The Immune Defense of the Honey Bee (*Apis mellifera*)
NKFI K 120140 Project Closing Report

The western honey bee (*Apis mellifera*) contributes significantly to the food supply for mankind. They are essential in plant pollination and producing several health benefits such as honey, propolis, and royal jelly. The honey bee is a social insect that lives in highly structured colonies composed of three castes: the worker, the drone and the queen, and develops with complete metamorphosis. Its development consists of five larval stages, followed the pupal and the adult stage (Winston, 1991). The honey bee colonies are threatened by different stress factors, as bacteria, viruses, parasitic mites and pesticide poisoning, which often act in combination all around the world, including Hungary (Forgách et al., 2008, Tapaszti et al., 2009, Genersch et al., 2010, Moritz et al., 2010, Levy et al., 2011). Chronic increases in individual morbidity and mortality may lead directly to hive collapse or cause long term reductions in hive-level fitness resulting in productivity loss, denoting serious threats to agriculture and the ecosystem. The parasitic mite *Varroa destructor*, which not only feeds on the host hemolymph thus weakening the individual but also serves as a vector of different viruses and has been proposed to be responsible for extensive colony losses (Farkas et al., 2001, Bakonyi et al., 2002, Chen et al., 2007, Rosenkranz et al., 2010, de Miranda et al.,2010, Martin et al., 2012). The American foulbrood, which is a severe bacterial disease affecting bee larvae, caused by *Paenibacillus larvae* and the emergent microparasite fungus *Nosema ceranae* affects the bee gut; both are serious threats to *A. mellifera* (Potts et al., 2010, Genersch et al., 2010, Csáki et al., 2015).

Social insects, including the honey bee, developed group-level as well as individual immunity-related strategies against pathogenic microbes and parasites (Evans et al., 2006). They have fewer canonical immunity-related genes relative to solitary insects, which may be a consequence of their “antiseptic behavior” based on communal defense systems like grooming, hygienic behavior, glandular secretions and use of resins in the nest (Barribeau et al., 2015; Boutin et al., 2015, Doublet et al., 2017, van Alphen and Fernhout 2020). The individual defense in the honey bee is based on humoral and cellular immune responses to prevent andfight pathogen infection and transmission of the host and shares many similarities with solitary insects. Immunity is a machinery of inborn structures and mechanisms for defense against infection by parasites and microorganisms. This innate immune system is based on evolutionarily conserved factors, receptor molecules, signaling pathways and mechanisms characterized extensively in the model organism *Drosophila melanogaster* (Hoffmann et al., 1999; Hultmark 2003). After microbes and parasites enter the body cavity humoral and cellular immunity is manifested through the generation of antimicrobial peptides, phagocytosis of microorganisms or encapsulation of larger invaders by different types of hemocytes (Vilmos and Kurucz 1998, Hoffmann et al., 1999, Honti et al., 2014). Wounding, or pathogens entering the body upon septic injury, immediately trigger the activation of proteolytic cascades, leading to coagulation of hemolymph, clot formation and melanization (Theopold et al., 2002, Cerenius and Söderhall 2011, Dudzic et al., 2015;). The clot prevents the loss of body fluids and stops spreading of microorganisms into the hemocoel by immobilizing the bacteria at the wound site (Bidla et al., 2005).

In the best studied organism *D. melanogaster* three main classes of hemocytes have been described, plasmatocytes, crystal cells and lamellocytes. The plasmatocytes are phagocytic cells, but besides engulfing microbes they produce antimicrobial peptides, extracellular matrix proteins and blood clotting components (Stuart and Ezekowitz 2008, Goto et al., 2001). In response to parasitic wasp infection special cell types are formed: called lamellocytes in *D. melanogaster* (Rizki and Rizki 1992), nematocytes in *Zaprionus indianus* (Kacsoh et al., 2014) and multinucleated giant hemocytes in spp. of the *ananassae* subgroup of Drosophilidae (Markus et al., 2015). A minor population of the blood cells, the crystal cells in *D. melanogaster* (Meister 2004), oenocytoids in Lepidoptera and Hymenoptera (Lavine and Strand 2002) secrete components of the phenoloxidase cascade and are involved in the melanization reaction. Insect hemocytes have been classified on the basis of their morphological, histochemical

and functional characteristics (Gupta 1986; Jiravanichpaisal et al., 2006; Lavine and Strand 2002), as granular cells, plasmatocytes, spherule cells, oenocytoids and prohemocytes. The classification of hemocytes initially relied on morphological criteria (Rizki 1957).

Previously in our laboratory significant progress has been made in understanding cell-mediated immune reactions in *D. melanogaster* and *D. ananassae*. We identified specific marker molecules expressed on different subsets of blood cells in the larva as well as in the adult (Kurucz et al., 2007a Acta). A panel of immunological reagents was developed against molecules expressed in functionally different hemocyte populations and immune compartments, and the application of a reverse genetic approach led to the identification of several genes involved in immune functions. We characterized the first hemocyte specific gene Hemese, encoding a transmembrane receptor, a glycophorin family member (Kurucz et al. 2003) and the high molecular weight isoform of Filamin (Filamin- 240) (Rus et.al 2006) involved in the regulation of hemocyte differentiation. We identified the gene for a bacterium binding protein NimC1 (Kurucz et al., 2007b, Zsámboki et al., 2013). We found *nimC1* gene is part of a cluster of ten nimrod related genes (Somogyi et al., 2010), which are also expressed beside Drosophilidae in other insect species, including *A. mellifera* (Kurucz et al., 2007b). The honey bee *nimA* and *draper* genes encode proteins with an EMI domain, a possible protein-protein interaction module that was first named after its presence in proteins of the EMILIN family (Callebaut et al., 2003). In the *nimA* gene the EMI domain is followed by a single NIM repeat, two copies of another atypical EGF-like repeat with eight cysteines, a putative transmembrane region, and a relatively large intracellular domain. By contrast, the *nimB* gene product has fourteen NIM repeats and lacks membrane anchor thus is probably exported. The *nimC1* and *nimC2* gene products are transmembrane proteins with forty-eight and sixteen NIM repeats, respectively.

