produce exosomes, which cargo proteins, lipids, and nucleic acids either in their internal compartment or displayed on their surface. It is feasible that exosomes and anucleated structures released by the MGHs of *D. ananassae* and *Z. indianus* mediate signaling to other tissues to maintain homeostasis after parasitoid infection, or directly contribute to the killing of parasitoids.

Transcriptome and comparative analysis of *Z. indianus* and *D. ananassae* hemocytes with special emphasis on MGHs, and selection of putative structural and functional molecules by *in silico* analysis

Blood cells of naïve and parasitoid wasp induced *D. ananassae* and *Z. indianus* larvae were subjected for transcriptome analysis. As following wasp infection, several morphological and functional changes occurred in the blood cells, gene expressional changes were coherent. In the immune induced samples of both species, the overexpression of several genes was detected (https://doi.org/10.1159/000520110 and manuscript in preparation) which, in other organisms, have been recognized to be involved in different cellular events.

The transcriptomic findings underlayed the extremely fast cellular growth, the high motility, and the efficient defense against parasitoids. In both species, we observed significantly higher expression of the *Rattus norvegicus* CD63 orthologs, which is known to function as cell surface receptor, involved in reorganization of the actin cytoskeleton, cell adhesion, spreading and migration. Furthermore, the orthologs of *Drosophila melanogaster* lamellocyte-specific (cells responsible for the encapsulation process in this species) marker genes as *atilla* and the integrin betanu subunit (itgbn) were highly expressed by MGHs of *D. ananassae*, and also by the immune induced blood cells of *Z. indianus*. This suggests that *atilla* and *itgbn* orthologs could function in MGH-mediated encapsulation reaction. Moreover, the gene encoding for the ortholog of G-protein coupled receptor moody, was overexpressed in both species, suggesting that these cell surface receptor could be involved in recognition and activation of MGHs.

The *D. melanogaster trehalase* orthologs are expressed at high level in the MGHs of *D. ananassae* and also in immune induced hemocytes of *Z. indianus*. This is suggestive for extensive sugar metabolism in the MGHs, required for the dynamic movements, microtubule rearrangements and vesicle transport. A suggestion for the considerable involvement of this system in the defense mechanism is, that a giant hemocyte marker detected by the 7C5 monoclonal antibody could be a giant cell specific trehalose transporter, possessing 12 transmembrane domains, suggesting a high demand for energy in the encapsulation reaction. Furthermore, the high expression of L-lactate dehydrogenase suggests that an enhanced aerobic glycolysis (Warburg effect) is taking place in the MGHs, which allows cells to convert nutrients such as glucose more efficiently into biomass, thus resulting in a larger cell size.

Although MGHs are non-phagocytic, a large number of genes involved in autophagy were highly expressed by these cells in. Hence, the orthologs of Croquemort (a *D. melanogaster* protein,

promoting apoptotic cell clearance in the plasmatocytes), and Vps39 (a *Mus musculus* vesicle-mediated protein involved in autophagic pathways) were also overexpressed in the immune induced blood cells of *Z. indianus*. This suggests that after immune induction an extensive lysosomal self-degradation is taking place, which might serve as a part of a scenario for cell remodeling. Further gene expression analysis revealed high expression of multiple genes that encode Vacuolar-type ATPase subunits, which could contribute to intensified proton pumping activity and thus acidification. For example, the ortholog of *D. melanogaster* GC8177, encoding for a protein similar to Slc4a3 in vertebrates, an anion exchange protein 3, is highly expressed in both, the MGHs of *D. ananassae* and also the immune induced blood cells of *Z. indianus*, which suggests the important role of the protein in acidification processes possibly also involved in efficient elimination of the parasitoids. Orthologs of *D. melanogaster* Syngr (involved in exocytosis) and mammalian Synaptotagmin-1 (having a regulatory role in the membrane interactions during trafficking of synaptic vesicles at the active zone of the synapse) were expressed at high level in both, the *D. ananassae* MGHs and also immune induced blood cells of *Z. indianus*.

