University of South Bohemia

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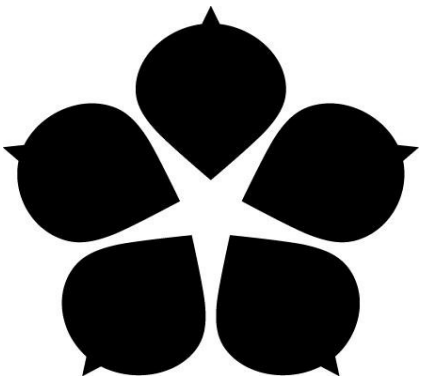

Drosophila macrophages – opening gates for immunometabolic research in insects

HABILITATION THESIS

Mgr. et Mgr. Adam Bajgar, Ph.D.

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CONTENT

Acknowledgment

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Heart, soul, mind in strife,
Macrophages, mighty knights,
Amoeboid healers.
A.B. 2024

Abstract of the thesis

Macrophages are the most functionally versatile cells in the animal body. Their characteristic functional repertoire ranges from protection against invading pathogens to clearance of dysfunctional cells, wound healing, and maintenance of tissue and metabolic homeostasis. Additionally, virtually every tissue in the body contains populations of tissue-resident macrophages that adopt specific features essential for the functioning of a particular organ.

Macrophages' impressive functional versatility relies on their characteristic features comprising perception of external signals, amoeboid motility, phagocytosis, adoption of diverse metabolic regimes, and the production of a wide spectrum of signaling factors. That allows macrophages to rapidly identify the source of the stress and coordinate the recovery of previously lost homeostasis.

Particular attention deserves the metabolic polarization of macrophages. Although it is a key to macrophage functional plasticity, metabolic induction of certain polarization phenotypes in inadequate context is on the basis of many human non-communicable diseases. Excessive macrophage activation can thus gradually lead to the development of many syndromes, of which non-alcoholic fatty liver disease, atherosclerosis, arthritis, neurodegenerations, diabetes, and cancer affect most significantly people in Western civilization. I believe that a better understanding of macrophages in the whole diversity of their natural functions may help us to better understand also their role in pathology.

In my work, I investigate the biology of macrophages in their non-canonical roles in the insect model organism *Drosophila melanogaster*. I study their role in the regulation of metabolism in infection, their nutritional roles in metamorphosis, starvation, as well as obesity. Currently, we expand our studies also to other insect species and we plan to study amoeboid cells even in more basal animal groups. Macrophages do not cease to fascinate us by their diverse features.

Here, I would like to present the work I have made over the last ten years in *Drosophila* and persuade readers that using this model organism may advance substantially our knowledge of macrophage biology. In addition, I present here a hypothesis about the evolutional origin of macrophages' functional versatility and put it in the context of the seemingly counterintuitive pathological behavior of macrophages. Finally, I marginally report here about my efforts to develop novel experimental tools to facilitate the investigation of macrophages in *Drosophila* and other non-model insect species.

I hope that my scientific endeavors will lead to a better understanding of macrophage physiology and pathophysiology and that my sometimes tiring and insignificant journey will finally lead to future significant discoveries.

Foreword

In this habilitation thesis, I will introduce macrophage biology seen from different angles to explain my motivation to dedicate my post-graduate scientific career to the study of these fascinating cells. Although some conclusions and ideas may be seen as speculative and not satisfactorily supported by the current knowledge, they represent my open-minded conviction and should explain my logic behind the planning and realization of particular projects. In my work, I aim to better comprehend the role of macrophages in various adaptive and pathological processes with an emphasis on their ability to govern systemic metabolism and thus promote the recovery of homeostasis.

I have been working on this topic for the last ten years together with my friends and colleagues, of whom my Ph.D. student Gabriela Krejčová is the most involved in the progress of our work and Tomáš Doležal stood with me and mentored me at the beginning of this scientific journey. We have shown that analogically to the situation in mammals, also *Drosophila* macrophages undergo metabolic polarization towards aerobic glycolysis which is an essential adaptation for their bactericidal function [1]. However, while this metabolic polarization allows macrophages to be highly efficient in killing bacteria, it is also associated with significant disadvantages as macrophages become functionally dependent on nutrients from external sources. We found that to ensure the nutritional supply, macrophages produce several signaling molecules that affect systemic metabolism and induce the redistribution of nutrients within the body.

We identified two of these factors which also represent two distinct strategies of how a change in cellular metabolism and enhanced nutritional demands may be reflected in the production of systemic signals. The first of these, extracellular adenosine (eAdo), is produced by cells as a reflection of the dramatically changing ATP:ADP:AMP:Ado ratio, and the released eAdo thus directly carries information about increased ATP consumption and enhanced metabolic stress [2,3]. The production of the second signaling factor, *Imaginal morphogenesis protein late 2* (*ImpL2*), is coupled with the adoption of aerobic glycolysis, which represents the metabolic program required for the phagocytic and bactericidal function of macrophages. Aerobic glycolysis is triggered by the central metabolic regulator *Hypoxia-inducible factor 1α* (*Hif1α*), which controls the expression of many metabolic genes involved in this metabolic program and along with this triggers also expression of *ImpL2* [4]. The resulting interorgan signaling, mediated either by eAdo or IMPL2, leads to the redistribution of carbohydrates and lipids from the central metabolic organs toward the nutritionally demanding immune system. While the mechanism behind eAdo activity is not completely resolved, we have revealed that IMPL2 acts via the intervention of insulin signaling in the central metabolic organ of flies and thus triggers the *forkhead box O* (FOXO) transcriptional program controlling the production of lipoproteins.

Our data further indicate that the *ImpL2* role may be conserved also in mammals as mammalian *ImpL2* homolog IGFPBP7 is produced by infection-activated liver macrophages. Moreover, increased production of IGFBP7 by liver macrophages increases the release of lipoproteins from

neighboring hepatocytes [4]. However, the adaptive meaning of this process for mobilization of nutrients for an activated immune system remains to be further experimentally approved.

The connection between aerobic glycolysis and the production of cytokines intervening in systemic insulin signaling is of particular interest. That raises the question of whether the adoption of aerobic glycolysis by macrophages, always inevitably leads to the production of cytokines causing insulin resistance in their surroundings. Such interconnection would have fundamental importance for understanding of adaptive meaning of insulin signaling during infection, hypoxia, or starvation, as well as its pathological aspects during obesity and other chronic inflammatory states. We found that *ImpL2/IGFBP7* production is strongly increased by macrophages in obese flies, but also mice, primates, and human patients, and that enhanced production of these factors is central for the development of systemic metabolic changes and subsequent metabolic syndrome [5].

Our data indicate that although insulin resistance is predominantly perceived as a pathological state accompanying obesity and other chronic inflammatory diseases, it may originally evolve as a metabolic adaptation for the mobilization of nutrients to be available for activated immune systems [6]. That may explain why the occurrence of pro-inflammatory macrophages is so common for many metabolic diseases and why inflammation is so closely interconnected with metabolism.

The hallmark of severe obesity and progressive metabolic syndrome is the infiltration of adipose tissue by macrophages. Although *Drosophila* is a widely used model organism for the study of human diseases including metabolic syndrome and diabetes, the interaction of macrophages with adipose tissue has been reported only in a few records with negligible importance. This represents the serious limits of *Drosophila* as a model for the study of obesity and obesity-related diseases.

We found that the fat body of adult flies is strongly infiltrated by macrophages in response to metabolic stress, such as starvation, infection, genetically induced lipodystrophy, or metamorphosis. Investigating the interaction of macrophages with adipocytes during metamorphosis, we found that macrophages engulf moribund larval adipocytes and convert a matter of dying cells, leaking lipids, and protein granules into storage peptides and lipoproteins that can be further exploited within the body. In this situation, macrophages transiently combine their ability to engulf and digest foreign objects in the phagolysosome with secretory metabolic properties previously attributed exclusively to adipocytes. That allows them to contribute to the recirculation of cellular fragments and nutritionally support other ongoing physiological processes such as the maturation of reproductive organs [7].

Our work indicates that even in simple organisms such as *Drosophila*, macrophages can adopt many diverse roles that are often far from their immune-related protective function. Moreover, as documented in the case of IMPL2/IGFBP7 signaling, the production of the same factor may have a beneficial role in stress response while it becomes pathogenic if chronically activated. That led

us to the question of where in the evolution macrophage-like cells emerged in the animal kingdom and when acquired their characteristic features.

We have compiled a literature review bringing several pieces of evidence indicating that macrophage pro-inflammatory polarization originates in unicellular predatory ancestors of multicellular animals and that already first multicellular animals possess macrophage-like amoeboid cells. Macrophages seem to be necessary for the coherence of multicellular organisms, inter-cellular signaling, regeneration, and maintenance of homeostasis. We speculate that macrophage-like amoebocytes stand for the functionalities of the not-yet-developed nervous and endocrine system and coordinate the function of distinct cellular lineages. Based on that, we proposed that macrophages attained many of their crucial features in conditions and environments far from that observed in human tissues and that the ancient origin of macrophage features may in some cases explain their pathological behavior [8].

We believe that our observations made in *Drosophila* represent only a tiny piece of not yet unexplored spectrum of non-canonical functions of immune cells in insects, particularly if we consider a broad ecological and morphological variability in this clade. To facilitate the experimental investigation of immune cells even in non-model insect species, we developed many diverse modifications of glucan microparticles. That allowed us to establish a label-free method for the isolation of macrophages on the magnetic columns and develop tools for macrophage-specific delivery. These tools can be used for macrophage-specific regulation of transcription (via delivery of recombinant transcription factors) enzymatic activity (via delivery of small metabolic inhibitors), and as indicated by our preliminary data also expression (via delivery of siRNAs) [9–11]. These tools will hopefully enable us to be spectators of the many fascinating and surprising processes in which macrophages participate.

List of authored publications used as a basis for the habilitation thesis:

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Authorship code - [*]Corresponding author; [+]First author; [#]Responsible for *Drosophila* part of the project

1[*] Krejčová, G., Danielová, A., Nedbalová, P., Kazek, M., Strych, L., Chawla, G., Tennessen, J.M., Lieskovská, J., Jindra, M., Doležal, T., **Bajgar A.** (2019). Drosophila macrophages switch to aerobic glycolysis to mount an effective antibacterial defense. Elife 8. 10.7554/eLife.50414.

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Discovery of macrophages

Macrophage role in immunity was discovered by the embryologist and evolutional biologist Ellie Metchnikoff (Ilya Ilyich Mechnikov) in the late 1890s (Fig. 1)[12]. Although it may seem clear to us today that macrophages phagocytose foreign objects and try to protect the organism from pathogens, several very original ideas led to this discovery.

Metchnikoff like many of his colleagues in the 19th century was a universal scientist who was interested in many fundamental unanswered questions. He was devoted to the study of how primitive animals lacking digestive systems gather nutrients, how is maintained the homeostasis between different cellular lineages, how animals change over metamorphosis, and how the first multicellular organism looked like [13]. Interestingly, all these questions are interconnected by topics of current cellular biology and to a large extent related to macrophages.

During the study of starfish metabolism, Metchnikoff's attention was soon attracted by motile amoeboid cells crawling in mesoglea and showing striking capability to internalize injected pigment granules. These amoeboid cells were activated by the presence of foreign material, were attracted to its vicinity, and tended to encapsulate and internalize the foreign matter to isolate it from the rest of the body. Moreover, these amoeboid cells were able to distinguish between parts of themselves and foreign material [14]. Metchnikoff speculated about the nutritive importance of these cells in primitive multicellular organisms and called these fascinating cells "macrophages ambulatoires" which can be translated as traveling big-eaters. After several experiments using different foreign objects and bacteria, he realized their huge potential in protecting the organism against pathogenic organisms and established the "phagocytic theory of immunity"[15]. Along with this, Metchnikoff highlighted the importance of these cells also for other processes within the multicellular body such as nutritive phagocytosis formation of developing individuals and clearance of damages and senescent cells [13].

Metchnikoff believed that the first animals were similar to amoeboid phagocytic cells and that the first multicellular organism was formed by aggregation of these cells. He called this hypothetical organism "*Phagocytela*", thus sparking several years of dispute with his contemporary Ernst Haeckel [16]. Metchnikoff perceived multicellular organisms as "inherently disharmonious" and composed of distinct cellular lineages completing different tasks and trying to outcompete the others. Based on that, he believed that a functioning multicellular organism requires harmonizing power, which he saw in macrophages [17]. Metchnikoff thus discovered not only the macrophage role in the immune response but also recognized that the macrophage role resides in their ability to restore and maintain tissue homeostasis.

Figure 1. *Metchnikoff in his laboratory in 1913, five years after being awarded the Nobel Prize. (Source: Gallica Digital Library digital ID btv1b6926750k/f1).*

To fully appreciate Metchnikoff's genius, we must remember that the discovery of macrophages in protecting organisms from bacterial infection was revealed in the era of Koch's newly formulated postulates, which fully defined the "germ theory of diseases" [18].

The original ideas of Metchnikoff's theory were thus formulated at a time when the prevailing explanation of the origin of diseases was attributed to bad air, the so-called miasma theory (Fig. 2) [19]. Metchnikoff, as a big admirer of Charles Darwin, described the protective role of phagocytic cells not only in starfish, but also in insects, worms, and many vertebrate species and proposed evolutional conservation of their functions. Despite his discovery of macrophage protective immune function attracted immense attention, he never relayed his initial ideas of the nutritive role of phagocytosis and macrophages as maintainers of tissue homeostasis [17]. Metchnikoff postulated that the concept of nutritive phagocytosis could extend to higher multicellular organisms and studied in this context the role of macrophages in the retraction of the tadpole tail in metamorphosis. Metchnikoff proposed that the processes he observed during the protection of the organism against bacteria can be to some extent applied also in healthy tissues and introduced the term "homeostatic inflammation" as a process promoting tissue regeneration [20].

By mere observation and excellent experimental logic, Metchnikoff was able to set the base not only for modern immunology but also for our modern conception of macrophage roles in organisms that have been during the following one hundred years vastly omitted. Currently, we know that macrophages form an important part of the reticulum of the mononuclear phagocyte system eliminating pathogens, clearing damaged cells and toxic compounds from the body, and also playing a central role in tissue regeneration and response to metabolic stress [21].

Compared to other cells within the body, macrophages are the ultimate multitaskers that can adjust their behavior plastically to the actual context [22]. Moreover, tissue-resident macrophages, present in virtually every tissue in the body, often exhibit highly specialized and unexpected features [23]. More than a century later, equipped with modern techniques of molecular and cell biology, we are trying to fully understand Metchnikoff's ideas, in which he recognized macrophages as the dominant harmonizing force controlling homeostasis in otherwise "inherently disharmonious multicellular organisms".

Figure 2. *Lithography painted in 1831 by Robert Seymour, depicting cholera spreading through a war field like a tainted air. Source: U.S. National Library of Medicine Digital Collections.*

Macrophages - truly exceptional

Compared to other cells in the animal body, macrophages display several specific features that underpin their phenotypic plasticity and functional versatility. These properties make macrophages truly exceptional and to some extent superior to ordinary cells, which often perform only one repeated task function during their whole lifetime.