The individual immunity of honey bee defends the homeostasis on the organism by the aid of immune cells, therefore it is particularly important to gain knowledge on the cellular immunity of this species too. Up till now, various approaches have been employed to identify the hemocyte subtypes of *A. mellifera*, based on histochemical staining, and lectin binding. Size and morphological features classified hemocytes into several subtypes, as phagocytic granular cells, plasmatocytes, oenocytoids, coagulocytes, binuclear cells, permeabilized cells and prohemocytes (de Graaf et al., 2002, El-Mohandes et al., 2010, Marringa et al., 2014, Negri et al., 2014, Schmid et al., 2008, Van Steenkiste 1988). Due to the lack of the molecular markers however, these results are difficult to comprehend and in addition the terminology for hemocyte subsets differs from laboratory to laboratory.

To address the issue of molecular and functional heterogeneity in honey bee hemocytes we took a combined immunological and functional approach and developed antibodies with a high specificity for functionally different hemocyte subsets and found restricted reactions in larvae and in adults of each cast.

One set of the antibodies reacted with the hemolymph clot and with a subpopulation of non-phagocytic hemocytes, named plasmatocytes. We found that these cells form aggregates, *in vivo* adhere to large foreign particles which cannot be taken up by phagocytosis. Using a reverse genetic approach, mass spectrometry analysis and subsequent RNAi studies we revealed that the molecule defined by the 4E1 antibody is the *A. mellifera* Hemolectin (AmHml) (Gábor et al., 2017). It is the major protein in the insect hemolymph soft clot, possessing von Willebrand factor homology domains, characteristic for proteins involved in blood coagulation and platelet aggregation in mammals (Goto et al., 2003, Lesch et al., 2007). The *Hemolectin (Hml)* homolog *Hemocytin* (*Hmc*) gene has been described from *Bombyx mori* (Kotani et al.,1995) and a *Hml* related gene was identified in the genome of *A. mellifera* (Honeybee Genome Sequencing Consortium 2006, Lesch et al., 2007).

In contrast to *D. melanogaster*, where a subset of hemocytes, the phagocytic plasmatocytes express *Hml* (Williams 2007) in the honey bee both in the larva and in the adult, we observed a clear division of cells and complementarity with respect to phagocytosis and AmHml expression. In the larva the majority of the circulating

hemocytes exhibit phagocytic function, while AmHml is expressed in about 30% of the hemocytes. In the young adult 80% of circulating hemocytes express AmHml and a non-overlapping 15-20% of hemocytes are phagocytic. As there is no overlap between phagocytosis and AmHml expression it is conceivable that there is a functional heterogeneity within circulating hemocytes (e.g. a minor population exhibits phagocytic function while the majority is involved in the production of a coagulation factor), as revealed by antibody staining. This dichotomy of cell populations is supported by our finding that the majority of hemocytes in the hemolymph coagulum and the cells involved in the demarcation and encapsulation of the foreign particles are the AmHml expressing hemocytes. As AmHml is expressed in a subset of hemocytes we carried out combined functional and serological analysis by correlating the AmHml expression with phagocytic capacity of the cells. We injected fluorescein isothiocyanate conjugated *Escherichia coli, Staphylococcus aureus, Enterobacter cloacae* and the honey bee pathogen *Melissococcus pluton* into young adults, bled the animals on slide, let the cells adhere, stained with anti-AmHemolectin antibody (a-AmHml) and found the majority of circulating hemocytes expressed AmHml protein, moreover these AmHml positive hemocytes formed aggregates and did not engulf FITC labelled bacteria. Analysis of the correlation between AmHml expression and melanization revealed that AmHml expressing cells did not melanize and melanizing oenocytoids did not express AmHml. The adhesion to foreign particles, and the encapsulation reaction (Andrade et al., 2010, Brandt et al., 2016, Cox-Foster and Stehr 1994, Renault et al., 2002) was also demonstrated on adults by inserting a nylon fiber between the third and the fourth abdominal segments. The fiber was removed after 3 h and stained for the presence of AmHml. We found that AmHml positive cells were embedded into an AmHml positive matrix as a clot formed around the plastic fiber (Gábor et al., 2017).

The other hemocyte sub-population of *A. mellifera* hemocytes, called oenocytoids, are involved in the production of components of the melanization and clotting cascades (Gábor et al., 2020). These cells react with another set of antibodies. These cells also aggregate around large foreign particles, and a fiber matrix of the coagulum. One of the antibodies (2.28) gave two bands in Western blot, a 150 and a 75 kDa protein. The 75 kDa band is corresponding to the predicted molecular mass (74.4 kDa) of the *A. mellifera* prophenoloxidase (AmPPO) (Lourenço et al., 2005, Zufelato et al., 2004). We found, that the double band is corresponding to the zymogen and the activated forms of AmPPO (Gábor et al., 2020). The *D. melanogaster* genome contains three prophenoloxydase (PPO) encoding genes (*PPO1, PPO2* and *PPO3*) (Binggeli et al., 2014, Dudzic et al., 2015). PPO1 and PPO2 proteins are produced by the crystal cells, while PPO3 is synthesized by the lamellocytes. The PPOs are the initiators of the PPO-cascade, which causes melanization, the rapid synthesis of melanin, which is a major immune response of insects to infection and injury (Biedermann and Morit, 1998, Cerenius et al., 2008, Kanost and Gorman 2008). We observed that the 2.28 oenocytoid specific antibody cross reacted with crystal cells and a subset of lamellocytes in *D. melanogaster* wild type larvae, but did not react with hemocytes from prophenoloxidase deficient $PPO1^{\Delta},2^{\Delta},3^{1}$ (Binggeli et al., 2014, Dudzic et al., 2015, Gábor et.al 2020) triple mutant larvae. From these results we propose that the 2.28 *A. mellifera* oenocytoid specific antibody reacts with PPO (Gábor et al., 2020).

