Project closing report - K 120142

Title: Characterization of a novel cell type in innate immunity, the multinucleated giant hemocyte

Generation and analysis of novel blood type specific molecular markers for further analysis of the multinucleated giant hemocytes (MGHs)

By the aid of K 120142 grant we selected five antibodies reacting with multinucleated giant hemocytes (MGHs) of *D. ananassae*, one antibody to MGHs in *D. bipectinata* (https://doi.org/10.1159/000369618) and eight antibodies to multinucleated giant cells of *Zaprionus indianus* (https://doi.org/10.1159/000502646). Also, several antibodies were found to mark plasmatocyte subsets. This suggests that the plasmatocyte (macrophage) population is heterogeneous in respect of cell surface antigen expression and commitment to differentiate effector plasmatocytes or encapsulating cells, as we found previously in *D. melanogaster* and in the honey bee, *Apis mellifera* (Anderl et al.,2016: https://doi.org/10.1371/journal.ppat.1005746 Gabor et al., 2017: https://doi.org/10.1016/j.dci.2017.07.013 and 2020: https://doi.org/10.1016/j.dci.2020.103701). To reveal the complexity of the plasmatocyte and the giant hemocyte populations we developed for the first time single cell mass cytometry for multi-dimensional immuno-profiling of *Drosophila* hemocytes by using *D. melanogaster*, the best studied Drosophilid with respect to composition and function of hemocyte subsets and compartments (DOI: 10.1101/2020.06.10.144584). We optimized this platform to analyze the cellular elements of the *Drosophila* innate immune system. A comparative FACS and SCMC analysis with antibodies to discriminative cell surface and intracellular antigens expressed in hemocyte subsets of both IgG and IgM type showed a good accordance of results, in terms of positivity of hemocytes. Further, we investigated the antigen expression profile of single cells and hemocyte populations in naive, in immune induced states, in tumorous mutants as well as in stem cell maintenance defective hdcΔ84 mutant larvae (https://doi.org/10.3390/genes10030173). Multidimensional analysis enabled the discrimination of the functionally different major hemocyte subsets, lamellocytes, plasmatocytes, crystal cells, and delineated the unique immunophenotype of the mutants. Our results demonstrated for the first time, that mass cytometry, a recent single cell technology coupled with multidimensional bioinformatics analysis represents a versatile and powerful tool to deeply analyze at protein level the regulation of cell mediated immunity of *Drosophila*. This system can be used to reveal the functional heterogeneity and stem cell composition of the Drosophilids differentiating MGHs in response to infection by parasites.

Gene expression profile and correlation with morphological features

We carried out transmission electron microscopic and, in parallel, transcriptome analysis experiments of plasmatocytes and giant hemocytes. The results revealed that the MGHs possess a characteristic cellular ultrastructure, a finding, which is sustained by the gene expression profile (manuscript in preparation). In addition, we found that the cytoplasm of the MGHs carry a large number of intracellular canals, sinuses and vesicles, which permit a considerable metabolic advantage and a fast contact with the environment, hence this remarkable structural and molecular organization might contribute to the mechanism of the effective immune response against parasitoid wasps. Furthermore, we observed that the MGH localized on the capsule around the parasitoid wasp became polarized and the acidic vesicles developed in their cytoplasm accumulate at the basal surface of the cell where they form an acidic and electron dense layer on the surface of the parasitoid. MGHs carry a large number of nuclei at diverse

positions in their cytoplasm and we observed that all the nuclei were transcriptionally active (https://doi.org/10.1159/000502646).