Macrophages are equipped on their surface with a broad spectrum of receptors that enable them to perceive different signals from their micro-environment. These receptors, spread over the whole surface of the cell, can perceive even tiny differences in the titer of the signaling molecules and provide thus macrophages with directional information about the source of the signal [24]. Macrophages thus have information about changes in extracellular space and also about the direction these signals are coming from. Macrophages are chemotactically guided through the environment against the concentration gradient of diverse stimuli, such as chemokines, polyunsaturated fatty acids, leukotrienes, eicosanoids, folic acid, components of the complement cascade, formyl peptides, etc. [25]. However, macrophages do not react only to the presence or absence of a specific signal, but their activation is driven by any significant change in the physicochemical conditions of the external environment. Thus, they react to changes in ions, pH, extracellular matrix composition, or oxygen availability [25]. According to the "disharmonious continuum theory", it is assumed that any change in external conditions leads to macrophage activation, which seems to be an important prerequisite for macrophages to become the guardians of tissue homeostasis.

Wile ost of the time, macrophages reside within tissue as sentinel cells, perception of any danger signal leads to their activation and enhanced motility. Macrophages are exceptionally motile cells that can crawl over the surface of other cells and extracellular matrix. Stimulation of surface G-protein coupled receptors (GPCRs) and subsequent signaling via second messengers triggers stress response-related signaling cascades, such as PKC, PI3K-AKT-mTOR, MAPK-ERK, AP, and JAK-STAT*. These cascades overall enhance the expression of genes predominantly involved in the remodeling of cytoskeleton, actin polymerization, generation of lysosomal enzymes, and phagocytosis [25]. Macrophages can thus sense tissue disharmony or stress signal production in relatively distant tissues. These signals lead to their awakening from a quiescent metabolic state and activate their motility throughout the body toward the source of the stress signal.

Once macrophages approach the source of chemotactic signals, they are exposed to factors in the local microenvironment and gather information about the ongoing processes through tactile sensing and micropinocytosis. Macrophages use their pattern recognition receptors (PRRs) to detect pathogen and damage-associated molecular patterns (PAMPS and DAMPS) [26]. Macrophages exhibit a broad spectrum of PPRs, categorized into several classes according to their

* PKC – proteinkinase C; PI3K - Phosphoinositide 3-kinase; AKT - Protein kinase B; mTOR – mammalian target of rapamycin; MAPK – mitogen activated protein kinase; ERK extracellular signal regulated kinase; AP – activing protein; JAK – Janus kinase; STAT - signal transducer and activator of transcription proteins.

structure. Many of these receptors, such as toll-like receptors, scavenger receptors, c-type lectins, or NOD-like receptors, are evolutionarily conserved and are of ancient origin [27]. Macrophages' ability to recognize PAMPs has evolved over billions of years of coevolution between pathogens and macrophages [28], and their ability to sense DAMPs has evolved with the increasing complexity of the body plan of multicellular organisms [29].

Infiltrating macrophages thus stand at a crossroads and "decide" how to react according to the external signals. This decision is also strongly influenced by chemical signals - cytokines, produced in a given place by their counterparts as well as other immune cells. This brings us to a very interesting situation in which the mutual interaction of many individual units that can make certain decisions leads to either the acceleration or resolution of the whole physiological process. Macrophages in a pathological situation, often outnumber the original cells of the tissue, thus functioning to some extent as a computing device, with hardly predictable outcomes (Fig. 3).

During infection, macrophages detect the presence of pathogens or pro-inflammatory cytokines and adopt pro-inflammatory polarization. Antigen binding to PRRs activates macrophage immune-related cascades, such as NFκB, ERK, JNK, and p38**, which initiate complex cellular response resulting in local remodeling of the macrophage cytoskeleton and formation of membrane invaginations to engulf the particle and form a phagosome [30]. Subsequently, the primary phagosome fuses with acidic lysosomes, which contain a mixture of enzymes that cleave the phagocytosed material. Activation of specific mechanisms for acidification and generation of reactive oxygen species into the lumen of phagolysosome later ensures the elimination of bacterial threat [31].

** NFκB – nuclear factor kappa B; JNK - c-Jun N-terminal kinases; p38 -mitogen-activated protein kinase

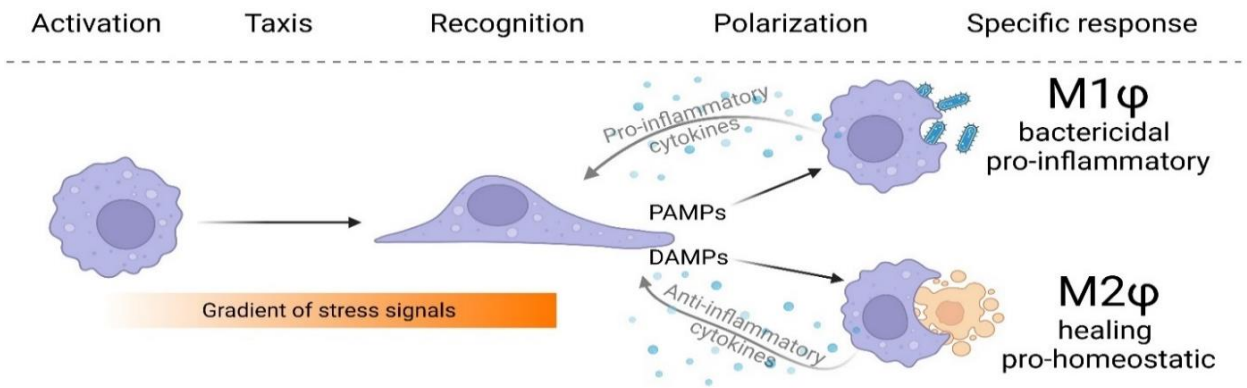

Figure 3. *Macrophages are activated by even tiny changes in extracellular space and migrate against the concentration gradient toward the source of activating signal. There they perceive local pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs). Based on extrinsic cues and intrinsic predeterminations, macrophages functionally polarize to diverse functional polarization states from which M1 or M2 polarizations represent two extremes of the whole spectrum. Functional polarization is accompanied with production of characteristic cocktail of cytokines influencing polarization of other cells and tissues. Adopted from: "On the origin of the functional versatility of macrophages". Adam Bajgar, Gabriela Krejcova 2023* [8].

That is achieved by the activity of *natural resistance-associated macrophage proteins* (NRAMP) transporters, which pump divalent ions into the phagolysosome lumen and by the activity of mitochondria attached to the surface of phagolysosome [32]. Bactericidal (pro-inflammatory) macrophages along with this process release a cocktail of proinflammatory cytokines such as interleukins (Il-1; Il-6), tumor necrosis factors (TNFα), and interferons (IFN-γ) [33]. The whole nature of infiltrating macrophages thus changes dramatically and has to be supported by adjustment of virtually all major metabolic pathways of the cell, as will be more thoroughly described in the following chapter. Importantly, macrophages can adopt pro-inflammatory polarization even in the absence of a pathogen. In this scenario, antigens naturally occurring within the tissue trigger activation of immune-related cascades leading to pro-inflammatory polarization that is further amplified by the release of pro-inflammatory cytokines resulting thus in sterile inflammation [34].

In situations of tissue or organ failure, infiltrating macrophages can locally recognize signals produced by damaged, senescent, and apoptotic cells known generally as DAMPs, which induces macrophage healing polarization phenotype [35]. Healing macrophages clear damaged cells and their remnants by a process called efferocytosis and produce factors promoting angiogenesis, remodeling of extracellular matrix, and proliferation of progenitor cells. Healing macrophages can uptake and deposit all the major components of the extracellular matrix, such as collagen, elastin, reticulin, fibronectin, laminin, and proteoglycans [36]. Healing macrophages are thus indispensable for processes such as regeneration, wound healing, and embryonic morphogenesis, but play also a fundamental role in the immune response to viral and nematode infections [37].

Healing macrophages function in the body as a kind of counterbalance to the pro-inflammatory macrophages and contribute significantly to the resolution of inflammation and the restoration of tissue homeostasis after an inflammatory response [38]. Healing macrophages thus represent the fundamental force for the maintenance of immunological tolerance, i.e., the prevention of an immune response to its antigens, and keeping of the symbiotic microbiota [39].

Macrophages are not only masters in the perception of signals from the environment but also in their production. Macrophages generate many signaling factors that effectively regulate the functioning of neighboring cells and shape the surrounding extracellular matrix. Macrophage-derived cytokines regulate many important processes such as tissue metabolism, apoptosis, regeneration, proliferation, etc. [40]. For simplification, macrophages are commonly distinguished as pro-inflammatory and anti-inflammatory, however, currently, the whole macrophage polarization spectrum is recognized [41]. Nevertheless, both these polarization phenotypes are required within the organism. For instance, during infection, both pro-inflammatory and healing macrophages are present on the site of infection, and their ratio changes throughout the immune response [42]. While pro-inflammatory macrophages are essential during the acute phase of infection, healing macrophages mediate the resolution of infection, tissue regeneration, and wound healing. Interestingly the breaking point in which accelerating inflammation turns back to its resolution and restoration of homeostasis is not well understood (Fig. 4).

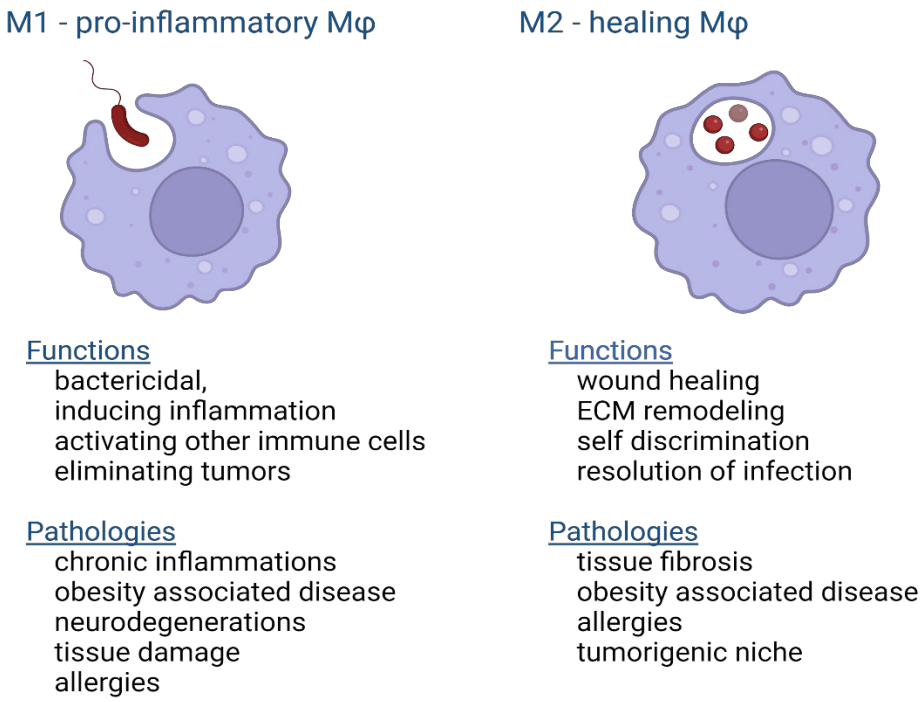

Figure 4. *The polarization states of macrophages M1 - pro-inflammatory and M2 - healing allow different macrophage functions, but can also cause the development of serious human pathologies.*

It can be assumed that neither of these macrophage polarization phenotypes is good or bad for our health. What makes an organism healthy is the ability to efficiently transition from one polarization state to another, thus maintaining macrophage plasticity within their various populations at maximum levels. Because of the enormous influence of macrophages on the function of all tissues and organs in the body (which will be addressed later in this work), macrophage plasticity is what broadly determines the health of the overall organism. The remaining question is how macrophage plasticity can be trained. The answer can be found in the controlled induction of metabolic stress in these cells, which throws them out of balance for a short period and thus activates the mechanisms restoring their recovery. I believe that many of the common practices that are generally used to heal the body, such as fasting, cold water bathing, special diets, and aerobic exercise (resulting in anaerobic states in tissues) fundamentally affect the metabolism and subsequent polarization of macrophages, which thus represent a possible connection between these practices and their healing effects [43]. The interplay between the metabolic polarization of macrophages and their functional polarization will be further addressed in the following chapter.

Metabolic polarization - key to macrophage phenotypic plasticity

As mentioned above, macrophages can adopt different polarization phenotypes based on the assessment of internal and external signals. This ability allows them to perform a variety of functions in different biological situations, which also applies to a wide range of different biological situations and tissues [43]. To disentangle something so complex would be impossible and therefore scientists resorted to a reductionist conception of this issue and developed *in vitro* protocols to set up two extremes on the otherwise continuous spectrum of macrophage polarizations. Macrophages activated according to "classic protocol" by using lipopolysaccharides (LSP) and interleukin IL1β were considered proinflammatory, also known as M1. Contrary to that macrophages activated by their exposure to interleukins IL4 and IL13 were established as healing macrophages, alias M2 [44]. Although these artificial experimental systems do not fully reflect the natural situation in tissues, they have made it possible to identify important differences between the two polarization states and thus gain insight into their role in the context of different physiological processes.

It was found that M1 and M2 macrophages differ primarily in their cellular metabolic program[45]. This wouldn't be so surprising, as each cell type must produce different metabolic intermediates necessary for the production of effector molecules, but it turned out that cellular metabolism itself directly determines macrophages' polarization [46]. In addition, inhibition of the ability to enter one metabolic state often leads to the activation of the other and thus it seems that both states are to some extent exclusive and mutually interconnected. If the oxidative phosphorylation is inhibited at the level of AMPK or mitochondrial metabolism itself, macrophages automatically polarized into the proinflammatory M1 phenotype. Conversely, if glycolysis or the metabolism of pyruvate to lactate is intervened, anti-inflammatory polarization predominates [47].

The interconnection of macrophage metabolism with their phenotypic polarization is crucial for several reasons. Firstly, it shows that the metabolic imbalance within the tissue can consequently lead to a certain polarization and potential pathology. On the other hand, it opens the door to relatively natural possibilities for intervention of macrophage polarization by targeting their metabolism. At the same time, it may explain the reaction of macrophages to various immune-unrelated stimuli such as hypoxia, over-nutrition, or malnutrition leading often to activation of pro-inflammatory states [48].

The metabolism of M1 pro-inflammatory macrophages is known as aerobic glycolysis. The primary goal of this metabolic program is to produce a large amount of energy in the form of ATP and reducing equivalents necessary for the production of reactive oxygen species and acidification of the phagolysosome. The pro-inflammatory polarization begins with the stabilization of *Hypoxia-inducible factor 1α* (*Hif-1α*), which is the main transcription factor responsible for the rewiring of cellular metabolism towards aerobic glycolysis [49]. HIF-1α is constantly translated and

degraded by protein ubiquitination and its stabilization requires the intervention of *prolyl-hydroxylase dehydrogenase* (*Phd*) function. PHD's ability to hydroxylate HIF-1α depends on several cofactors such as the availability of oxygen, metabolites of the TCA cycle such as α-ketoglutarate, and ferrous ions (Fe^{2+}). HIF-1α is therefore stabilized under situations of hypoxia, during sequestration of cytosolic iron, or in case of an interrupted standard course of the cycle of tricarboxylic acids (TCA) [50].