We investigated the phylogenic conservation of epitopes defined in *A. mellifera* hemocytes. We tested *D. melanogaster* and *Bombix mori* larval hemocytes with the antibodies generated against *A. mellifera* hemocytes. We found that plasmatocyte markers were not expressed on the hemocytes of *D. melanogaster* or *B. mori*, however the anti-oenocytoid mAbs reacted with the crystal cells and with a subpopulation of the lamellocytes in *D. melanogaster* and with the oenocytoids, granulocytes and a subpopulation of the plasmatocytes of the *B. mori*. These data strongly indicative for the identification of a phylogenetically conserved immunological epitope on a molecule expressed by cells involved in aggregation, clotting and/or melanization, which could lead to the development of a novel immunodiagnostic antibody, applicable across phylogenetically distant insect species.

To reveal the functional role of the adult hemocyte subpopulations in the phagocytosis of microbes FITC-labeled *E. coli* bacteria were injected into adults, after which hemocytes were isolated and an indirect immunofluorescence assay was carried out. We found that AmHml expressing plasmatocytes and 2.28 expressing oenocytoids did not take up bacteria, while 4.70 marker expressing hemocyte subset, dubbed in *A.mellifera* as the granulocytes were phagocytic (Gábor et al., 2020). We observed that the proportion of phagocytic cells in the social honey bee was much lower than in the solitary *D. melanogaster* (over 95%), which has only individual immunity (Rizki and Rizki 1984). Considering the alternative defense strategies of social insects, it is possible that fewer microorganisms reach the body cavity of individual bees. In fact, only a few parasites and microbes are described that affect the honey bee cellular immune response. *Spiroplasma melliferum* infection results in a change in the proportion of plasmatocytes and granulocytes, as detected by Wright staining (Shen PF and Patterson 1983), while *Serratia marcescens sicaria* infection caused a decrease in the total hemocyte number compared to uninfected animals (Burritt et al., 2016, Yang et al., 2017). The phagocytosis of the non-pathogenic *E. coli* and the bee-pathogenic bacteria *Melissococcus pluton* and the endospore-forming *Paenibacillus larvae* was also compared. The phagocytic capacity of granulocytes was comparable *in E. coli* and *M. pluton,* however they were able to engulf much less *Paenibacillus larvae* bacteria both in the larva and in the adult (Gábor et al., unpublished observation).

Using the aforementioned molecular markers of *A. mellifera* hemocyte sub-populations, we were able to characterize the hemocyte subpopulations of different castes throughout development, but also defined the subpopulations involved in the response against various immune threats (Gábor et al., 2012, Gábor et al., 2013a, Gábor et al., 2013b, Gábor et al.,2014, Gábor et al., 2015, Gábor et al., 2020). Furthermore, our results help to elucidate the similarities and differences between the cellular immune responses of social and solitary insects. We also found, that prophenoloxidase function in the honey bee is similar to that in *D. melanogaster*, and prophenoloxidase plays an indispensable role in melanization. We believe that our newly identified markers will help to further identify the components of cellular immunity, as well as analyze the composition of the honey bee immune compartments.

We studied the effects of different risks factors on the defined hemocyte sub-populations by investigating the cell-mediated immune responses (phagocytosis, encapsulation, coagulation and hemocyte aggregation) and the expression pattern of hemocyte markers of adults collected from hives kept on sunflower fields grown from neonicotinoid treated seed (Department of Microbiology and Infectious Diseases, University of Veterinary, Hungary). Also, we collected samples from individuals from hives before and after amitraz treatment, a drug used to fight *Varroa destructor* mite invasion (Immunology Unit, BRC-HAS, Hungary). The hemocyte phenotype of the pupae and larvae, infected with *Varroa destructor* mites (University of Pannonia Georgikon Faculty, Hungary) and from colonies which were selected for Varroa sensitive hygiene (VSH) behavior (Agricultural Biotechnology Center, National Agricultural Research and Innovation Centre, Hungary; University of Pannonia Georgikon Faculty, Hungary) was determined. The blood cells were collected from all tree casts, in the summer and during winter hibernation. The ratio of different blood cell sub-populations was not affected by the amitraz treatment, or by the neonicotinoid treatment of the seed. We found no significant difference in the phenotype of hemocytes in Varroa sensitive hygiene (VSH) behavior colonies compared to the non-sensitive colonies. The hemocyte sub-populations of the summer and winter workers were comparable.

To reveal genetic factors, receptors and mechanisms involved in anti-parasite response and in response to septic injury we analyzed the blood cell populations of the individuals of colonies infected with an ectoparasite, *Varroa destructor*, and the fungus *Ascopphora apis* and compared the parameters of the circulating blood cell pool to that of the healthy colonies. The external parasitic mite *Varroa destructor* (Varroa mite) feeds on the fat body

tissue of the larvae and also a vector for other common bee infections. Chalkbrood disease is caused by the fungus *Ascosphaera apis*. The fungus weakens the colonies and can lead to reduced susceptibility to other bee pests and diseases. For the identification of plasmatocytes we used the a-AmHml antibody, the oenocytoids were defined by the melanization reaction product brown melanin and the rest of the hemocyte population was termed as granulocytes. We counted the ratios of the three blood cell populations and compared the values with that of uninfected colonies. We did not find any significant differences between the proportion of the different blood cell populations between the infected and uninfected colonies, revealing a remarkable difference between the defense mechanisms of solitary and sociable organisms.