While there are many similarities between the expression pattern of several genes in the hemocytes of parasitoid wasp induced *D. ananassae* and *Z. indianus*, we also detected a major difference between these two species. Until it is known that in *D. melanogaster* prophenoloxidase 3 (PPO_3), is essential for the melanization in the capsule formed around the parasitoids, and this process participates in killing the invaders (https://doi.org/10.1186/s12915-015-0193-6), we found that the genome of *D. ananassae* does not encode for a PPO_3 ortholog. Furthermore, transcriptional analysis of *D. ananassae* blood cells revealed that neither PPO1 nor PPO2, which could also facilitate melanization, was expressed by the MGHs. In addition, neither of the orthologs of MP1, Sp7, and Hayan, which activate the melanization cascade in *D. melanogaster*, were expressed by MGHs of *D. ananassae*, confirming that melanization is not involved in the encapsulation reaction mediated by MGHs of this species. Meanwhile, in the immune induced blood cells of *Z. indianus* two genes encoding for phenoloxydase activity factor like proteins, and seven genes encoding for serine proteases are overexpressed, all involved in melanization reaction. In agreement with these expression data, in *Z. indianus* the capsule melanized, while in *D. ananassae* the melanization of the capsule never occurred.

Based on the *D. ananassae* MGH specific transcriptome data (https://doi.org/10.1159/000520110) we identified a family of 12 genes, which represent members of the Hemolysin E family. Hemolysins are pore-forming toxins that can lyse erythrocytes and mammalian cells and are encoded by several bacterial species (https://doi: 10.1016/s0092-8674(00)81564-0). These genes have no orthologs in *D. melanogaster*, hence they could be important for the highly efficient elimination of the parasite. The scattered occurrence of the hemolysin-like genes in the phylogenetic tree of *Drosophilidae* is indicative for multiple cases of horizontal transfer.

The horizontal transfer of another toxin gene, encoding for the cytolethal distending toxin B from prokaryotes or phages to the genomes of several species in the *ananassae* subgroup (https://doi: 10.1093/molbev/msz146), and *Zaprionus* sp. but not into the genome of *D. melanogaster*, has been shown (submitted for publication). Cytolethal distending toxins are eukaryotic genotoxins, characteristic for Proteobacteria, Actinobacteria and bacteriophage genomes. They function as tripartite (CdtA, CdtB, CdtC) holotoxins, which attack eukaryotic cells leading to cellular distension, cell cycle arrest and cell death. Interestingly, the aphids carrying the *Hamiltonella defensa* bacterial symbiont, with the APSE-2 bacteriophage encoding for the CdtB toxin, were resistant to parasitoid wasps (https://doi:10.1126/science.1174463; https://doi:10.1146/annurev-

ento-112408-085305). It was shown that the CdtB encoded by the *D. ananassae* genome exhibited a strong DNase activity, hence we propose that in this species CdtB might be involved in the efficient protection against parasitoids. We first analyzed the expression of the *cdtB* using quantitative RT-PCR, which revealed high gene expression in embryonic and early larval stages. Parasitoid wasp infection caused a six-fold increase in the gene expression profile in larvae, which suggested the involvement of this gene is the defense against parasitoid wasps (submitted for publication).

Generation of recombinant proteins for further studies and development of immunological reagents for immunochemistry and functional assays

Further, we expressed the *D. ananassae* CdtB protein in recombinant form and developed CdtB specific monoclonal antibodies for immunological studies. During *D. ananassae* development, the CdtB protein was expressed in embryonic and early larval stages, and endoparasitoid wasp infection caused increase in the expression of the protein in larvae. Indirect immunofluorescence experiments targeting CdtB revealed that the protein is expressed in embryonic macrophages, cells, involved in remodeling of the nerve chord, depositing extracellular matrix proteins and retaining innate immune functions through development (https://doi.org/10.1242/jcs.129700). Expression of the CdtB was detected in the fat body of *L. boulardi* infected larvae. The generated null mutant for the *cdtB* gene had embryonic developmental defects and the larvae were more susceptible to *L. boulardi, L. heterotoma* and *L. victoriae* infection, suggesting that the gene is differentially involved in regulation of embryonic development, tissue-remodeling and in immune defense. Hence, we posit that the CdtB toxin is involved in the highly effective immune mechanism (submitted for publication). Furthermore, we identified two other *cdtB* genes in the genome of *D. ananassae*, which are in fusion with the *aip56*, another gene with prokaryotic origin encoding for an exotoxin. These genetic elements drive the evolution of innate immunity via domestication of phage toxins, revealed by us, for the first time (manuscript submitted for publication). Expressional and functional analysis of the newly identified candidates will be carried out in collaboration with Noah Whiteman's laboratory (Berkeley University, US).

Our findings obtained in the framework of the K 128762 grant enable us to understand the basic elements of the extremely effective innate immune response. The identified molecules provide insights, and illuminate a path toward the biological properties of the MGHs and the elimination of the parasitoid. Identification of the horizontally transferred genes, and discovering their involvement in the immune defense mechanisms of *Drosophila* species possessing MGHs, allowed us to trace how the newly acquired elements form novel modules, how they integrate and may interact to provide a highly effective immune response.