A common course of HIF-1α stabilization upon detection of bacterial antigens is via activation of the *toll-like receptor 4* (TLR4) and downstream *nuclear factor kappa B* (*NFκB)* signaling pathway. That leads to increased expression of *Ferritin* and its accumulation in cytosol. Ferritin binds free ferrous ions thus inducing the initial stabilization of HIF1α (Fig. 5) [51]. HIF1α is a transcription factor enhancing the expression of many genes via binding specific sites in their promoter called hypoxia response element (HRE) [52]. Virtually hundreds of metabolic genes involved in the adoption of aerobic glycolysis are under the control of HRE [53]. The most prominent changes in the cellular metabolism of pro-inflammatory macrophages are increased glycolysis and pentose phosphate pathway, conversion of pyruvate into lactate instead of its transport to mitochondria, and broken TCA cycle utilizing glutamine as an essential precursor [54]. All these changes lead to enhanced production of ATP and NADPH required for active motility of macrophages, phagocytosis, and acidification of phagolysosome. Mitochondria is no longer used for the production of energy and engaged to phagolysosome forms reactive oxygen species for bacterial killing. Itaconate, succinate, and fumarate accumulate in the cytosol, while significantly less of α-ketoglutarate is produced [55]. In addition to being frequently associated with their direct antibacterial effects, changes in TCA products lead to the secondary stabilization of HIF1α via mitochondrial retrograde signaling and further maintenance of the entire metabolic program of the cell [56].

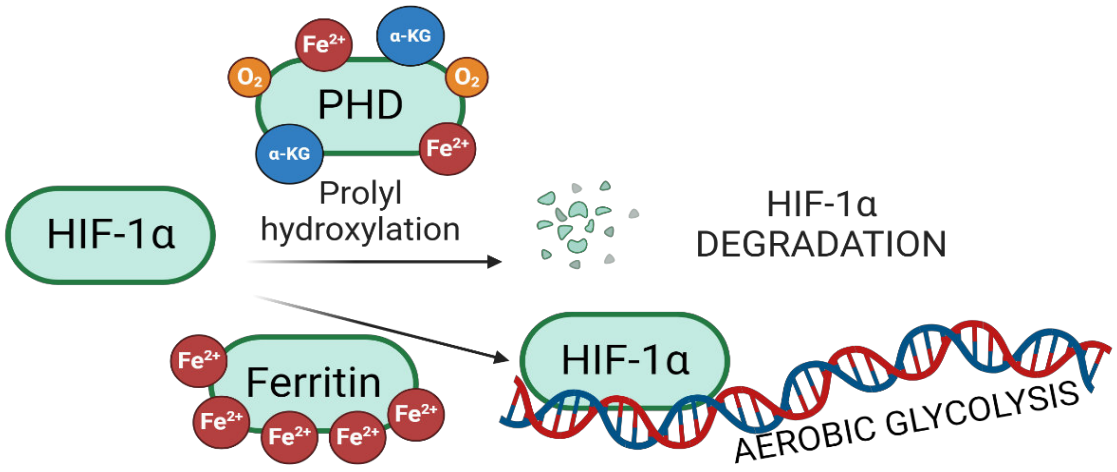

Figure 5. *Model of HIF-1α stabilization in normoxia. Infection-activated macrophages enhance expression of Ferritin by transcriptional activity of NFκB factor resulting in sequestration of cytosolic Fe^{2+} ions and intervention of function of prolyl hydroxylase dehydrogenase (PHD). HIF-1α is later stabilized by mitochnodiral retrograde transport by lack of α-ketoglutarate (α-KG). Aerobic glycolysis is finally stabilized by epigenetic remodeling influenced by availability of TCA products as cofactors for this process.*

Mitochondria and *NADPH-dependent oxidoreductase (NOX)* generate a large amount of reactive oxygen species into the phagolysosome, and ATP is used to cover the enormous energy demands associated with active motility and phagocytosis. In parallel, NADPH and arginine serve as substrates for *inducible nitric oxide synthase* (*iNOS*) generating reactive nitric species [57].

A very interesting aspect of aerobic glycolysis is the apparent preference for speed over the economical utilization of sources. Activated cells thus rapidly deplete their cellular carbohydrate reserves and rely on external sources of glucose, amino acid glutamine, but also cholesterol, and phospholipids [58].

Contrary to M1 pro-inflammatory polarization, cells adopting the M2 healing phenotype primarily rely on generating ATP through the classical pathway of oxidative phosphorylation. This metabolic regime is governed by *AMP-activated protein kinase* (*AMPK*) and is also largely dependent on the utilization of fatty acids as an energy source [59]. The primary task of healing macrophages is the detoxification of tissues, removing dead and damaged cells through efferocytosis, and remodeling the extracellular matrix [60].

The metabolism of M2 macrophages thus generates the required precursors for these processes. That may be demonstrated by the metabolism of arginine, which is entirely different from pro-inflammatory macrophages. Arginine is metabolized into ornithine and urea, which further serve for the synthesis of putrescine, spermidine, spermine, proline, and collagen. These compounds are essential for extracellular matrix remodeling and tissue regeneration (Fig. 6) [61].

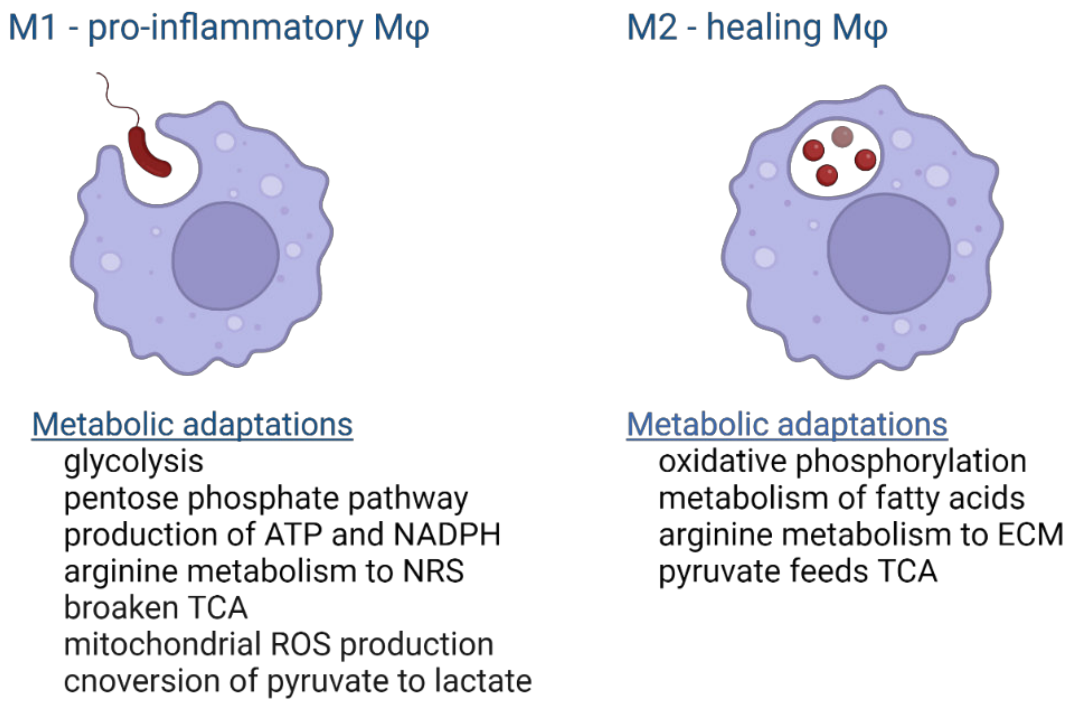

Figure 6. *M1 and M2 macrophages substantially differ in their cellular metabolism. While metabolism of M1 is adjusted for production of enough of ATP, NADHP and reactive oxygen and nitric species, M2 metabolism supports processing of engulfed mater of dying cells and generate components of extracellular matrix.*

Once the macrophage chooses one polarization, it must be fixed for some time. This can be achieved by epigenetic modifications. Several TCA intermediates serve as cofactors or substrates for epigenetic modifying enzymes. Mitochondrial retrograde transport of these metabolites likely represents a mechanism of stabilization of macrophage metabolic polarization and characteristic TCA products influence the epigenetic setup of polarized macrophages [62].

These fascinating findings provided fundamental information for understanding the principles of macrophage function. However, it can be expected that there are a large number of subtle deviations from these metabolic programs. To this day, a whole range of polarization types are distinguished and recognized, which are characteristic of specific processes in the body (M1, M2, M2a, M2b, M2c, Mhem, Mox, M4) [63]. However, this is by no means an exhaustive description of the functional versatility of these fascinating cells, whose full functional repertoire can be admired in the context of tissue-resident subpopulations.

Tissue-resident macrophages – revealing the full spectrum of functionalities

As already described in previous chapters, macrophages display a whole range of abilities that enable them to fulfill many diverse roles within the organism. They can perceive changes in the environment very sensitively, move toward the source of chemical signals, and enter different metabolic and functional polarizations. In addition to these properties that are common to all macrophages in the body, there are many extraordinary, tissue-specific, tasks that macrophages can perform that are absolutely essential to the function of the body [64].

Common macrophages of the reticuloendothelial system originate mostly in the bone marrow and their continuous production in bone marrow is essential for the replacement of senescent macrophages and maintenance of their number with aging of individual [23]. However, in addition to these cells, virtually every tissue in the body contains a unique population of tissue-resident macrophages. Tissue-resident macrophages infiltrate particular organs and tissues already during embryonal development from the yolk sack and fetal liver and the renewal of individual subpopulations takes place by their self-replication directly in a given organ [65]. Today, the whole range of tissue-resident macrophages is recognized that perform very diverse functions in the organism. Macrophages are thus responsible for specialized functions such as nutritional support for neurons, clearance and production of surfactants in lungs, detoxification process in the liver, control of hormonal signaling in adipose tissue, control of ovulation, supporting production of breast milk, maintaining hair bulb cells, building and degrading bone matter, etc. (Tab. 1) [66].

Table 1. List of recognized tissue-resident macrophages and their functions within tissue.

Tissue-resident macrophages	Function
Kupffer cells (liver)	Clearance of pathogens and debris from the bloodstream.
Alveolar macrophages (lung):	Responsible for clearing inhaled particles, pathogens, and mucus.
Microglia (brain)	Immune defense, nutritional support to neurons, hypoxic response.
Langerhans cells (skin)	Antigen-presenting, regeneration, hair growth support.
Splenic macrophages (spleen)	Red blood cell clearance, regulation of insulin production.
Peritoneal macrophages	Tissue repair and host defense against intra-abdominal pathogens.
Renal macrophages (kidney)	Clearance of waste products, and maintenance of tissue integrity.
Intestinal macrophages (gut)	Homeostasis, immune defense, regulation of mucosa.
Synovial macrophages (joints)	Inflammation and tissue repair in synovial joints.
Adipose tissue macrophages	Regulation of lipid metabolism, insulin sensitivity, and regeneration.
Mammary gland macrophages	Regulation of function and development of mammary gland.
Cardiac macrophages (heart)	Tissue repair, regulation of cardiac tonus, and cardiac remodeling.
Adrenal macrophages	Regulate the production of hormones and inflammation.

Certainly, it is worth noting that individual populations of tissue-specific macrophages are dedicated to their functions by both extrinsic and intrinsic factors. Macrophages in a given tissue are exposed to factors whose action leads to the determination of a specific functional type. Moreover, by changing these factors, we can alter the specialization of macrophages under experimental conditions, which often results also in a change in the whole cellular morphology [67]. The distinct populations of tissue-resident macrophages differ significantly from each other, especially in their epigenetic landscape, which defines their characteristic morphological and functional properties [66]. Nonetheless, all tissue-resident macrophages possess general macrophage features, which they extensively utilize as support for their specialized roles. This refers to their ability to be activated from a resting state by cytokines, to be mobile, and to polarise metabolically towards pro-inflammatory or anti-inflammatory polarisation states [68]. Prolonged metabolic polarization of tissue-resident macrophages may intervene in their function which consequently leads to the development of tissue-specific pathologies.

Macrophages were identified to be responsible for the development of diseases such as metabolic syndrome, diabetes, cardiovascular diseases, neurodegenerative diseases, cancer, cachexia, autoimmune diseases, etc [69]. All these diseases share in common that they belong to a group of non-communicable diseases for which it seems to be particularly difficult to develop effective treatment. Modern medicine is thus currently focused on regulating macrophage polarization through intervention in their metabolism, however, even this strategy seems not to be easy. The complexity of such diseases can be illustrated by the example of obesity, where macrophages play a crucial role at several levels.

The role of macrophages in the pathogenesis of obesity-related diseases

Macrophages play an important role in the pathogenesis of obesity. During obesity, the body suffers from excessive caloric intake that results in the accumulation of lipids in adipose tissue, and the liver, which later leads to ectopic lipid deposition in peripheral tissues [70]. Macrophages are involved in the pathogenesis of obesity on several layers. First, tissue-resident macrophages react to stress situations and try to recover lost homeostasis. This is followed by massive infiltration of the affected organ by macrophages from the circulation, which often outnumber the original cells in the tissue. Macrophages in reflection of excessive amounts of lipids cause tissue inflammation, which further accelerates the pathology. However, to fully understand obesity, we need first to understand the role of macrophages in these tissues in healthy individuals [71].

A common role of adipose tissue macrophages relies on removing dying adipocytes, turnover of lipids, and stimulation of angiogenesis [72]. Additionally, they can initiate the regeneration of adipose tissue and promote the replacement of dying adipocytes with new ones regulating the neoplasia of the tissue. That is particularly important for animals in which the volume of adipose tissue is periodically changing and in which hyperplasia and neoplasia of adipocytes can substantially increase the volume of lipid stores. Recently, adipose tissue macrophages were found to control hormonal signaling in adipose tissue and govern thus lipogenesis and even thermogenesis [73].

Liver macrophages, known as "Kupffer cells", naturally play a crucial role in the detoxification of the body. Kupffer cells remove a large number of damaged and senescent cells and modified lipids from circulation. Moreover, they serve as sensitive sensors of metabolic stress and promote the production of nutritionally rich compounds from the liver to circulation. That can be achieved by the release of cytokines affecting the metabolism of hepatocytes in their close vicinity [74].

Arterial macrophages are indispensable for the metabolism of cholesterol in healthy individuals. Lipids are released into the bloodstream from the liver in the form of low and very-low-density lipoproteins (LDLs and VLDLs) that are water soluble and can be thus easily transported to the periphery [75]. Besides large amounts of triglycerides, sphingomyelin, and ceramide, LDLs, and VLDLs also carry cholesterol, which is their essential structural component [76]. While triglycerides from lipoproteins are consumed on the periphery, cholesterol must be recycled back to the liver via "reverse cholesterol transport". This recycling of cholesterol is fundamental for the formation of new lipoprotein molecules in the liver and the functioning of lipid metabolism in the body. Arterial macrophages are essential for the whole process by extracting cholesterol from lipoproteins in arteries and loading it into high-density lipoproteins (HDLs) to be transported back to hepatocytes [77].

We can see that even in healthy individuals, macrophages are strongly entangled with the metabolism of lipids in the body. However, while their capacity is sufficient for most situations, it is not the case for obesity. As obesity progresses, we can observe how adipose tissue, liver, and

arterial walls are gradually overwhelmed by the products of lipid metabolism and how these lipids affect macrophages residing in these tissues (Fig. 7) [78,79].