The host defense system in different insect shares many of the basic characteristics at the level of the coordinate control of gene expression. We used *Paenibacillus larvae* (a spore-forming bacterium, the causative agent of a bacterial disease of honey bee brood, also known as American foulbrood) in a septic injury assay in *Drosophila* (Kari et al., 2013) for the identification of genes involved in host-pathogen interaction. Previously we confirmed that some immunity related genes are involved in the response to septic injury and identified two factors, instrumental in host defense (Kari et al., 2016). Using this test system, we tested possible involvement of different components of the Toll, the immune deficiency and the JAK/STAT pathways in the pathogenicity of *P. larvae* in wild type and the immunodeficient *D. melanogaster* stocks Our results showed strict host-specificity of *P. larvae*, e.g. none of the *Drosophila* immune gene mutants showed symptoms of the infection and showed difference in the proportion and number of different blood cell types.

We investigated the capacity of *A. mellifera* recombinant Nimrod proteins to bind bacteria. In *D. melanogaster* the Nimrod superfamily is characterized by the presence of a special EGF repeat, the NIM repeat (Kurucz et al., 2007a). Previously it was described, that several members of the superfamily function as receptors in phagocytosis or binding bacteria (Zsámboki et al., 2013, Melcarne et al., 2019), which indicates an important role in the cellular immunity and in the elimination of apoptotic cells. Most of the genes of the Nimrod superfamily are a part of an evolutionary conserved gene cluster, the Nimrod cluster including among others *nimA, nimB, nimC,* and *vajk* genes and even. This cluster can be found in the genome of *Apis mellifera* too (Somogyi et al., 2008, Somogyi et al., 2010, Cinege et al., 2017).

To investigate the bacterial binding properties and specificity of the honey bee Nimrod proteins we used the Bac-to-Bac™ Baculovirus Expression System to produce FLAG tagged honey bee NimA, NimB, NimC1 and NimC2 recombinant proteins in Sf9 lepidopteran cell line. We used the *D. melanogaster* Draper and NimC1 recombinant proteins (Kurucz et.al., 2007a, Zsámboki et al., 2013) as positive, and recombinant Vajk4 protein (Cinege et al., 2017) coding construct and the empty bacmid transfected the Sf9 lepidopteran cells as negative controls. We standardized the amount of the corresponding recombinant proteins in the SF9 cell lysates with Western blot using anti-FLAG antibody and fluorescein isothiocyanate labeled *E. coli, Paenibacillus larvae, Melissococcus pluton, Staphylococcus epidermidis, Serratia marcescens, Xanthomonas campestris, Pseudomonas aeruginosa, Bacillus subtilis, Bacillus cereus var. mycoides, and Micrococcus luteus* bacteria (Kurucz et al., 2007b Suppl.1). The bacteria were co-incubated with the Sf9 cell lysates containing the corresponding FLAG-tagged recombinant proteins. After overnight incubation the FLAG-tagged recombinant protein-bacterium complexes were reacted with a monoclonal mouse anti-FLAG antibody and Alexa Fluor 633 goat-anti-mouse IgG, and analyzed by flow cytometry with a FACSCalibur equipment (BecktonDickinson) for fluorescence intensity. In the FACS analysis the FITC-labeled bacteria were identified by their FITC fluorescence in the FL1 green (530/30 nm) channel, the bacterium-binding recombinant proteins were detected on the basis of their Alexa Fluor 633 fluorescence in the FL4 far-red (661/16 nm) channel. The bacterium-binding capacity of the recombinant proteins was determined by calculating the relative mean fluorescence intensity of the samples, i.e. the ratio of the mean fluorescence intensity of the bacterial binding of protein of interest and the mean fluorescence intensity of the negative control protein (Zsámboki et al.,

2013). The binding capacity of Sf9 cell lysates and empty bacmid transfected Sf9 cell lysates was also determined. We showed that *A. mellifera* recombinant NimA, NimB, NimC1 and NimC2 proteins possess higher preference for binding Gram-negative than Gram-positive bacteria. Furthermore, NimA has the highest binding capacity with a preferential binding of *S. marcescens* and *X. campestris*. Recently it has been recognized that *S. marcescens* is an opportunistic pathogenic in the honey bee workers (Raymann et. al., 2018) and that it may be highly virulent under certain conditions e.g., the presence of Varroa mites that puncture the cuticle, thereby enabling entry of the bacteria into the hemocoel.

In the framework of the present project we produced a panel of monoclonal antibodies detecting reference markers for *A. mellifera* blood cells and by the aid of the antibodies we identified functionally distinct hemocyte subsets (Gábor et.al., 2020). In order to refine the technology of detection of *A. mellifera* hemocyte subsets, we have set up to test whether a novel detection system, the single cell mass cytometry can be applied for insect blood cells too. In these studies, we used the model insect, *D. melanogaster*, in combination with the standard panel of anti-hemocyte antibodies (Kurucz et. al., 2007b). Single cell mass cytometry combines features of traditional flow cytometry with mass spectrometry and allows the measurement of several parameters at the single cell level, thus permitting a complex analysis of cellular elements, as the hemocytes of the insect innate immune system. We constructed six, metal-conjugated antibodies against cell surface and cytoplasmic antigens and studied the antigen expression profile of single cells and hemocyte populations in naive, in immune induced states and in tumorous mutants. This multidimensional analysis enabled the discrimination of the functionally different major hemocyte subsets for the first time and delineated the unique immune phenotype of the mutants (Balogh et. al, 2021). The method can be applied for both cell surface and cytoplasmic antigens. As the majority of the *A. mellifera* hemocyte antigens are cytoplasmic as detected by the antibodies (Gábor et.al., 2020), the method could be extended to the further analysis of the honey bee hemocyte subsets too.