The gene expression profile of the respective cells was carried out by a single cell based comparative transcriptome analysis of hemocytes isolated from wasp-induced *D. ananassae*, and analysis of uninduced hemocyte pools. Immune induced plasmatocytes and giant hemocytes were isolated from *Leptopilina boulardi* infected *Drosophila ananassae* larvae. Cytosol of the blood cells was aspired with micromanipulator using a glass pipette and the samples were stored at -80 °C until further processing. cDNA library was prepared and analyzed on the Fragment Analyzer. Sequencing was done in an Illumina NextSeq500 instrument and ZingerR-DESseq2 pipeline had been used for data normalization and to determine the differential gene expression among the two hemocyte types. We compared the transcriptome of MGHs to the transcriptome of plasmatocytes and also the gene expression profile of the uninduced hemocytes.

Compared to the uninduced hemocyte pools, the expression of 1730 genes were significantly higher, and of 2760 genes were significantly lower in MGHs, while 1332 genes showed a significantly higher expression and 2766 genes exhibited significantly lower expression in induced plasmatocytes. The comparative transcriptome analysis resulted in 482 genes significantly overexpressed, and 224 genes significantly downregulated in MGHs, compared to activated plasmatocytes.

Gene Ontology (GO) enrichment analysis was performed using GO::TermFinder open source software. Enrichment analysis was performed against the background of all of the annotated *D. melanogaster* genes on www.flybase.org database. Results were summarized and visualized with REVIGO. Compared to the uninduced hemocyte pool, activated MGHs have an overrepresentation of genes involved in actin filament organization (Arp2/3 complex-mediated, actin cytoskeleton reorganization, regulation of filopodium assembly), vesicles (vesicle and endosome transport, vesicle fusion, SNARE complex assembly, multivesicular body sorting pathway, protein localization of vacuole, lysosomal transport), exocytosis and secretion, clathrin-dependent endocytosis, autophagosome maturation, dsRNA transport, ATP synthesis, positive regulation of TORC1 signaling (involved in the activation of translation, cell growth, cell proliferation, cell motility), gene expression (transcription by RNA polymerase II, mRNA splicing), mitotic cytokinesis, regulation of JNK cascade (cell differentiation and proliferation, inflammatory conditions), cellular response to starvation. There is an underrepresentation of genes related to metabolic processes (lipid metabolism, carbohydrate biosynthesis, chitin-based embryonic cuticle biosynthetic process etc.) and larval development (wing disc pattern formation, hindgut development, salivary gland development etc.).

These findings are in congruence with the observations by transmission electron microscopy, as the cytoplasm of MGHs are saturated with vesicles, some of which are acidic in nature. Genes involved in actin filament organization and cell motility provide these cells with high motility, which allows them to react and move quickly to the site of the parasitoid larvae. The high number of receptors allow MGHs a short reaction time. The mechanism of action of MGHs may involve the exocytosis and secretion of effector molecules.

In case of activated plasmatocytes (macrophages), compared to the uninduced hemocyte pool, there was a higher expression of genes related to the phagocytic function of these cells: regulation of actin filament polymerization, which is necessary for phagocytosis (regulation of lamellipodium assembly), late endosome to vacuole transport, protein degradation necessary for the degradation of the phagosome (proteasomal ubiquitin-independent protein catabolic process, proteasome assembly), and the abundance of mitochondrial-related genes suggest a high energy demand (mitochondrial translation, mitochondrial electron transport). Genes related to transcription by RNA polymerase I, tRNA transcription by RNA

polymerase III, or maturation of large subunit ribosomal RNA suggest that there is a need for a high number of ribosomes, as an intensive translation is taking place in these cells, possibly for antimicrobial molecule secretion and other signaling immune functions. Genes, related to mitotic cytokinesis suggest that they actively divide. Similar to MGHs, in plasmatocytes there is a significantly lower expression in genes related to metabolism and organ development.

Furthermore, comparison between the gene expression profiles of MGHs and plasmatocytes resulted in a significantly higher expression of genes in MGHs related to vesicle-mediated transport, vesicle fusion, endocytosis, exocytosis, signaling, and a lower expression of genes related to metabolic processes (including reactive oxygen species metabolic process, organonitrogen compound biosynthetic process), proteasome-mediated ubiquitin-dependent protein catabolic process, proteasomal ubiquitin-independent protein catabolic process. These findings show that MGHs may have a pivotal role in immune signaling and in the coordination of the immune response. The abundance of vesicle-related genes suggest that they have an essential role in the effector functions of these cells.