Excessive intake of dietary lipids is initially buffered by adipose tissue. This tissue is very well equipped to store unnecessary dietary lipids in the form of triglycerides. Prolonged intake of excessive calories, however, exceeds the limits of entire adipose tissue [80]. Adipocytes unable to further store excessive nutrients become hypertrophic and leaky for lipids. They lose contact with the extracellular matrix, initiate autophagy, and undergo apoptosis [81]. These signals activate adipose tissue macrophages and invoke the infiltration of macrophages from circulation [82]. Macrophages engulf fragments of dying adipocytes and lipids to reduce ectopic lipid deposition. Macrophages typically become filled with lipid droplets and acquire a characteristic appearance known as "foamy" or "lipid-laden" macrophages. That is accompanied by their pro-inflammatory polarization and production of pro-inflammatory cytokines such as IL1, IL6, and TNFα [83]. Inflammation in adipose tissue causes adipocytes insensitive to insulin signaling which makes the whole situation even worse. Insulin-resistant adipocytes further enhance the mobilization of lipids into circulation, in the form of exosomes (AdExos) and free fatty acids (FFAs) accumulating subsequently in the liver hepatocytes [84].

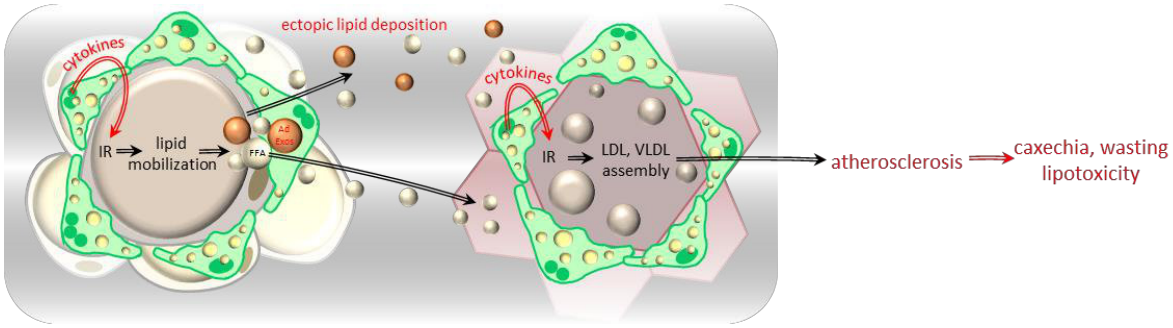

Figure 7. *Dual role of macrophages in obesity-related diseases. Obesity leads to progressive accumulation of lipids in adipose tissue, liver, and arteries. In response to metabolic stress induced by excessive lipids all these tissues are infiltrated by macrophages. Infiltrating macrophages exposed to excessive lipids adopt pro-inflammatory polarization and produce factors intervening insulin signaling in particular tissues. Insulin resistance subsequently leads to the development of metabolic syndrome and type two diabetes. Design credit: Krejčová G., Bajgar. A.*

The large accumulation of lipids in the liver leads to lipotoxicity of this metabolic organ [85]. Excessive amounts of FFAs, often present as polyunsaturated fatty acids (PUFAS) or non-esterified fatty acids (NEFAS), are toxic to hepatocytes, and macrophages try to scavenge these lipids to protect the vital function of the liver. Accumulation of these lipids in the cytosol of cells can initiate lipid peroxidation, chain reaction eventually leading to the depolarization of the mitochondrial cell membrane and induction of cell death [86]. Kupffer cells together with infiltrating macrophages become pro-inflammatory and release pro-inflammatory cytokines inducing insulin resistance in hepatocytes [87]. Insulin resistance has the opposite effect on hepatocytes than on most other cells

in the body, which is a phenomenon known under the term "liver insulin resistance paradox" [88]. Insulin-resistant hepatocytes enhance lipogenesis and lipolysis and generally increase the mobilization of energy-rich substances. Eventually, exhausted and damaged hepatocytes are removed by liver macrophages and tissue is restored by deposition of extracellular matrix leading to irreversible tissue fibrosis [89].

Mobilized lipids from the liver enter circulation mainly in the form of lipoproteins. Lipoproteins circulate in the bloodstream and are captured in vessels by endothelial cells expressing *lipoprotein receptors* and *lipoprotein lipase* [90]. However, an excessive titer of lipoproteins in circulation called hyperlipidemia results in the accumulation of lipoproteins within the arterial intima. Aggregates of acetylated and oxidized lipoproteins are surrounded by macrophages which tend to clear the arterial walls from lipid deposits [91]. Macrophages take up lipoproteins through scavenger receptors and metabolize them in lysosomal compartments. Lipoproteins are very rich in cholesterol and triglycerides, ceramides, and sphingomyelin. Lipoproteins are cleaved by macrophages to their basal components and while most lipid species are stored within their cytosol, cholesterol is expelled into extracellular space, where in high concentration forms cholesterol crystals. Their accumulation eventually leads to arterial stiffness and atherosclerosis [92].

Similarly to the situation in adipose tissue and liver, also arterial macrophages undergo pro-inflammatory polarization. Combined production of pro-inflammatory cytokines by lipid-exposed macrophages in all these tissues leads to systemic insulin resistance called type 2 diabetes and the collapse of metabolic homeostasis known as metabolic syndrome [93]. Recently, the surprising similarity between lipid-handling macrophages within all three tissues was described which gives rise to a unique subpopulation recognized as "lipid-associated macrophages" (LAMs) also assigned as "Trem2-positive" according to their characteristic surface marker.

It is interesting to look closer at why macrophages in adipose tissue, liver, and arteries react to lipids by strong pro-inflammatory polarization. Lipids are present in excess in all of these tissues and often in their damaged unnatural forms [94]. Although macrophages are enzymatically well-equipped to handle these lipids and their primary intake induces rather M2 polarization, the accumulation of products from lysosomal lipid metabolism leads to dysfunction of mitochondrial metabolism. Macrophages with dysfunctional mitochondria depend metabolically on aerobic glycolysis leading to macrophage pro-inflammatory polarization and production of pro-inflammatory cytokines [95]. Moreover, macrophages carry a variety of receptors on their surface that recognize FFAs and lipoproteins and subsequently activate characteristic pro-inflammatory immune cascades and inflammatory polarization [96]. Although the reason why macrophages respond to external lipids by proinflammatory polarization has not been fully elucidated, we bring one explanation rooted in the evolutionary origin of bactericidal macrophage polarization. We suggest that the bactericidal function of macrophages originates from the nutritional phagocytosis of free-living amoebae, where the lipids produced occur as secondary metabolites of bacteria and serve as microbial traces. This hypothesis will be further explained below.

Evolutional origin of macrophage functional versatility

In previous chapters, we described macrophages as ultimate multitaskers. For this purpose, they utilize their exclusive features such as signal perception from the environment, amoeboid motility, phagocytosis, and lysosomal metabolism of engulfed matter. The spectrum of their functions is further expanded by the ability to enter various metabolic states and adopt different functional polarizations [97]. However, macrophages are like a double-edged sword for the organism and while they protect and nurture, they can also harm and cause pathologies. Moreover, it often happens for reasons that are incomprehensible and puzzling to us as observers.

Pro-inflammatory polarizations are triggered in response to endogenous substances even under sterile conditions, and pro-inflammatory polarization may result simply from the metabolic imbalance of the tissue. Pro-inflammatory macrophages are thus associated with arthritis, atherosclerosis, liver dysfunction, obesity, neurodegenerative disorders, and autoimmune diseases. Conversely, excessive polarization of macrophages towards a healing phenotype leads to chronic bacterial infections, liver fibrosis, and tolerance of tumor growth [98]. For instance, tumors re-program macrophages in their vicinity via cAMP signaling to adopt tolerogenic polarization and support tumor growth by production of growth factors, providing it with nutrients and promoting angiogenesis [99].

When we compare the features of mammalian macrophages with macrophage-like amoebocytes of lower animals, we can find striking similarities even at the molecular level. We can assume that many characteristic macrophage features evolved in an environment highly different from the body of a complex multicellular organism. Human macrophages thus acquired a wide range of their features by repurposing their original ones which inevitably carry certain rudimentary imprints of their origin that can play a fundamental role in their pathological behavior. This concept will be discussed in more detail in the following section.

Origin of (M1) pro-inflammatory polarization of macrophages

In an attempt to trace the evolutionary origin of the basic properties of macrophages, we first asked in which animal phagocytic amoeboid cells first appeared. We progressed from humans through the dominant phylogenetic taxa to the most primitive multicellular animals and found that there is compelling evidence in the literature for the presence of amoeboid phagocytic cells, in virtually all multicellular animals [100]. If we follow this idea further to unicellular animals, we realize that many unicellular lines at the base of the animal clade display intriguing life cycles that always go through the stage of highly motile and phagocytic amoeboid cells [101].

Every good zoologist should point out that we know very well that the first animal cells are choanocytes or collar cells and not some amoebocytes [100]. However, that was the case only until recently. In 2000, a professor at Howard Hughes Medical Institute, Nicole King with her team published an exciting story, experimentally documenting that choanocytes exposed to stressful conditions can change their cellular type and become amoebocytes. This transient switch of cell

type was experimentally demonstrated for a wide range of primitive animal clades, suggesting that we should reconsider our ideas about the appearance of the first multicellular animal to evolve. As I mentioned in the introduction, Metchnikoff was in dispute with Haeckel about whether the first animals originated from aggregations of phagocytic amoebocytes or filtering choanocytes. Currently, the prevailing opinion is that both were right since the first animals were most likely organisms capable of switching between both these states during their life cycle according to external conditions [100].

This has several fundamental implications for our thinking. Firstly, it seems that macrophages, similar to amoeboid cells, are inherent to all animals. Secondly, we can look for their key characteristics much further in the evolution, even to *Amoebozoa*. Thirdly, if we consider that amoebocytes were the first type of animal cells, choanocytes, specialized for food acquisition, represent the second choice, which could theoretically open the door for macrophages to develop their secondary functions that were independent of gathering nutrients.

In our search for key features of macrophages such as motility, phagocytosis, sensing of bacteria, and cytokine signaling, we came to free-living amoebae such as *Acanthamoeba*. *Acanthamoeba* is a ground-dwelling primitive unicellular organism that hunts bacteria and small unicellular eukaryotes for nutritive reasons. However, in a closer look, we may find that these unicellular organisms exhibit absolutely fascinating similarities with macrophages. Indeed, macrophages and Acanthamoeba show almost identical morphological and molecular bases of processes such as amoeboid movement, mechanism of actin polymerization, perception of bacteria and intracellular transduction of those signals, as well as molecular mechanisms of phagocytosis, and bacterial digestion in the phagolysosome (Fig. 9) [102,103].

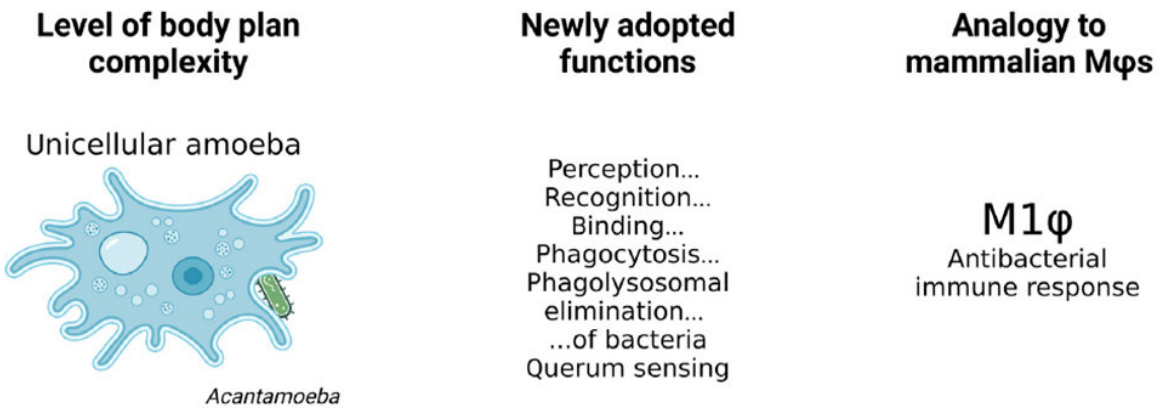

Figure 9. *The characteristic features of M1 - pro-inflammatory macrophages probably originate from a common ancestor of animals and amoebae. The bactericidal immune function of macrophages probably has its origin in bacterial hunting for nutritional reasons. Adopted from: On the origin of macrophage functional versatility… , Bajgar A. and Krejčová G. 2023* [8].

The similarity of these processes reaches such a level that it is assumed that a whole range of intracellular bacteria such as *Mycobacterium*, *Legionella*, or *Listeria* has trained in coevolution with *Acanthamoeba* how to evade phagolysosome of macrophages making them dangerous pathogens for humans [104].

From our perspective, this means that if we exclude the very unlikely scenario of convergent evolution, the common ancestor of amoebas and animals was an amoeboid, free-living cell capable of detecting, tracing, and engulfing bacteria and digesting it in phagolysosome by a highly similar mechanism as we can observe in mice or human macrophages [105]. That indicates that the ability of mammalian macrophages to eliminate pathogenic bacteria evolved in evolution from hunting bacteria as prey and nutritive phagocytosis [106]. Importantly the nutritive role of phagocytosis in the digestive system can be still observed in many groups of Animalia with the exception of vertebrates and arthropods [107].

The origin of bactericidal pro-inflammatory polarization of macrophages in nutritive phagocytosis and hunting of bacteria might have serious consequences in the pathogenesis of many diseases. For instance, synovial macrophages play a central role in the pathogenesis of arthritis, where they invoke sterile inflammation when reacting to the body's own folic acid [79]. Even though there is no obvious reason why synovial macrophages react to folic acid in this situation, the folate receptor on their surface shows high homology to one found in *Acanthamoeba*, which uses it for tracing bacteria generating folate as a secondary metabolite[108]. The reaction of macrophages might be in this situation only a recall of their ancestral origin.

Origin of tolerogenic (M2) polarization of macrophages

In the previous paragraph, we consider the possibility that the evolutionary origin of pro-inflammatory polarization of macrophages has its roots in the nutritive phagocytosis of the common ancestor of *Amoebas* and *Animals*. However, can we similarly trace the origin of healing macrophages, which manifests primarily by tolerogencity, recognition of self and non-self, and remodeling of the extracellular matrix?

Upon deeper reflection, it occurs to us that these properties are particularly suitable for the formation and functioning of primitive multicellular organisms. Individual cells forming colonies by aggregation need to develop several novelties. They need to produce factors that increase their coherence, facilitating their communication and enabling them to distinguish themselves from cheating foreigners [109]. Self-recognition is one of the fundamental complications emerging already with multicellularity. In multicellular organisms, usually, only a subpopulation of the colony undergoes the sexual process and passes on its genetic material to the next generation. Therefore, it is essential to protect the colony from cheating alien invaders who try to optimize their reproductive potential at the expense of the host [110]. To identify genes that evolved along with the emergence of multicellularity, Ruiz-Trillo and his colleagues compared genomes of primitive unicellular and multicellular animals at the base of *Animalia* [111]. Between the compared groups

were multicellular *Porifera* (lacking true tissues) and unicellular animal clades such as *Filasterea*, *Ichthyosporea*, *Coralochatrea,* and *Choanoflagellata*. Such analysis has revealed several clusters of genes devoted to cell adhesion, intercellular communication, remodeling of extracellular matrix, and signaling. Many of these genes represent characteristic expression repertoire of healing macrophages. This leads us to consider that a whole range of healing properties of M2 macrophages originated at the inception of multicellularity [111].