Irodalom:

Andrade GS, Serrão JE, Zanuncio JC, Zanuncio TV, Leite GL, Polanczyk RA. (2010) Immunity of an alternative host can be overcome by higher densities of its parasitoids Palmistichus elaeisis and Trichospilus diatraeae. PLoS One. 5:e13231. doi: 10.1371/journal.pone.0013231.

Bakonyi T, Farkas R, Szendrői A, Dobos-Kovács M, Rusva, M. (2002). Detection of acute bee paralysis virus by Rt-PCR in honey bee and Varroa destructor field samples: rapid screening of representative Hungarian apiaries. Apidologie, 33, 63-74.

Balog JÁ, Honti V, Kurucz É, Kari B, Puskás LG, Andó I, Szebeni GJ. (2021) Immunoprofiling of Drosophila Hemocytes by Single-cell Mass Cytometry. Genomics Proteomics Bioinformatics. 10:S1672-0229(21)00055-3.doi: 10.1016/j.gpb.2020.06.022.

Barribeau SM, Sadd BM, du Plessis L, Brown MJ, Buechel SD, Cappelle K, Carolan JC, Christiaens O, Colgan TJ, Erler S, Evans J, Helbing S, Karaus E, Lattorff HM, Marxer M, Meeus I, Näpflin K, Niu J, Schmid-Hempel R, Smagghe G, Waterhouse RM, Yu N, Zdobnov EM, Schmid-Hempel P. (2015) A depauperate immune repertoire precedes evolution of sociality in bees. Genome Biol. 16:83. doi: 10.1186/s13059-015-0628-y.

Bidla G, Lindgren M, Theopold U, Dushay MS. (2005) Hemolymph coagulation and phenoloxidase in Drosophila larvae Dev. Comp. Immunol., 29:669-679. doi: 10.1016/j.dci.2004.11.007.

Biedermann W, Moritz P. (1998) Beiträge zur vergleichenden Physiologie der Verdauung. Pflüger, Arch. 73, 219-287. https://doi.org/10.1007/BF01796256.

Binggeli O, Neyen C, Poidevin M, Lemaitre B. (2014) Prophenoloxidase activation is required for survival to microbial infections in Drosophila. PLoS Pathog. 10 e1004067. https://doi.org/10.1371/journal.ppat.1004067.

Boutin S, Alburaki M, Mercier PL, Giovenazzo P, Derome N. (2015) Differential gene expression between hygienic and non-hygienic honeybee (Apis mellifera L.) hives. BMC Genomics. 16:500. doi: 10.1186/s12864-015-1714-y.

Brandt A, Gorenflo A, Siede R, Meixner M, Büchler R. (2016) The neonicotinoids thiacloprid, imidacloprid, and clothianidin affect the immunocompetence of honey bees (Apis mellifera L.). J Insect Physiol. 86:40-7. doi: 10.1016/j.jinsphys.2016.01.001.

Burritt N., Foss NJ, Neeno-Eckwal EC, Church JO, Hilger AM, Hildebrand JA, Warshauer DM, Perna,NT, Burritt JB, (2016) Sepsis and hemocyte loss in honey bees (Apis mellifera) infected with Serratia marcescens strain sicaria. PLoS One 11, e0167752. https://doi.org/10.1371/journal.pone.0167752.

Callebaut I, Mignotte V, Souchet M, Mornon JP. (2003)EMI domains are widespread and reveal the probable orthologs of the Caenorhabditis elegans CED-1 protein. Biochem Biophys Res Commun. 2003 Jan 17;300(3):619-23. doi:10.1016/s0006-291x(02)02904-2.

Chen YP and Siede R. (2007) Honey bee viruses. Adv Virus Res. 70:33-80. doi: 10.1016/S0065-3527(07)70002-7. PMID: 17765703.

Cerenius L, Lee BL, Söderhäll K. (2008) The proPO-system: pros and cons for its role ininvertebrate immunity. Trends Immunol. 29, 263-271. https://doi.org/10.1016/j.it.

Cerenius L and Söderhäll K. (2011) Coagulation in invertebrates. J. Innate Immun. 3:38. https://doi.org/10.1159/000322066.

Cinege G, Zsámboki J, Vidal-Quadras M, Uv A, Csordás G, Honti V, Gábor E, Hegedűs Z, Varga GIB, Kovács AL, Juhász G, Williams MJ, Andó I, Kurucz É. (2017) Genes encoding cuticular proteins are components of the Nimrod gene cluster in Drosophila. Insect Biochem Mol Biol. 2017 Aug;87:45-54. doi: 10.1016/j.ibmb.2017.06.006.

Cox-Foster DL, Stehr JE. (1994) Induction and localization of FAD-glucose dehydrogenase (GLD) during encapsulation of abiotic implants in Manduca sexta larvae. J. Insect Physiol. 40:235e249. http://dx.doi.org/10.1016/0022-1910(94)90047-7.

Cuttel L, Vaughan A, Silva E, Escaron CJ, Lavine M, Van Goethem E, Eid JP, Quirin M, Franc NC. (2008) Undertaker, a Drosophila Junctophilin, links Draper-mediated phagocytosis and calcium homeostasis. Cell 135: 524-534. doi: 10.1016/j.cell.2008.08.033. PMID: 18984163.

Csáki T, Heltai,M, Markolt F, Kovács B, Békési L, Ladányi M, Péntek-Zakar E, Meana A, Botías C, Martín-Hernández R. et al. (2015) Permanent prevalence of Nosema ceranae in honey bees (Apis mellifera) in Hungary. Acta Vet. Hung. 63:358-369. doi: 10.1556/004.2015.034. PMID: 26551426.

de Graaf D, Dauwe R, Walravens K, Jacobs F. (2002) Flow cytometric analysis of lectin-stained haemocytes of the honeybee (Apis mellifera). APIDOLOGIE 33:571-579.

de Miranda JR and Genersch E. (2010) Deformed wing virus. J. Invertebr. Pathol. 103 Suppl 1, S48-S61.