Among the genes highly expressed in MGHs, there are 63 which have no orthologs in *D. melanogaster*, therefore these might be unique to those organisms that differentiate MGHs and should also be explored further.

These results are being processed in a manuscript. The further research of these genes might shed light on the molecular mechanisms of MGHs functions.

Vertebrate homologs of the newly identified immune-genes/molecules

Drosophila MGHs share several features in development and ultrastructure with giant cells of vertebrates. In mammals, similarly to invertebrate MGHs, giant cells form by either fusion of macrophages (foreign body giant cell, Langhans giant cell, Touton giant cell, osteoclasts) or by endomitosis in cells of hematopoietic origin (megakaryocyte).

In our transcriptome data, among the genes which have a higher expression in MGHs compared to plasmatocytes, 86% possess an ortholog in *D. melanogaster*, while 76% of the genes have an ortholog in both *Mus musculus* and *Homo sapiens*.

Our transcriptome data suggest that *Drosophila* MGHs formation may have a similar molecular mechanism to the formation of mammalian multinucleated giant cells (MGCs). Mammalian MGCs are formed by the fusion of monocytes, in which the tetraspanin superfamily of proteins (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2691789/) has a pivotal role. Specifically, CD9, CD63 and CD81 are all involved in MGC formation. We found that the orthologs of these genes, Tsp2A, Tsp42Ea and Tsp42Ee are all expressed at a high level in *D. ananassae* MGHs.

There are other similarities at gene expression level. It has been shown that foreign body giant cells and osteoclasts both express similar levels of carbonic anhydrase 2 (CAII) and vacuolar-type H+-ATPase (v-ATPase), two important genes for lowering the pH (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4591016/). Both cell types contain numerous acidic vacuoles in their cytosol, the acidification of which are driven by H+ transport by membrane-bound v-ATPase proton pumps. Osteoclasts need these proteins to dissolve hydroxyapatite, while in case of foreign body giant cells their function is still unknown. Similarly, in *D. ananassae*, we have detected an abundance of acidic compartments in the cytosol with LysoTracker staining, and there is an upregulation of vacuolar-type H+-ATPase subunits, and of the CAH2 gene, the ortholog of the mammalian carbonic anhydrase 2.

The formation of multinucleated giant cells is a widespread phenomenon in the animal kingdom, with similar differentiation mechanisms. Therefore, studies on *Drosophila* multinucleated giant cells can help us gain deeper knowledge of human multinucleated giant cells to understand their significance in immunity in general.

Generation of genetic markers and transgenic lines in *D. ananassae* as tools for further analysis of MGH differentiation

For tissue specific overexpression or silencing of genes encoding for marker molecules (GFP and RFP) and selected MGH specific genes we first inserted an *attP* landing site in the white gene of *D. ananassae*. Hml-GAL4, *attB* and UAS-GFP, *attB* carrying plasmid constructs were generated and, based on *attP*/*attB* recombination, were attempted to be targeted to the *attP* landing site of the transgenic line. We made several efforts to achieve recombination, also including insertion of the integrase coding gene near the *attP* landing site, but we observed that not the lack of the internal integrase source was the problem for a successful recombination. According to these results we concluded that the *attP*/*attB* recombination does not function in *D. ananassae*. As the *attP* landing site was inserted with the CRISPR Cas9 technique we will generate the overexpression/silencing genetic system with this method. We designed and achieved a plasmid construct carrying the Hml-GAL4 and one carrying the UAS-GFP targeted to be inserted with CRISPR in the white gene of *D. ananassae*. We plan to change the GFP marker gene with the appropriate sequences of the genes of interest, fused to an HA tag for expression analysis. Furthermore, generation of a transgenic *D. ananassae* line carrying endogenous Cas9, localized on the second chromosome is in progress. This line will be used for the more efficient insertion using CRISPR Cas9 of the UAS-gene of interest-HA constructs.