An alternative system where similar phenomena can be studied is facultative multicellular organisms such as *Dictyostelium discoideum*. *Dictyostelium* is an organism from the group *Amoebozoa*. A large part of the life cycle of this organism consists of individually moving cells - amoebocytes, which behave very similarly to *Acanthamoeba* [112]. They search in their surroundings for bacteria to hunt and phagocytize for nutritional reasons. However, under the circumstance of nutritional scarcity amoebocytes start to generate a self-amplifying cascade of cAMP signaling that spreads through the whole population in fascinating spiral waves. This signal leads to the aggregation of individual cells and the formation of facultative multicellular formation called "slug" [113]. The slug can move in a coordinated manner in a certain direction, allowing the entire population of individual amoebocytes to relocate from the place of scarcity towards sources or places where it forms the fruiting bodies and eventually spores (Fig. 10) [114].

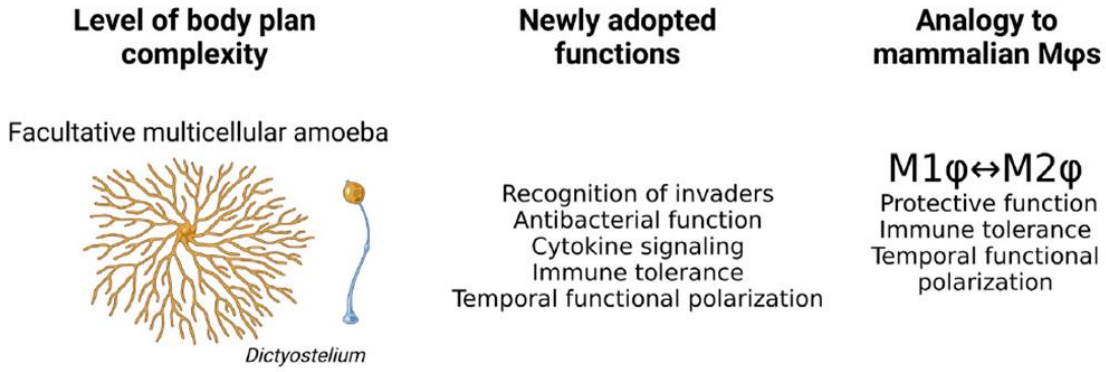

Figure 10. *The characteristic features of M2 - healing macrophages appeared in evolution along with multicellularity. The subpopulation of amoebocytes in the aggregation phase of the dictyostelium life cycle shows features that resemble healing macrophages in many ways. Adopted from: On the origin of macrophage functional versatility… , Bajgar A. Krejčová G. 2023* [8].

However, the slug is not just an aggregate of random cells from the surroundings. The amoebocytes recognize their relatives, and if we mix two populations of amoebocytes, two individual slugs will form. Within this facultative multicellular entity, individual cells take on various functions and differentiate accordingly [115]. Thus, we can find cells that become some sort of immune system, called sentinel cells, cells that form the stalk of the reproductive organ, other cells destined for sporulation, and cells responsible for the coherence of the entire organism [116]. We are particularly interested in the sentinel cells and cells responsible for the coherence of the slug.

Sentinel cells indeed greatly resemble cells of the innate immune system. To eliminate potential pathogens, they apply strategies well known from the human immune system. They eliminate bacteria within phagolysosomes, produce large amounts of reactive oxygen species into the environment, use netosis, etc. Moreover, for danger signaling and mutual communication, they use cytokine signaling and receptors for their recognition, which are homologous to these receptors in mammals. An intriguing aspect is the coordination of these cells in hunting larger cells and organisms, which are subsequently surrounded by the slug, and the captured prey is enzymatically digested by the exocytosis of lytic enzymes from the phagolysosome [117].

The second population of cells responsible for the coherence of the multicellular slug is no less interesting. These cells specialize in the production of components of the extracellular matrix and remove old and unnecessary cells that could become a threat to the entire organism. They also regulate, through signaling factories, the differentiation of individual cells for specific tasks, thus playing a certain regulatory role Interestingly, these cells develop tolerance to other cells within the slug and are able to maintain a small population of commensal bacteria such as *Klebsiella*, which is occasionally used as a nutritional reserve [118].

Along the transition from unicellular amoebocytes into slugs, individual cells undergo also remodeling of their cellular metabolism from predominant aerobic glycolysis towards oxidative phosphorylation. This metabolic switch is coordinated by sulfur sequestration in mitochondria a change of mitochondrial metabolism, resembling thus healing macrophages in mammals, however, this phenomenon remains to be studied thoroughly in the future [119,120].

Already in *Dictyostelium*, we can observe a large number of characteristics of M2 healing or tolerogenic macrophages, and we can speculate that at some stage in the evolution of multicellularity, the evolutionary origin of these properties was probably absolutely crucial. The tolerogenic setting of individual amoebas is induced by a chain of cAMP signaling and sulfur sequestration in mitochondria. It is interesting that cAMP signaling is also associated with the tolerogenic setting of the immune system in humans and is a key signal produced by neoplastic tumors in an attempt to reverse macrophages from M1 to M2 in the tumor microenvironment. M2 macrophages then produce nutritional substances and factors that support tumor growth and angiogenesis. It is therefore possible that the adaptation that was at the beginning of the origin of multicellularity in animals lay in the tolerogenic setting of macrophages and currently represents a possibility of how the tumor can avoid elimination by pro-inflammatory macrophages and natural killer cells [121]. Similarly, sulfur sequestration has been recently identified as a fundamental mechanism by which macrophages change their pro-inflammatory polarization towards the healing phenotype by forming cysteine persulfides which subsequently inhibits the action of pro-inflammatory cytokines [122].

Archaeocytes – versatile functions of amoebocytes in sponges

As we continue to observe the functional versatility of macrophages, we can see significant development in their roles already in primitive multicellular organisms such as sponges. Sponges are multicellular organisms composed of four types of cells that do not form true tissues as known in higher animals [123]. While other cell types such as choanocytes, porocytes, and spiculocytes have clearly defined functional roles that do not change during their lifetime, the remaining amoeboid cells called archaeocytes are absolutely different in this aspect [124]. Archaeocytes are totipotent cells that can differentiate into any other cell type and play a fundamental regulatory role in sponges. Archaeocytes are motile amoeboid professional phagocytes that fulfill various tasks within an individual according to current needs. Certain subpopulations of archaeocytes are responsible for the transport of nutrients from choanocytes to other cells residing in mesoglea, playing thus fundamental nutritional function [125]. Other archaeocytes are responsible for the

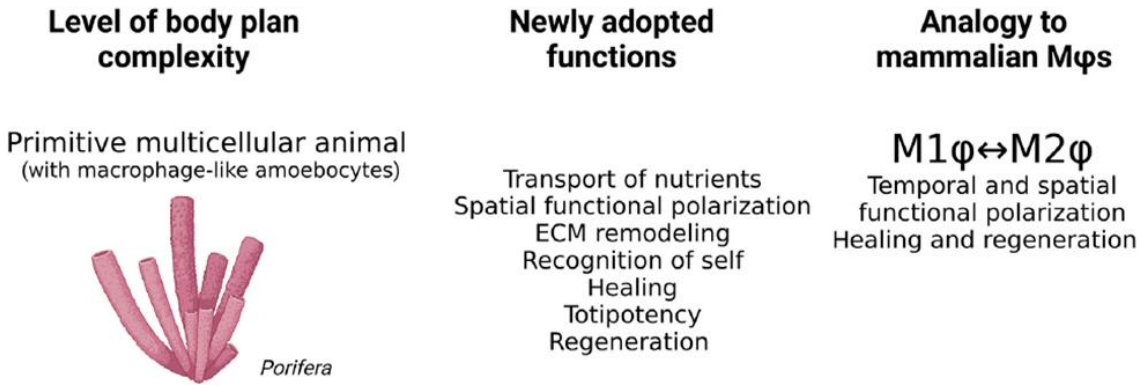

Figure 11. *Most of characteristic features of mammalian macrophages can be observed to some extent already in primitive multicellular animals lacking true tissues, indicating their ancient evolutionary origin. Adopted from: On the origin of macrophage functional versatility… , Bajgar A. Krejčová G. 2023* [8].

elimination of old and damaged cells. Moreover, archaeocytes play a protective immune function, participate in self–recognition, and govern regeneration [126]. The whole organism can be restored only from a population of archaeocytes and if we mix together two individual organisms they can recognize their identity and form two genetically defined individuals [127]. Archaeocytes also produce a whole spectrum of signaling molecules and are responsible for the coordination of stress response [128]. Interestingly, sponges are often described as holobionts and represent virtual colonies of several organisms. Porifera cells live frequently in close ecological relationships with photosynthetic algae and particularly archaeocytes are responsible for generating the tolerogenic niche for the maintenance of these symbionts (Fig. 11) [129].

Therefore, we can say that macrophage-like cells, already in sponges, possess a wide range of highly diversified functions and can dynamically switch between individual tasks according to the specific context and situation. Indeed, in archaeocytes, we can observe most of the basic functional elements, whose repurposing has established the entire functional versatility well described in

human macrophages. The remaining question is what induced or better say allowed for the expansion of macrophage functional versatility already in these primitive animals? We offer two different explanations. The first lies in the deliberation of macrophages from their original duty of procuring food. One of the first cellular types to appear even in facultative multicellular organisms are choanocytes, or collar cells, specializing in food gathering by filtration of the surrounding fluid [130]. This allows macrophages to no longer remain solely in a bactericidal setting and gives them the opportunity to devote themselves to other tasks based on the different metabolic setups of these cells.

The second explanation can be found in the necessity of coordinating various relatively complex physiological tasks in the multicellular body of the organism, which, however, lacks well-developed sensory organs, a nervous system, or specialized endocrine cells [131]. Amoeboid cells well equipped by a whole spectrum of surface receptors and signaling factors, likely serve this function in *Porifera*, fulfilling central regulatory roles. Thus, we can even explain their certain superiority over other cell types in mammals and their often fundamental control and regulatory functions in tissue-specific contexts.

Considering the context in which macrophages acquired almost all of their key properties in evolution, it is evident that to understand their often seemingly incomprehensible pathological behavior, we must study their role in much more primitive organisms and search for their original, non-pathological, and adaptive significance of these processes (Fig. 12).

In our work, we focused on the role of macrophages in bacterial infection in the model organism *Drosophila melanogaster*. However, we did not directly study the mechanisms by which these cells eliminate pathogens themselves, but rather their metabolic adaptations associated with combating bacteria and the production of signaling factors through which these cells regulate systemic metabolism. In the following chapters, I will describe how the metabolism of insect macrophages changes during infection, how macrophages influence systemic insulin signaling to obtain a sufficient supply of energetically valuable substances, and how these mechanisms causing originally adaptive changes in metabolism can lead to metabolic diseases in human patients.

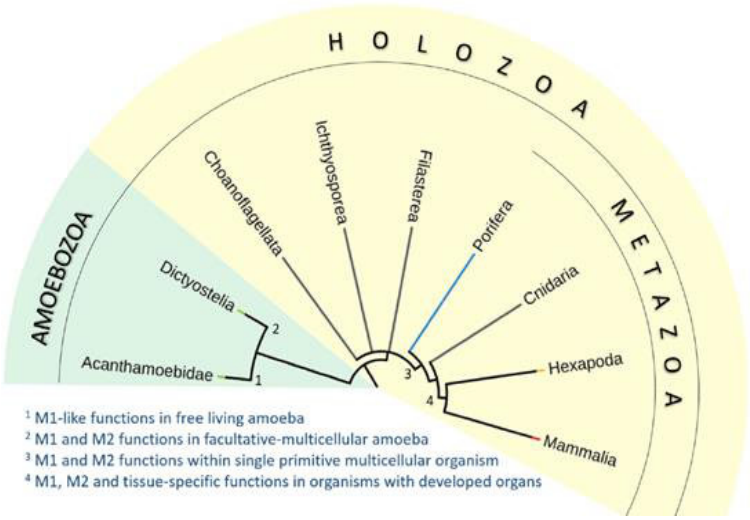

Figure 12. *Summary of our hypothetical model, according to which key features of macrophages are of ancient evolutionary origin and their current functions in humans have evolved by repurposing. Adopted from: On the origin of macrophage functional versatility… , Bajgar A. Krejčová G. 2023* [8].

Drosophila melanogaster as a model organism for the study of macrophages

Drosophila is an absolutely exceptional model organism, which in many respects has no adequate comparison with any other model. *Drosophila* has been used as a model organism for fundamental cellular, genetic, and physiological processes for over a hundred years [132]. The community of scientists dedicated to *Drosophila* has carried out a whole range of projects over this time, turning this organism into an invaluable experimental model (Fig. 13).

Among these projects, it is worth mentioning several rounds of mutant line production by random insertion and subsequent mapping of the P-element, or several rounds of preparation of transgenic lines carrying RNA-interfering constructs for all known transcripts in *Drosophila* genome [133]. Collections of such transgenic lines allow for rapid characterization of the role of a given gene without the need for costly production of new transgenes. They also allow for the screening of many lines in an experimental assay, which enables us to relatively quickly and inexpensively identify new key players in the process under study [134]. An inherent feature of this model is a well-annotated genome and transcriptomes with knowledge of the function of a large portion of genes involved in various processes. The list of possible tools and databases is almost endless and the details can be found in a unifying database called FlyBase (www.flybase.org).

To prevent damage to transgenic constructs by the process of recombination, lines carrying so-called balancer chromosomes were prepared. These chromosomes carry dominant markers and a whole series of inversions, which limit homology with the natural chromosome and thus the process of homologous recombination. Dominant markers then allow us to trace individual chromosomes through even complicated crosses without the need to genotype the resulting combinations in filial generations [135].

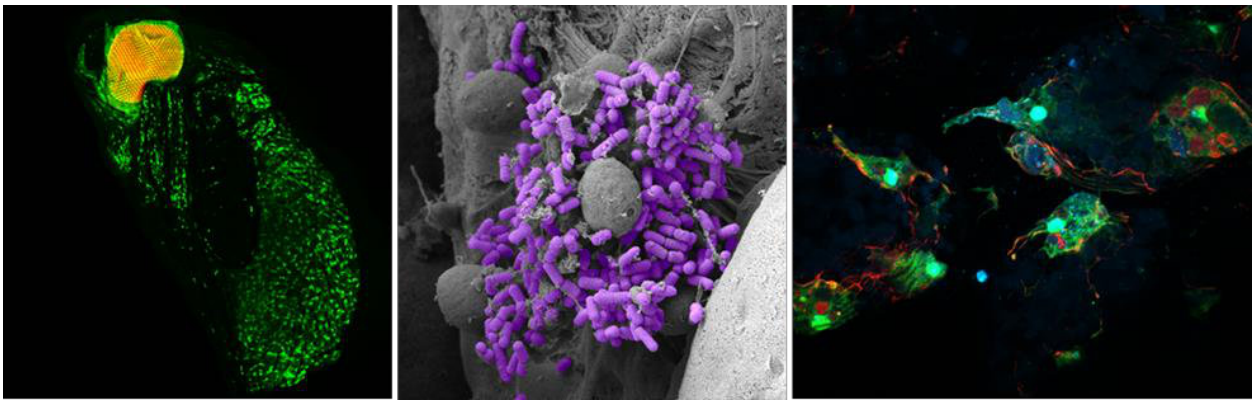

Figure 13. *Beauty of Drosophila macrophages. Image on the left shows macrophages within pupa, where macrophages play important role in digestion of larval histolyzing cells. Midle image from scanning electron microscope display drosophila macrophage with Streptococci in violet attached to its surface. The image on right shown macrophages infiltrating adipose tissue (Image credit: Krejčová G., Bajgar A.).*

However, the truly groundbreaking innovation represented the introduction of the Gal4>UAS system [136]. This system allows the utilization of tissue-specific promoters to induce any construct under the control of the UAS promoter. The Gal4 transcription factor is not natural for *Drosophila* and is introduced from yeast. Gal4 itself does not affect the expression of other genes in the organism. However, once we cross fly lines carrying the tissue-specifically expressed Gal4 transcription factor with lines carrying the UAS promoter, the construct in the target tissue is activated in the progeny. This system allows, for instance, tissue-specific knockdown of any gene of choice, label-specific cell lines, overexpressing any kind of construct or reporter of the ongoing process. Crosses are very easy as flies are simple to maintain and their generation time is about ten days in laboratory conditions [137].