Dudzic JP, Kondo S, Ueda R, Bergman CM, Lemaitre B. (2015) Drosophila innate immunity: regional and functional specialization of prophenoloxidases. BMC Biol. 13:81. https://doi.org/10.1186/s12915-015-0193-6.

El-Mohandes SS, Nafea E., Fawz AM. (2010) Effect of different feeding diets on the haemolymph of the newly emerged honeybee workers Apis mellifera L. Egypt. Acad. J. Biol. Sci. 3:213e220.

Evans JD, Aronstein K, Chen YP, Hetru C, Imler J-L, Jiang H, Kanost, M, Thompson GJ, Zou Z, Hultmark D. (2006) Immune pathways and defence mechanisms in honey bees Apis mellifera. Insect Mol. Biol. 15:645-656. doi: 10.1111/j.1365-2583.2006.00682.x.

Farkas R, Bakonyi T, Börzsönyi L, Rusvai M. (2001). A mézelő méh (Apis mellifera L.) Varroa jacobsoni Oudemans fertőzöttségével kapcsolatos kérdőíves vizsgálat hazai méhészetekben. Magyar Állatorvosok Lapja 123.348-353.

Forgách P, Bakonyi T, Tapaszti Z, Nowotny N, Rusvai M. (2008) Prevalence of pathogenic bee viruses in Hungarian apiaries: situation before joining the European Union. J Invertebr Pathol. 98:235-238. doi: 10.1016/j.jip.2007.11.002.

Gábor E, Török T, Csordás G, Honti V, Kuruc É, Andó I. (2012) A házi méh vérsejtjeinek heterogenitása. Immunológiai Szemle 4(3) 11.
Gábor E, Török T, Csordás G, Honti V, Kurucz É, Andó I. (2013a) Molecular and functional heterogeneity of honeybee hemocytes. Immunológigi Szemle 5(3) 25.
Gábor E, Török T, Csordás G, Zsámboki J, Kurucz É, Andó I. (2013b). The immunity of the honeybee (Apis mellifera). Tudomány a vidék mindennapjaiban ISBN: 978-963-306-245-6, 29-34.
Gábor E, Török T, Zsámboki J, Cinege Gy, Csordás G, Honti V, Kurucz É, Andó I. (2014) Spring, summer, autumn, winter: Seasonal changes in hemocyte number and type in the honey bee. Immunológiai Szemle, 6 (3-4) 22.
Gábor E., Zsámboki J, Cinege Gy, Török T., Csordás G, Honti V, Kurucz É, Andó I. (2015) Molecular definition of hemocyte subsets in the honey bee (Apis mellifera). Immunológiai Szemle, 7 (3) 27.
Gábor E, CinegeG, Csordás G, Török T, Folkl-Medzihradszky K, Darula Z, Andó, Kurucz É. (2017) Hemolectin expression reveals functional heterogeneity in honey bee (Apis mellifera) hemocytes. Dev. Comp. Immunol. 76: 403-411. https://doi.org/ 10.1016/j.dci.2017.07.013.
Gábor E, Cinege G, Csordás G, Rusvai M, Honti V, Kolics B, Török T, Williams MJ, Kurucz É, Andó I. (2020) Identification of reference markers for characterizing honey bee (Apis mellifera) hemocyte classes. Dev Comp Immunol. 109:103701. doi: 10.1016/j.dci.2020.103701.
Genersch E. (2010a) American Foulbrood in honeybees and its causative agent, Paenibacillus larvae. J. Invertebr. Pathol. 103 Suppl 1, S10–S19.
Goto A, Kumagai T, Kumagai C, Hirose J, Narita H, Mori H, Kadowaki T, Beck K, Kitagawa Y. (2001) A Drosophila haemocyte-specific protein, hemolectin, similar to human von Willebrand factor. Biochem. J. 359:99e108. doi: 10.1042/0264-6021:3590099.
Goto A, Kadowaki T, Kitagawa Y. (2003) Drosophila hemolectin gene is expressed in embryonic and larval hemocytes and its knock down causes bleeding defects. Dev. Biol. 264:582e591. doi: 10.1016/j.ydbio.2003.06.001. PMID: 14651939.
Gupta AP. (1986) Arthropod immunocytes. In: Gupta, A.P. (Ed.), Hemocytic and Humoral Immunity in Arthropods. John Wiley and Sons, New York, pp. 13e59, 3-59.
Honeybee Genome Sequencing Consortium (2006). Insights into social insects from the genome of the honeybee Apis mellifera. Nature 443:931-949. doi: 10.1038/nature05260.
Hoffmann JA, Kafatos FC, Janeway CA, Ezekowitz RA. (1999) Phylogenetic perspectives in innate immunity. Science 284:1313-1318. doi: 10.1126/science.284.5418.1313.
Honti V, Csordás G, Kurucz É, Márkus R, Andó I. (2014) The cell-mediated immunity of Drosophila melanogaster: hemocyte lineages, immune compartments, microanatomyand regulation. Dev. Comp. Immunol. 42:47-56. doi: 10.1016/j.dci.2013.06.005.
Hultmark D. (2003) Drosophila immunity: paths and patterns. Curr. Opin. Immunol. 15:12-19. doi: 10.1016/s0952-7915(02)00005-5.
Jiravanichpaisal P, Lee BL, Söderhall K. (2006) Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. Immunobiology. 211:213-36. doi: 10.1016/j.imbio.2005.10.015.
Kacsoh BZ, Bozler J, Schlenke TA. (2014) A role for nematocytes in the cellular immune response of the drosophilid Zaprionus indianus. Parasitology 141:697e715. http://dx.doi.org/10.1017/S0031182013001431:
Kari B, Csordás G, Honti V, Cinege G, Williams MJ, Andó I, Kurucz É. (2016) The raspberry Gene Is Involved in the Regulation of the Cellular Immune Response in Drosophila melanogaster. PLoS One. 11:e0150910. doi: 10.1371/journal.pone.0150910
Kari B, Zsámboki J, Honti V, Csordás G, Márkus R, Andó I, Kurucz É. (2013) A novel method for the identification of factors involved in host-pathogen interactions in Drosophila melanogaster. J Immunol Methods. 398-399:76-82. doi: 10.1016/j.jim.2013.09.011.