Analysis of the effective anti-parasite immune response and killing mechanism

Previously, in several species of the *Drosophilidae* family we described the presence of multinucleated giant hemocytes (MGHs), which are highly motile syncytial formations, and involved in the encapsulation and killing of larger foreign particles as the parasitoid wasps. Recently we observed that species developing MGHs (as *D. ananassae, D. bipectinata*, as well as two phylogenetically distant species, *Zaprionus indianus* and *D. willistoni*) are more resistant to parasitoid wasps, when compared to the killing efficiency displayed by *Drosophila melanogaster*, which uses mononuclear lamellocytes for the encapsulation and killing the parasitoid wasps.

Interestingly, there is correlation between the high effectiveness of the immune response in the species with the involvement of MGHs and the expression of a cytolethal distending toxin, an eukaryotic genotoxin, characteristic to Proteobacteria, Actinobacteria and bacteriophage genomes. It was observed that the *cdtB* gene, encoding for the cytolethal distending toxin B, was transferred horizontally from prokaryotes or phages to some species of aphids (*Aphididae*) and vinegar flies (*Drosophilidae*) also including *D. ananassae* and *D. bipectinata* of the *ananassae* subgroup. The *cdtB* gene was not detected in the genome of *D. melanogaster* which has a much less effective immune defense than *D. ananassae* or *D. bipectinata*. It was shown that the cytotoxic CdtB subunit was sufficient to generate the respective phenotypes, meanwhile the CdtA and the CdtC subunits were required for binding the toxin to the target cell. The CdtB protein 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. As

CdtB could be a key element of the innate immune response, our findings would lead to get insights into the molecular basis of the highly efficient immune response of the respective *Drosophila* species against the parasitoid wasps.

We observed (unpublished data, collaborative study with Professor Noah Whiteman of Berkley University), that the *cdtB* gene expression was much higher in wasp infected *D. ananassae* larvae than in the naïve animals, showing an approximately six-fold induction in the third instar larvae. Furthermore, we found that the gene is developmentally regulated, with the highest expression in embryonic stages, followed by a gradual expression decrease, which is kept at higher levels in wasp infected animals. Moreover, we observed that in third instar larvae the fat body and the blood cells are responsible for the *cdtB* expression. Recently in the genome of *D. ananassae*, a new *cdtB* gene copy was identified (Verster and Whiteman et al., unpublished), which is fused to the *aip56* gene (encoding for an exotoxin, the apoptosis inducing protein of 56 kDa) and the encoded CdtB protein region shows 55% identity with the original CdtB. The *aip56* in the genome of the APSE2 phage is localized immediately downstream of the *cdtB*, which suggests that the two genes might be transferred horizontally together. Furthermore, we found that the *cdtB* fused *aip56* gene region in *D. ananassae* encodes only for the C-terminal domain of the microbial AIP56 protein, which is known to be responsible for the nuclear targeting of the toxin, hence we suppose that in this species it might facilitate the internalization of the CdtB. These preliminary data generated with the support of the present grant will allow the analysis and the role of the *D. ananassae cdtB* genes in the highly effective immune reactions with the involvement of the MGHs. By the aid of the present grant we generated polyclonal and monoclonal antibodies to recombinant *D. ananassae* CdtB protein (produced by Dr. Zoltán Lipinszki, Lendület Laboratory of Cell Cycle Regulation at Institute of Biochemistry, Biological Research Centre, Szeged). The specificity and the reaction pattern of the antibodies is being analyzed on different tissues of naive and immune induced *D. ananassae* larvae as well as on parasitic wasps attacked by MGHs of the host (*D. ananassae*) organism.