Drosophila as a model organism has contributed to a whole series of fundamental discoveries, as evidenced by many Nobel Prizes awarded to research teams from around the world [138]. It is therefore not surprising that even in the field of immunity, *Drosophila* has contributed to many fundamental discoveries, such as the identification of the *Toll* receptor [139].

In *Drosophila*, the immune response is primarily studied in the larval stage. It is fairly well established that *Drosophila* possesses both humoral and cellular branches of the immune system. While the humoral immune system consists of antimicrobial proteins and other immune factors produced into circulation, cellular immunity consists of four types of cells [140]. These types include prohemocytes, crystal cells, plasmatocytes, and lamellocytes. These cells are involved in various tasks within the body, mounting immune responses to different pathogens and participating in wound healing [141]. Moreover, immune cells, especially in the embryonic stage, also participate in tissue morphogenesis, tissue remodeling, and the regulation of homeostasis during metabolic stress (Fig. 14) [142]. Last but not least, we have to mention the important role of *Drosophila* in the study of the basic mechanisms controlling hematopoiesis [143].

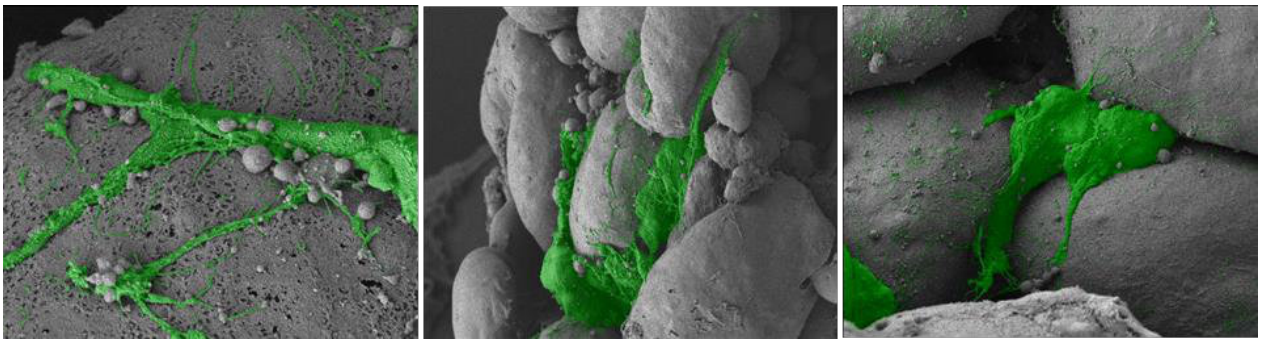

Figure 14. *Scanning electron micrographs of Drosophila macrophages infiltrating larval adipose tissue in freshly emerged adult flies. Infiltrating macropjgages tent to clear leaking lipids and cellular fragments (Image credit: Krejčová G., Bajgar A.).*

Immunological research on *Drosophila* has provided us with most of the knowledge about insect immunity, and *Drosophila* has contributed significantly to the understanding of the immune system of such fundamental insect species as the honey bees, mosquitoes, and tsetse flies, which have a major impact on human society [144].

In our work, we focused primarily on the stage of immune response in adult fruit flies. This scientific niche is not sufficiently explored and enables to study of many interesting aspects of insect immunity without the effect of ongoing development. In adult fruit flies, we essentially find only one type of immune cells called plasmatocytes. However, it may be assumed that this population is not homogenous [145]. Plasmatocytes share many properties with mammalian macrophages, both in terms of fundamental immune cascades and in the regulation of basic cellular processes such as phagocytosis, elimination of pathogen in phagolysosome, actin polymerization, wound healing, synthesis of antimicrobial peptides, etc (Fig. 15).

In adult *Drosophila* individuals, research primarily focuses on the role of immune cells in response to pathogenic infections, including bacterial, fungal, and viral pathogens. Infection in adult individuals can be achieved by adding pathogens to their food, which primarily induces gut immunity, and subsequently, after the pathogen penetrates into circulation, both cellular and humoral immunity [146]. An alternative method used in our research is the injection of a precisely defined amount of bacteria intra-abdominally directly into circulation. The injection itself is not a significant threat to the individual, and 100% of experimental individuals survive it. This approach allows for unprecedented control over the dose of the pathogen and thus also the progress and outcome of infection. Mostly, we are trying to achieve a 50% survival rate in the control genotype. Such a controlled infection allows us to infect thousands of individuals within one infection assay and thus analyze many genetic manipulations within one experiment.

Although it may seem that *Drosophila* as one of the best-studied organisms no longer offers any surprises in terms of its immunity, the opposite is true. There are still many unanswered questions regarding the function of macrophages in this organism. For instance, it is not entirely clear whether there are tissue-resident subpopulations in *Drosophila*, and until recently it was enigmatic, whether *Drosophila* macrophages adopt diverse metabolic polarization states in response to activating stimuli. Here, we are getting to the core of the research that we have focused on in recent years, which I will present in the following chapters.

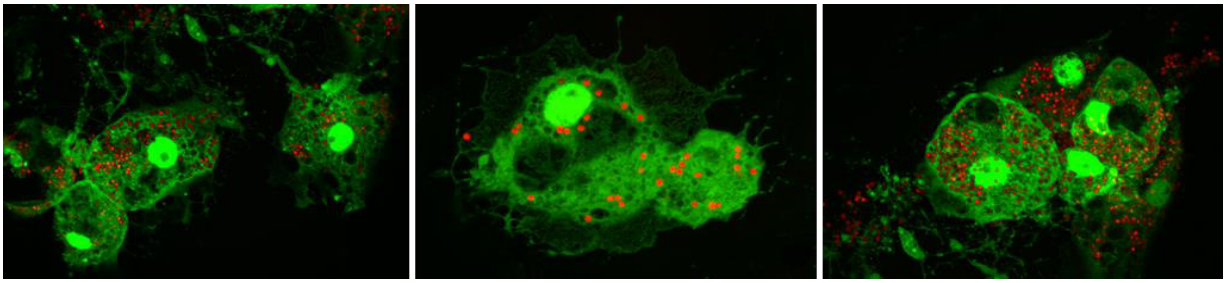

Figure 15. *Phagocytosis (S.a. pHrodo – red) in Drosophila macrophages. Activated macrophages display surprising capacity of phagocytosis. Image credit: Krejčová G., Bajgar A.*

Bioenergetics of immune response

To understand our research, it is first necessary to introduce certain views and perspectives through which we perceive the holistic concept of the organism's defense mechanisms against pathogens. In the previous chapters, I have attempted to present the importance of immune cells in the organism and the wide array of roles that these cells can fulfill. Immune cells are entirely indispensable for the organism, and their presence in almost every tissue is a necessary condition for maintaining tissue and metabolic homeostasis. However, maintaining a numerically sufficient population of immune cells is also a rather energy-intensive process for the organism [147]. Organisms thus try to circumvent this dilemma in several ways. Most of the time, immune cells in tissues reside in a quiescent state, with minimal metabolic activity. However, the identification of pathogens or threats from tissue damage leads to their rapid activation. An alternative strategy for organisms to avoid maintaining an excessively large number of immune cells is their rapid proliferation. In the case of a threat, cells of the immune system can rapidly multiply in hematopoietic organs, thus multiplying the protective potential of the whole organism [148].

In both cases, however, the activation of the immune system is associated with a sudden increase in the nutritional demands of the immune system. Activated immune cells require enormous amounts of glucose, amino acids, and lipoproteins. However, these nutritional needs cannot be covered merely from their intracellular reserves, and macrophages are therefore functionally dependent on external resources (Fig. 16) [149].

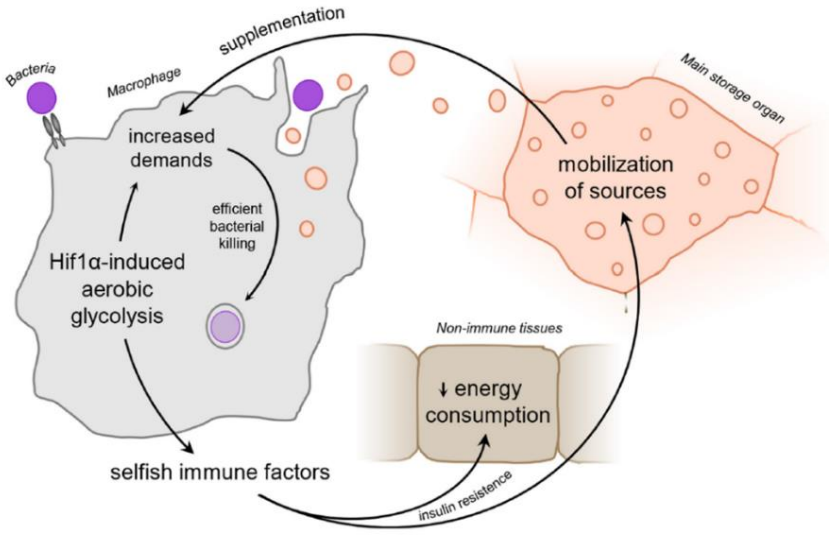

Figure 16. *Theoretical concept of selfish immune theory. Activation of immune system is connected with sudden energy and nutritional demands that need to be supplemented from external source. Activated immune cells produce signaling factors to ensure source on expense of other tissues and physiological processes. While this signaling is required during immune response, its activation in inadequate context may lead to development of insulin resistance and metabolic syndrome. Adopted from: Polarization of macrophage polarization in insects… Bajgar A., Krejčová G., Doležal T. 2021* [6].

This also reflects on the setting of the systemic metabolism of the whole organism. During infection, fundamental changes in metabolism occur [150]. Non-immune processes such as development, growth, sexual activity, and building of reserves are suppressed [151]. Nutrient-rich substrates, on the other hand, must be released into the circulatory system to be available for immune cells and tissues. One of the possible mechanisms to simultaneously achieve the mobilization of resources from central metabolic organs and their reduced consumption by peripheral tissues is insulin resistance [152]. It is relatively well known that insulin resistance leads to the suppression of metabolism in most tissues and prevents access to energy sources such as glucose or lipoproteins [153]. However, insulin resistance has the opposite effect on two tissues in the body. Insulin-resistant adipocytes lose their ability to continue storing lipids and, conversely, stimulate mobilization of free fatty acids [154]. Similarly, in hepatocytes, insulin resistance leads to accelerated metabolism and increased production of glucose and lipoproteins into circulation [155]. The result is a temporary increase in these nutritionally rich substances in circulation, which is recognized as a characteristic hallmark in patients suffering from severe bacterial infection or sepsis [156,157]. The close connection between an activated state of the immune system and systemic metabolic changes is evident, and adjustment of systemic metabolism is now widely recognized as an important component of the immune response [158]. However, it is not evident which signaling factors could reflect the degree of immune response activation and translate it into the observed systemic metabolic changes.

Infection-activated *Drosophila* macrophages adopt aerobic glycolysis

To better understand the bioenergetics of the immune response, we decided to examine the metabolism of activated immune cells during a severe bacterial infection. As a model for our research, we used adult *Drosophila* individuals injected with a high dose of Streptococcus *pneumoniae*. This infection leads to the rapid activation of the immune system, which attempts to quickly eliminate the rapidly multiplying pathogen. The main defense against streptococci is phagocytosis, and macrophages must clear the organism from bacteria within the first five days, otherwise it loses the chance to survive the infection.

In an effort to understand the metabolic adaptations of immune cells, we isolated macrophages from infected and control flies at two-time points: during the acute phase of infection (24 hours post-infection), and during the resolution phase of infection, when pathogenic bacteria are no longer present in the organism (120 hours post-infection). First, however, we had to establish a new method for isolating immune cells from adult *Drosophila* individuals. For this purpose, we introduced a protocol based on FACS in which we exploited strong endogenous expression of the gene encoding green fluorescent protein (GFP). The introduction of this method allowed us to obtain enough material to characterize immune cells in terms of their expression, their metabolic status, and the enzymatic activity of key metabolic cascades [1].

Detailed characterization of infected macrophages revealed that these cells, similar to their mammalian counterparts, undergo metabolic polarization to aerobic glycolysis in response to bacterial infection. Adopting aerobic glycolysis is crucial for fighting bacterial infection and elimination of pathogens. For this adaptation, the activity of transcriptional factor HIF1α and the enzyme converting pyruvate to lactate called lactate dehydrogenase, were essential. However, this adaptation is only temporary and lasts only over the acute phase of infection as macrophages isolated during the resolution phase of infection showed different metabolic setups (Fig. 17).