Kanost M and Gorma MJ. (2008) Phenoloxidases in insect immunity. Insect Immunol. 1:69-96.
Kotani E, Yamakawa M, Iwamoto S, Tashiro M, Mori H, Sumida M, Matsubara F, Taniai K, Kadono-Okuda K, Kato Y, et al. (1995) Cloning and expression of the gene of hemocytin, an insect humoral lectin which is homologous with the mammalian von Willebrand factor. Biochim Biophys Acta. 1260:245-58. doi: 10.1016/0167-4781(94)00202-e.
Kurucz E, Zettervall CJ, Sinka R, Vilmos P, Pivarcsi A, Ekengren S, Hegedüs Z, Ando I, Hultmark D. (2003) Hemese, a hemocyte-specific transmembrane protein, affects the cellular immune response in Drosophila. Proc Natl Acad Sci U S A. 2003 Mar 4;100(5):2622-7. doi: 10.1073/pnas.0436940100.
Kurucz E, Váczi B, Márkus R, Laurinyecz B, Vilmos P, Zsámboki J, Csorba K, Gateff E, Hultmark D, Andó I. (2007a) Acta Biol Hung. 2007;58 Suppl:95-111. doi: 10.1556/ABiol.58.2007.Suppl.8.
Kurucz E, Márkus R, Zsámboki J, Folkl-Medzihradszky K, Darula Z, Vilmos P, Udvardy A, Krausz I, Lukacsovich T, Gateff E, Zettervall CJ, Hultmark D, Andó I. (2007b) Nimrod, a putative phagocytosis receptor with EGF repeats in Drosophila plasmatocytes. Curr Biol. 17:649-54. doi: 10.1016/j.cub.2007.02.041.
Lavine MD, Strand MR. (2002) Insect hemocytes and their role in immunity. Insect Biochem. Mol. Biol. 32:1295e1309. doi: 10.1016/s0965-1748(02)00092-9.
Lesch C, Goto A, Lindgren M, Bidla G, Dushay MS, Theopold U. (2007). A role for Hemolectin in coagulation and immunity in Drosophila melanogaster. Dev. Comp. Immunol. 31:1255-1263. doi: 10.1016/j.dci.2007.03.012.
Levy S. (2011) The pollinator crisis: What's best for bees. Nature 479:164–165. doi: 10.1038/479164a.
Lourenço AP, Zufelato MS, Bitondi MG, Simões ZLP. (2005) Molecular characterization of a cDNA encoding prophenoloxidase and its expression in Apis mellifera. Insect Biochem. Mol. Biol. 35:541-552. https://doi.org/10.1016/j.ibmb.2005. 01.013.
Marringa WJ, Krueger MJ, Burrit NL, Burritt JB. (2014) Honey bee hemocyte profiling by flow cytometry. PLoS One 9. http://dx.doi.org/10.1371/ journal.pone.0108486.
Martin SJ, Highfield AC, Brettell L, Villalobos E., Budge GE, Powell M, Nikaido, Schroeder DC. (2012) Global honey bee viral landscape altered by a parasitic mite. Science 336:1304-1306. doi: 10.1126/science.1220941.
Márkus R, Lerner Z, Honti V, Csordás G, Zsámboki J, Cinege G, Párducz Á, Lukacsovich T, Kurucz É, Andó I. (2015) Multinucleated giant hemocytes are effector cells in cell-mediated immune responses of Drosophila. J. Innate. Immun. 7:40e353. http://dx.doi.org/10.1159/000369618.
Meister M. (2004) Blood cells of Drosophila: cell lineages and role in host defence. Curr. Opin. Immunol. 16:10e15. doi: 10.1016/j.coi.2003.11.002.
Melcarne C, Ramond E, Dudzic J, Bretscher A, Kurucz É, Andó I, Lemaitre B. (2019) Two Nimrod receptors, NimC1 and Eater, synergistically contribute to bacterial phagocytosis in Drosophila melanogaster. FEBS JOURNAL 286:2670-2691. doi: 10.1016/j.ibmb.2019.04.002.
Moritz RFA, Miranda J. de Fries I, Conte YL., Neumann P, Paxton RJ. (2010) Research strategies to improve honey bee health in Europe. Apidologie 41:227-242.
Negri P, Maggi M, Szawarski N, Lamattina L, Eguaras M. (2014) Apis mellifera haemocytes in-vitro, what type of cells are they? Functional analysis before and after pupal metamorphosis. J. Apic. Res. 53:576-589. http://dx.doi.org/10.3896/ IBRA.1.53.5.11.
Negri P, Maggi M, Ramirez L, Szawarski N, Feudis LD, Lamattina L, Eguaras M. (2015) Cellular immunity in Apis mellifera: studying hemocytes brings light about bees skills to confront threats. Apidologie 47:379-388. http://dx.doi.org/10.1007/s13592-015-0418-2.
Nunes FM, Simões ZL. (2009) A non-invasive method for silencing gene transcription in honeybees maintained under natural conditions. Insect Biochem Mol Biol. 39:157-60. doi: 10.1016/j.ibmb.2008.10.011.
Potts SG, Biesmeijer JC, Kremen C, Neumann P, Schweiger O, Kunin WE. (2010) Global pollinator declines: trends, impacts and drivers. Trends Ecol Evol. 25:345-53. doi: 10.1016/j.tree.2010.01.007.
Raymann K, Moran NA. (2018) The role of the gut microbiome in health and disease of adult honey bee workers.

Curr Opin Insect Sci. 26:97-104. doi: 10.1016/j.cois.2018.02.012.
Renault S, Petit A, Bénédet F, Bigot S, Bigot Y. (2002) Effects of the Diadromus pulchellus ascovirus, DpAV-4, on the hemocytic encapsulation response and capsule melanization of the leek-moth pupa, Acrolepiopsis assectella. J. Insect Physiol. 48:297-302. doi: 10.1016/s0022-1910(01)00174-3. PMID:
Rizki MTM. (1957) Alterations in the haemocyte population of Drosophila melanogaster. J. Morphol. 100:437-458. http://dx.doi.org/10.1002/jmor.1051000303.
Rizki RM and Rizki TM. (1984) Selective destruction of a host blood cell type by a parasitoid wasp. Proc. Natl. Acad. Sci. U.S.A. 81:6154-6158. doi: 10.1073/pnas.81.19.6154.
Rizki RM and Rizki TM. (1992) Lamellocyte differentiation in Drosophila larvae parasitized by Leptopilina. Dev. Comp. Immunol. 16:103-110. doi: 10.1016/0145-305x(92)90011-z.
Rosenkranz P, Aumeier P, Ziegelmann B. (2010). Biology and control of Varroa destructor. J. Invertebr. Pathol. 103 Suppl 1, S96–S119. doi: 10.1016/j.jip.2009.07.016.
Rus F, Kurucz E, Márkus R, Sinenko SA, Laurinyecz B, Pataki C, Gausz J, Hegedus Z, Udvardy A, Hultmark D, et al. (2006) Expression pattern of Filamin-240 in Drosophila blood cells. Gene Expr. Patterns 6:928-934. doi: 10.1016/j.modgep.2006.03.005.
Schmid MR, Brockmann A, Pirk CWW, Stanley DW, Tautz J. (2008) Adult honeybees (Apis mellifera L.) abandon hemocytic, but not phenoloxidase-based immunity. J. Insect Physiol. 54:439-444. http://dx.doi.org/10.1016/j.jinsphys.2007.11.002.
Shen PF and Patterson LT (1983) A simplified Wright's stain technique for routine avian blood smear staining. Science 62:923-924.
Somogyi K, Sipos B, Pénzes Z, Kurucz E, Zsámboki J, Hultmark D, Andó I. (2008) Evolution of genes and repeats in the Nimrod superfamily. Mol Biol Evol. 25:2337-47. doi: 10.1093/molbev/msn180.
Somogyi K, Sipos B, Pénzes Z, Andó I. (2010). A conserved gene cluster as a putative functional unit in insect innate immunity. FEBS Lett. 584:4375-4378. doi: 10.1016/j.febslet.2010.10.014.
Stuart LM and Ezekowitz RA. (2008) Phagocytosis and comparative innate immunity: learning on the fly. Nat Rev Immunol. 8:131-41. doi: 10.1038/nri2240.
Tapaszti Z, Forgách P, Kövágó C, Békési L, Bakonyi T, Rusvai M. (2009). First detection and dominance of Nosema ceranae in Hungarian honeybee colonies. Acta Vet Hung. 57:383-388. doi: 10.1556/AVet.57.2009.3.4.
Theopold U, Li D, Fabbri M, Scherfer C, Schmidt O. (2002) The coagulation of insect hemolymph. Cell Mol Life Sci. 59:363-72. doi: 10.1007/s00018-002-8428-4.
van Alphen JM, Fernhout BJ. (2020) Natural selection, selective breeding, and the evolution of resistance of honeybees (Apis mellifera) against Varroa Zoological Lett.6:6. doi:10.1186/s40851-020-00158-4
Van Steenkiste D. (1988) De hemocyten van de honingbij (Apis mellifera L.): typologie, bloedbeeld en cellulaire verdedigingsreacties (Ph.D.thesis). University of Ghent.
Vilmos P and Kurucz E. (1998) Insect immunity: evolutionary roots of the mammalian innate immune system. Immunol. Lett. 62:59-66. doi: 10.1016/s0165-2478(98)00023-6.
Yang D, Zha G, Li X, Gao H, Yu H. (2017) Immune responses in the haemolymph and antimicrobial peptide expression in the abdomen of Apis mellifera challenged with Spiroplasma melliferum CH-1. Microb. Pathog. 112:279-287. https://doi.org/10.1016/j.micpath.2017.10.006.
Zufelato MS, Lourenço AP, Simões ZLP, Jorge JA, Bitondi MMG. (2004) Phenoloxidase activity in Apis mellifera honey bee pupae, and ecdysteroid-dependent expression of the prophenoloxidase mRNA. Insect Biochem. Mol.Biol. 34:1257-1268. https://doi.org/10.1016/j.ibmb.2004.08.005.
Zsámboki J, Csordás G, Honti V, Pintér L, Bajusz I, Galgóczy L, Andó I, Kurucz É. (2013) Drosophila Nimrod proteins bind bacteria. Cent.eur.j.biol. 8:633-645.

Williams MJ, Ando I, Hultmark D. (2005) Drosophila melanogaster Rac2 isnecessary for a proper cellular immune response. Genes cells. 10:813-823. http://dx.doi.org/10.1111/j.1365-2443.2005.00883.x.