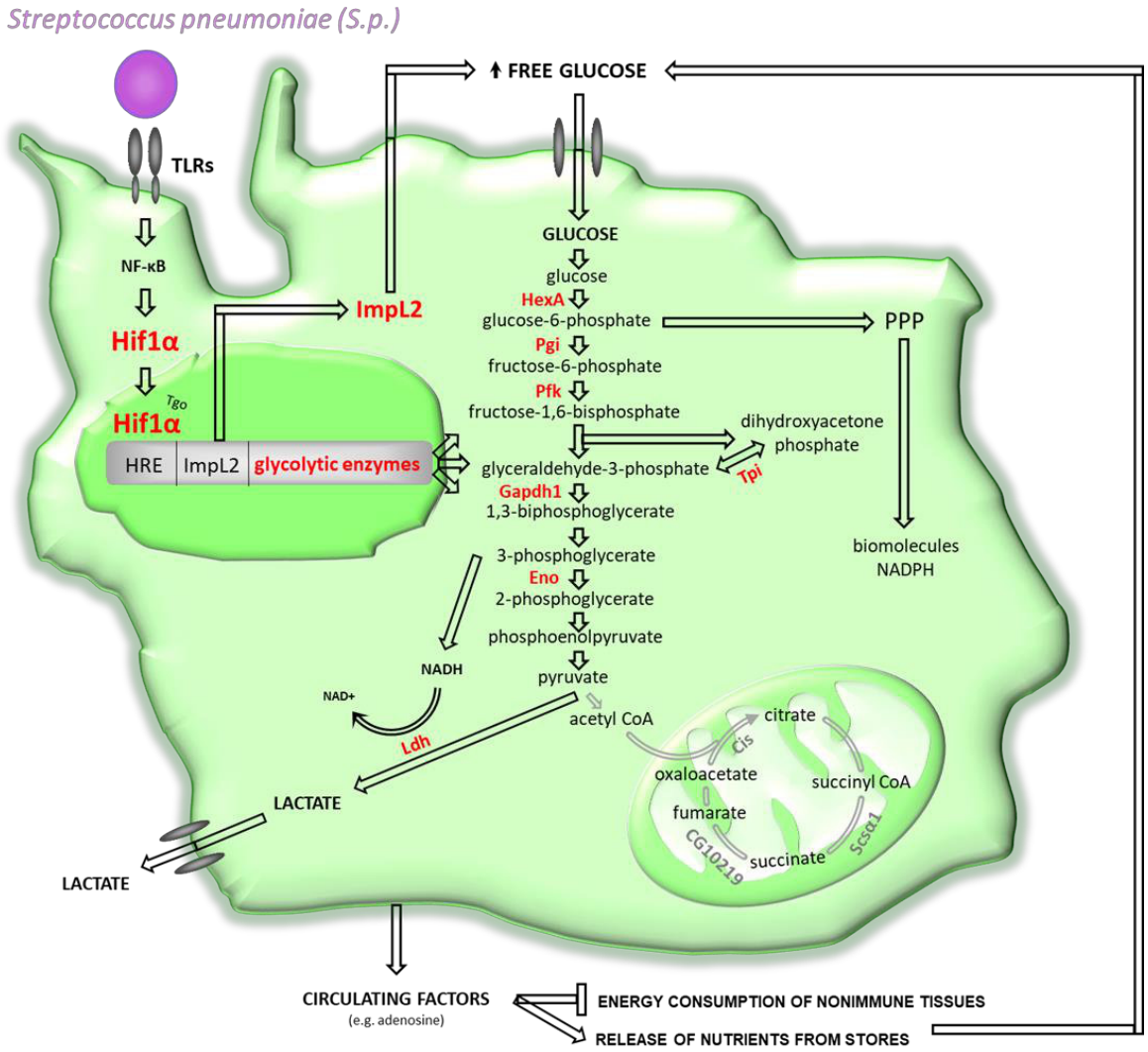

Figure 17. *Schematic representation of the cellular metabolic program in infection-activated macrophages in Drosophila. This metabolic regime closely resembles metabolism of M1 macrophages in mammals and is controlled by the same transcription factor HIF-1α. That indicate common origin of metabolic polarization for mammalian and insect macrophages and their evolutional conservation. Design credit Krejčová G.*

Our observations that the metabolic switch in *Drosophila* macrophages requires virtually the same transcription factor indicate the ancient origin of this metabolic adaptation by macrophages performing their bactericidal function. In our work, we have mainly focused on the characterization of glycolysis and the cycle of tricarboxylic acids. At that time, we did not have sufficient expertise to characterize mitochondrial metabolism in detail. Today I see this as a significant knowledge gap and it would certainly be nice to complete the characterization of bactericidal macrophages in this respect. Another important question that remains to be answered is the mechanism of HIF1α stabilization. As we discussed before, HIF1α can be stabilized by hypoxia, or under normoxia by sequestration of iron or via mitochondrial retrograde transport. I believe that *Drosophila* offers tools that can help to resolve this issue in the future.

Our observations indicate a highly conserved mechanism of metabolic polarization in macrophages even at the molecular level. Therefore, we can speculate whether aerobic glycolysis is an essential condition for phagocytosis and phagolysosomal elimination of bacteria in all phagocytic cells. It would be amazing to demonstrate this in primitive unicellular organisms such as *Acanthamoeba* and *Dictyostelium*, and I am considering dedicating a sabbatical to this topic.

Besides the characterization of the cellular metabolism of infection-activated macrophages, we also investigated systemic metabolic changes that occur during infection. We observed that infection is accompanied by a dramatic shift in systemic metabolism particularly of carbohydrates and lipids.

Inspired by the "Selfish brain theory" formulated by Prof. Achim Peters [159], we decided to investigate whether changes in systemic metabolism are interconnected with changes in the cellular metabolism of activated macrophages and which factors are responsible for coordinating these two processes.

Aerobic glycolysis is coupled with the production of signaling molecules

Aerobic glycolysis appears to be an indispensable adaptation of macrophages for their bactericidal function. This metabolic adaptation allows for the rapid generation of enormous amounts of ATP and NADPH, which are necessary to meet the energy demands for increased macrophage motility and phagocytosis, acidification of phagolysosomes, and generation of a sufficient amount of reactive oxygen species. Additionally, large amounts of metabolites such as succinate and itaconate, known for their bactericidal effects, are produced in the broken TCA cycle in excess and released to the external environment.

This metabolic adaptation requires a significant amount of external sources, especially glucose, glutamate, and lipoprotein [160]. Mobilization of resources must be closely linked to the current metabolic needs of activated immune cells, which can dramatically change over time. It can be assumed that there must be a sensitive mechanism that transmits the information between the cellular metabolism of immune cells and the central metabolic organs responsible for the induction of required metabolic changes. The absence of such a coordinating mechanism would

lead to the rapid depletion of resources stored by immune cells and bacterial bursting. Alternatively, the mobilization of excess nutrients would lead to wasting, cachexia, and the nutritional support of bacteria in the extracellular space.

When considering how the metabolic demand of immune cells can be measured, two possibilities arise. Firstly, the signaling molecule itself may be the metabolite produced in excessive amounts in relation to the given metabolic change, or secondly, the signal may be a cytokine whose production is connected to the given metabolic program.

In the first case, such a metabolite could be extracellular adenosine (eAdo). Research on this interesting metabolite was led by my former supervisor, Doc. Tomas Doležal Ph.D. Extracellular adenosine is an intriguing signaling molecule because its production is associated with a dramatic increase in ATP consumption. ATP is converted to ADP, AMP, and ultimately to adenosine, which is released into the extracellular space as a signaling metabolite activating the *Adenosine receptor* (*AdoR)*.

In our work, we found that adenosine is largely produced by activated immune cells and is released into circulation via the *Equilibrative nucleoside transporter 2* (*Ent2)*. In our infection assay, the production of eAdo by immune cells caused systemic metabolic changes in carbohydrate metabolism. If we allowed immune cells to produce eAdo, there was enhanced production of trehalose and glucose from the central metabolic organ of flies called the fat body. When we prevented eAdo production by silencing Ent2 expression specifically in immune cells, these metabolic processes did not occur and the organism was unable to resist bacterial infection. Given that the ability of immune cells to fight bacterial infection was significantly compromised, we concluded that activated immune cells produce eAdo as a selfish signal allowing them to obtain energetic resources at the expense of other tissues in the body.

Interestingly, eAdo is produced by mammalian tissues that are exposed to a hypoxic state. The lack of oxygen makes it impossible to use mitochondrial metabolism and these tissues are thus dependent on glycolysis as the only possible source of ATP. This suggests that this program may be generally associated with the production of this "alarmin" with a significant impact on the metabolic equilibrium of the organism.

An alternative to the mechanism described for eAdo is the production of the signaling factor *Imaginal morphogenesis protein late 2 (ImpL2)*. In our work, we showed that the production of the signaling factor *ImpL2* is closely associated with the adoption of aerobic glycolysis in infection-activated *Drosophila* macrophages. We demonstrated that the expression of the *ImpL2* is directly regulated by the transcription factor HIF1α. This factor is also recognized as a central regulator of the metabolic switch towards aerobic glycolysis controlling the expression of dozens of metabolic genes [161]. The adoption of aerobic glycolysis is thus inevitably associated with the production of this signaling factor. *ImpL2* is known as a crucial factor in the coordination of nutritional support and availability of oxygen with growth in larva [162] and the mechanism of *ImpL2* action is mediated via its high affinity to insulin and insulin-like peptides and intervention of insulin signaling [163].

Detailed analysis of *ImpL2* action during infection revealed its effects on the fat body. We found that macrophage-derived IMPL2 intervenes with insulin signaling in the fat body and triggers the expression program under the control of transcription factor FOXO. FOXO activation increases the expression of genes responsible for lipolysis and the mobilization of lipids in the form of lipoproteins (Fig. 18).

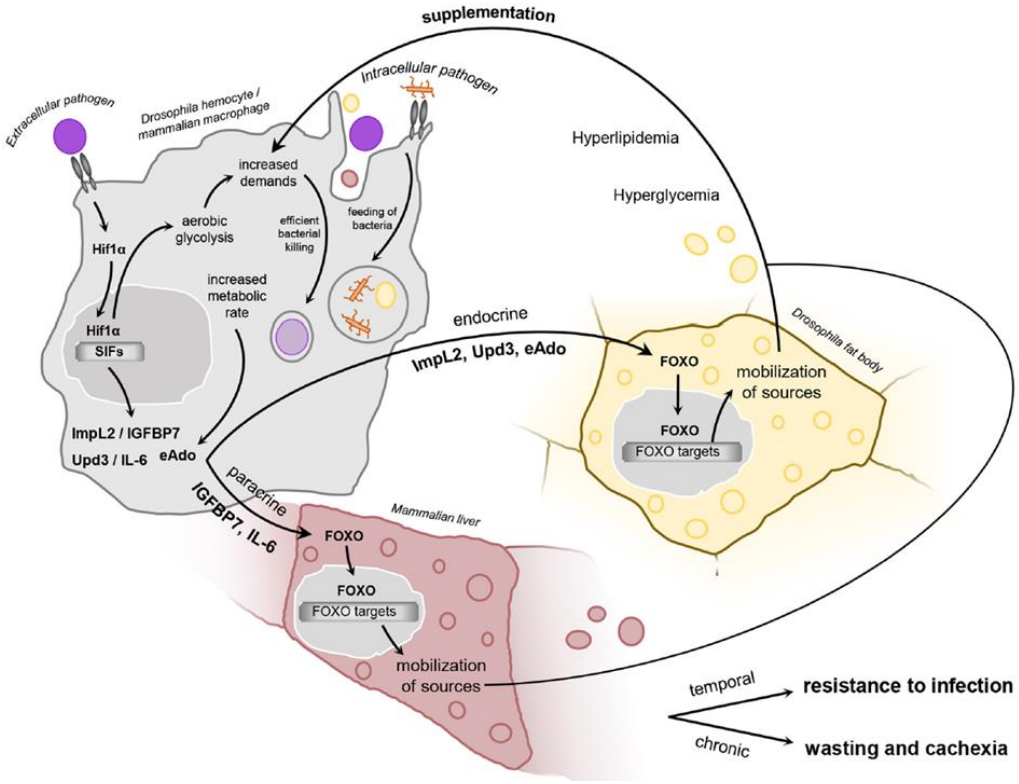

Figure 18. *Proposed role of signaling factors ImpL2 and eAdo in regulation of systemic metabolism. Both these factors are produced by activated immune cells as reflection of their increased nutritional demands connected with adoption of aerobic glycolysis. While production of these factors and induction of subsequent systemic metabolic changes is adaptive during infection, it may lead to pathology in obesity or another stress situation connected with pro-inflammatory polarization of macrophages. Adopted from: Polarization of macrophage polarization in insects… Bajgar A., Krejčová G., Doležal T. 2021* [6].

Activation of aerobic glycolysis is thus remotely coupled with induction of insulin resistance in the fat body and eventually mobilization of lipoproteins. When we prevented this signaling using macrophage-specific knockdown of *ImpL2*, the mobilization of lipids was missing. The intervention of ImpL2 signaling on several levels substantially limits the ability of macrophages to fight the pathogen resulting in suppressed resistance to infection. Importantly, the overexpression of *ImpL2* in macrophages under situations without bacterial infection was sufficient for the induction of lipoprotein mobilization and the accumulation of lipids in immune cells.

These data indicate that the activation of HIF1α should be associated with the production of IMPL2 and systemic changes even under sterile conditions. This is in line with other studies

describing increased expression of the *ImpL2* in neoplastic tumors, hypoxic tissues, and muscles with dysfunctional mitochondria [164–166]. In all these cases, stabilization of HIF1α and enhanced flow of glycolysis and pentose phosphate pathway is fundamental for the survival of these cells. Moreover, enhanced production of IMPL2 in these tissues leads often to loss of insulin sensitivity, and systemic metabolic changes.

The connection of the signaling role of the *ImpL2* gene with the activation of the transcription factor HIF1α and subsequent induction of insulin resistance has one fundamental significance. The activation of this signaling can easily occur in addition to bacterial infection during any situation in which metabolic stabilization of HIF1α occurs and consequently also the induction of insulin resistance. We brought experimental data that indicate this notion.

In an effort to reveal whether a similar signaling cascade may play a role in inducing metabolic changes during bacterial infection in mammals, we conducted several experiments aimed at analyzing the production of IMPL2 homolog IGFBP7 by liver macrophages. Liver macrophages have perfect potential to regulate hepatocyte metabolism and thus influence the mobilization of energy reserves in the form of lipoproteins [167]. Our data show that liver macrophages activated by LPS produce increased amounts of the signaling factor IGFBP7. Moreover, we found that enhanced production of IGFBP7 depends entirely on HIF1α activity in these cells.

Increased production of IGFBP7 induces the expression of apolipoproteins in hepatocytes and the production of low-density and very-low-density lipoproteins into the culture media. All these results collectively point to the conserved role of this signaling cascade between *Drosophila* and mammals and suggest the adaptive role of insulin resistance in the central metabolic organ during bacterial infection as a mechanism for mobilizing lipoproteins.

We further examined the role of this signaling in the context of diet-induced obesity. In *Drosophila*, we showed that macrophages in obese flies are exposed to an enormous lipid load. The cytosol of these cells is often filled with lipid droplets, and these cells in response to these stressful conditions also increase the expression of the *ImpL2* gene. These initial observations led us to an amazing collaboration with Professor Myriam Aouadi and her laboratory. Myriam involved us in an already ongoing study focusing on the role of liver macrophages in the induction of insulin resistance and metabolic syndrome in obese patients. Our research particularly intrigued her because we discovered via an independent approach in the *Drosophila* signaling factor that was the most upregulated gene in liver macrophages of obese patients when the expression was analyzed at the single-cell level. This fortunate coincidence not only led us to a very interesting and fruitful collaboration but also formed the basis for a strong friendship. The result of this study was the observation that the IGFBP7 gene is a key factor involved in the development of metabolic syndrome in obese patients.

Thus, our results show that while the IMPL2/IGFBP7 factor is necessary to provide sufficient nutritional support for activated immune cells, its production in obese patients may lead to negative effects on the insulin signaling pathway and the metabolic status of the individual.

Macrophages play a nutritive role in *Drosophila* postmetamorphic maturation

In the preceding paragraphs, I demonstrated how macrophages influence metabolism through the production of signaling factors. These factors subsequently regulate metabolic processes in adipose tissue and energy mobilization. The relationship between macrophages and adipocytes is truly fascinating and largely unexplored, especially in *Drosophila*. Our interest in the interaction between macrophages and adipocytes is also motivated by the fact that adipose tissue in obese patients is infiltrated by macrophages, whose presence is a hallmark of diabetes onset. In the following paragraphs, I will describe how far we have advanced in studying adipose tissue macrophages in *Drosophila*.

Macrophages play a fundamental role not only in the immune response but also in maintaining homeostasis. Every day in an individual's body, numerous old and damaged cells die, and need to be removed [168]. These processes are further accelerated in situations in which an organism undergoes substantial tissue and organ remodeling. This occurs particularly during embryonic morphogenesis, development, metamorphosis, or periodic changes such as in adipose tissue.

It is reasonably well-established that macrophages play a central role in removing unnecessary cells from the body through a process called efferocytosis [169]. During efferocytosis, a damaged or apoptotic cell is recognized, surrounded by macrophages in a crown-like structure, and digested by an enzymatic cocktail produced by macrophages via exocytosis. The individual fragments of cells are then endocytosed and digested in the phagolysosome [170].

However, what is the destiny of the ingested cellular material has not been sufficiently experimentally explored so far. During significant anatomical transformations, such as metamorphosis, the transformation may affect a substantial number of cells and tissues, and it is difficult to imagine that they would not be further utilized within the organism.

In our work, we focused on the role of macrophages during adipose tissue remodeling in metamorphosis. Metamorphosis in insects separates two life stages of an individual. In the larval stage, individuals seek to acquire as much nutrition as possible, which is used for their growth but also as reserves for the transition to adulthood [171]. Unlike many other tissues, the larval fat body does not undergo histolysis and remains in the individual's body until adulthood, serving as nutritional support for post-metamorphic development [172].

However, at the beginning of metamorphosis, this tissue undergoes significant changes under the influence of a hormone called ecdysone [173]. Initially, storage proteins are taken up from the hemolymph, forming RNA-protein granules in the cytosol of adipocytes. Subsequently, these hypertrophic adipocytes change their shape from sheet-like cells to rounded, and the tissue is massively infiltrated by macrophages (Fig. 19).

Infiltrating macrophages eventually cover the entire surface of adipocytes during metamorphosis and interact closely with them. However, during metamorphosis, there are no dramatic changes in this tissue. Nevertheless, the changes are accelerated around the time of

emergence, when roughly three thousand adipocytes in each individual disappear over the course of three days [174]. We decided to investigate the role of macrophages in this process. Macrophages have hardly been observed by other groups in this situation and hardly studied because a previous study recognized their role in metamorphosis as unnecessary [175]. This seemed suspicious to us because our confocal and electron microscopic images suggested that macrophages efferocytosed dying adipocytes and metabolized the material obtained in phagolysosomes.

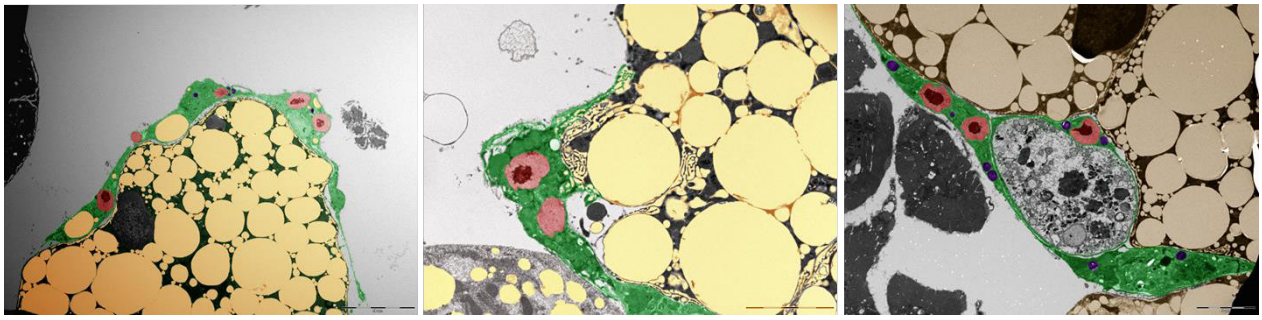

Figure 19. *Macrophages infiltrate larval adipose tissue over the course of metamorphosis, engulf leaking lipids and uptake moribund adipocytes via efferocytosis as documented here by transmission electron micrographs. Image credit: Krejčová G., Bajgar A.*

Nevertheless, our experiments disproved these initial claims. When we induced cell death in macrophages by overexpression of genes *Reaper* and *Hid* [176], genetically manipulated individuals were unable to survive beyond early pupal stages. When we triggered macrophage cell death after this critical time point of metamorphosis, individuals were able to complete their development but had significant problems with eclosion and delayed ovary maturation. Transcriptomic and proteomic analysis revealed that macrophages infiltrating adipose tissue significantly change the expression of genes involved in lysosomal metabolism but express highly expressed genes coding for lipoproteins and storage peptides. That was confirmed also by proteomic of hemolymph isolated from flies with macrophages-specific overexpression of biotin ligase. This construct allows us to distinguish which hemolymph proteins originate in macrophages [177].

Infiltrating macrophages become loaded by lipids and lysosomes containing fragments of adipocytes and we hypothesized that macrophages' role in this process may be the conversion of matter of moribund adipocytes into a suitable form of lipoprotein and storage peptides that can be further utilized within the body. To test this hypothesis, we prepared a fly strain with macrophage-specific knockdown of the *apolipophorin* (*apolpp*) gene, which is an essential component of lipoproteins in *Drosophila* [178]. Flies with inhibited production of *apolpp* in macrophages showed delayed ovary maturation when compared to controls which indicates an important role of these cells in providing nutritive support for post-metamorphic maturation in

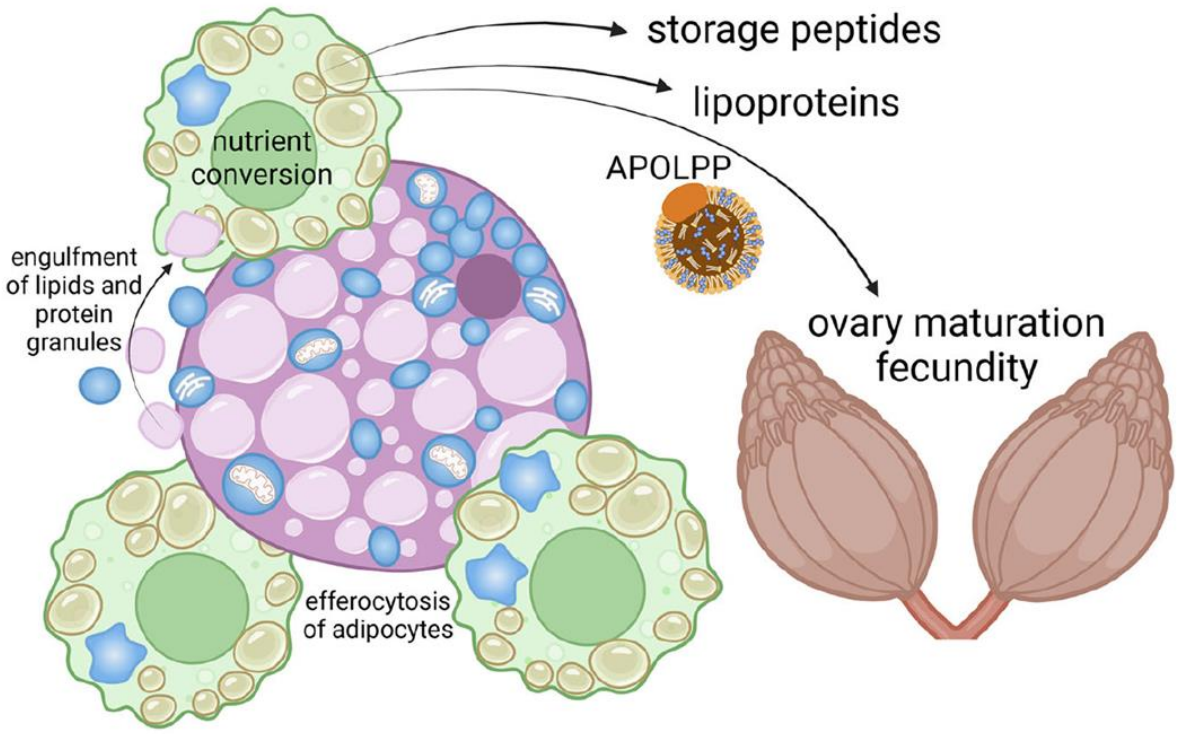

Figure 20. *Macrophage play nutritive role in Drosophila metamorphosis. Our data indicate that infiltrating macrophages combine features of macrophages and adipocytes to convert matter of moribund adipocyte into further exploitable compounds such as lipoproteins and storage peptides and support thus postmetamorphic maturation of individuals. Adopted from: Macrophages play nutritive role in …. Krejcova et al. 2024* [7].

Drosophila. Thus, macrophages transiently play a nutritional role and assume a function typically performed by adipocytes (Fig. 20).

The significance of these observations also lies in the fact that our work provides a large amount of descriptive data on the entire process and introduces a potential model for investigating the interaction of macrophages with adipocytes in a widely used molecular biology model. Our unpublished data further show that the adult fat body is heavily infiltrated by macrophages in response to situations associated with strong metabolic stress. Thus, we observe macrophage infiltration into adipose tissue in response to starvation, bacterial infection, as well as obesity or genetically induced lipolysis. However, this entirely new experimental system still requires more detailed characterization in the future. The aim of our further work is to understand the role of infiltrating macrophages in these processes and establish *Drosophila* as a new experimental model for research on adipose tissue macrophages.

Development of macrophage-specific delivery system in *Drosophila*

We have discussed that a key feature of macrophages is their ability to adopt a specific metabolic program, which also determines their functional polarization [46]. Therefore, the application of medicaments that affect the metabolic polarization of macrophages is considered a promising approach for intervening in many macrophage-related diseases, such as metabolic syndrome, cardiovascular diseases, neurodegenerative diseases, etc. [179]. More than three hundred substances carrying the potential to revert macrophage polarization have been identified. However, systemic administration of these metabolic inhibitors carries the significant risk of negative impacts of such treatment on other than the immune tissues. Therefore, we decided to try to develop tools that would serve for macrophage-specific delivery of various substances in *Drosophila* to test the effect of promising metabolism-regulating compounds.

With this idea, I contacted Professor František Štěpánek. Prof. Štěpánek was excited about extending his portfolio, which was mainly focused on preparing targeted cancer therapy and chemical robots releasing drugs in response to external signals, in a relatively less competitive research field. Moreover, while in common targeted therapy, the immune system causes problems as it tends to capture all foreign particles from circulation, in this case, this property becomes beneficial. I have therefore decided to undergo a kind of sabbatical under his supervision and attempt to test which microparticles could serve as suitable tools for targeted delivery to macrophages in insects.

After initial considerations and tests, we opted for glucan micro-particles. Glucan micro-particles are produced by derivatization of organic compounds from yeast [180]. The result of this process is a β-glucan shell, which allows easy modification of their surface and filling the core with chemicals of various nature. Glucan micro-particles are highly attractive bites to macrophages, which readily internalize them into their % of these particles by macrophages within phagolysosomes. Systemic administration of glucan micro-particles results in internalization of minutes and all particles are virtually cleared from circulation within minutes. Glucan particles are highly specific in targeting macrophages and under confocal and electron microscope, we have never observed their internalization by other cells. In addition, glucan microparticles show a low level of immunogenicity and basically no toxicity in the tested range of dilutions.

We designed many modifications of glucan microparticles that would help us to trace them within the organism and analyze them under an electron microscope or in a micro-CT instrument. In this regard, Dr. Ivan Saloň and Dr. Gabriela Ruphuy from Prof. Štěpánek group provided us with great service. Furthermore, we prepared glucan microparticles containing in their core paramagnetic nanoparticles which provide them magnetic features. This modification allows the isolation of macrophages (the only cells in insects capable of phagocytosing glucan particles) on magnetic columns. This new method for isolating macrophages from adult *Drosophila* individuals thus represents a gentle way to isolate macrophages of comparable quality to FACS sorting.

Moreover, their isolation does not require endogenous labeling with transgenic GFP construct or preparation of antibodies recognizing their surface antigens (Fig. 21).

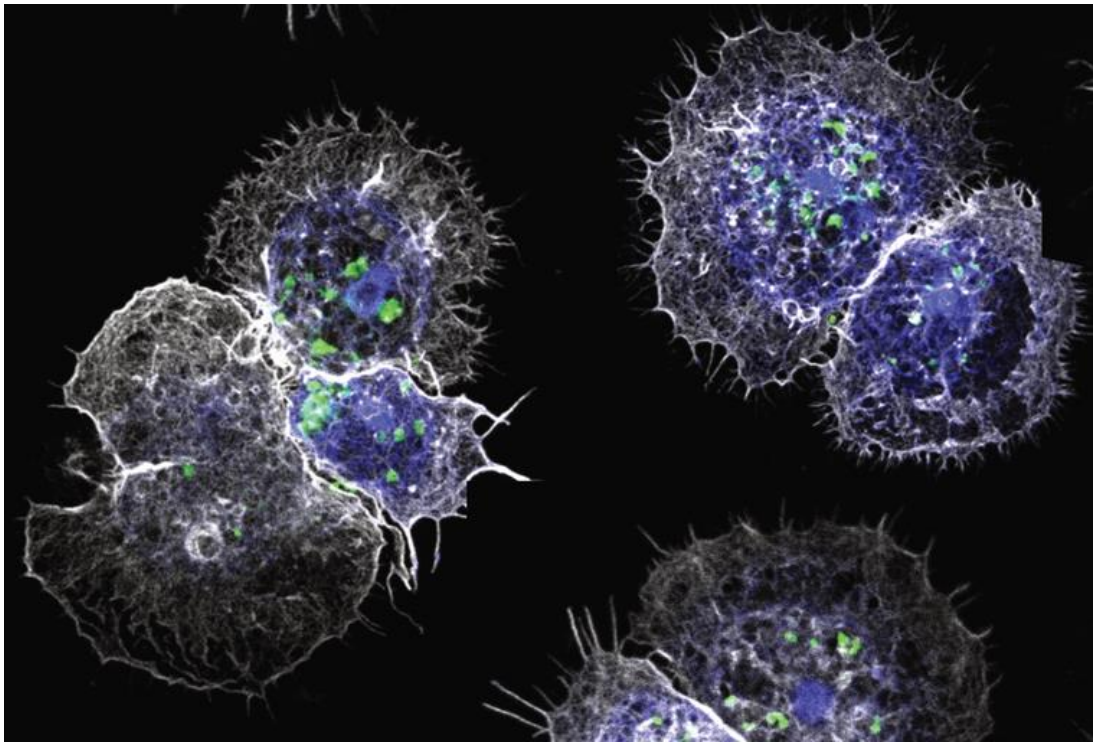

Figure 21. *Confocal image of Drosophila macrophages containing glucan microparticles in their cytosol. Actin filaments stained by Phalloidin are in white, Glucan microparticles in green. Image credit: Krejčová G., Bajgar A.*

To test glucan microparticles as a potential delivery tool, we prepared glucan microparticles carrying the transcription factor Gal4 and particles carrying atorvastatin, a widely used inhibitor of the mevalonate metabolic pathway. Our data indicate that these microparticles serve as a suitable delivery tool, allowing for the delivery of metabolic inhibitors or even small transcription factors to immune cells (Fig. 22).

In the currently ongoing project, we deliver double-stranded RNA through glucan microparticles and expand their use to non-model insect species. Our preliminary data suggest very encouraging results in this regard. Glucan particles have already been tested in the fruit fly, mosquito, honeybee, and mealworm beetle. We believe that once glucan particles are fully established in these insect species, they will be a highly valued experimental tool.

One of the ultimate research goals regarding glucan microparticles is to deliver orally administered antigens of viral and bacterial infectious agents. In this way, we would like to immunize vulnerable insect populations against viral and bacterial pathogens and turn the insect immune system against arboviruses, to which their vectors have developed immune tolerance.

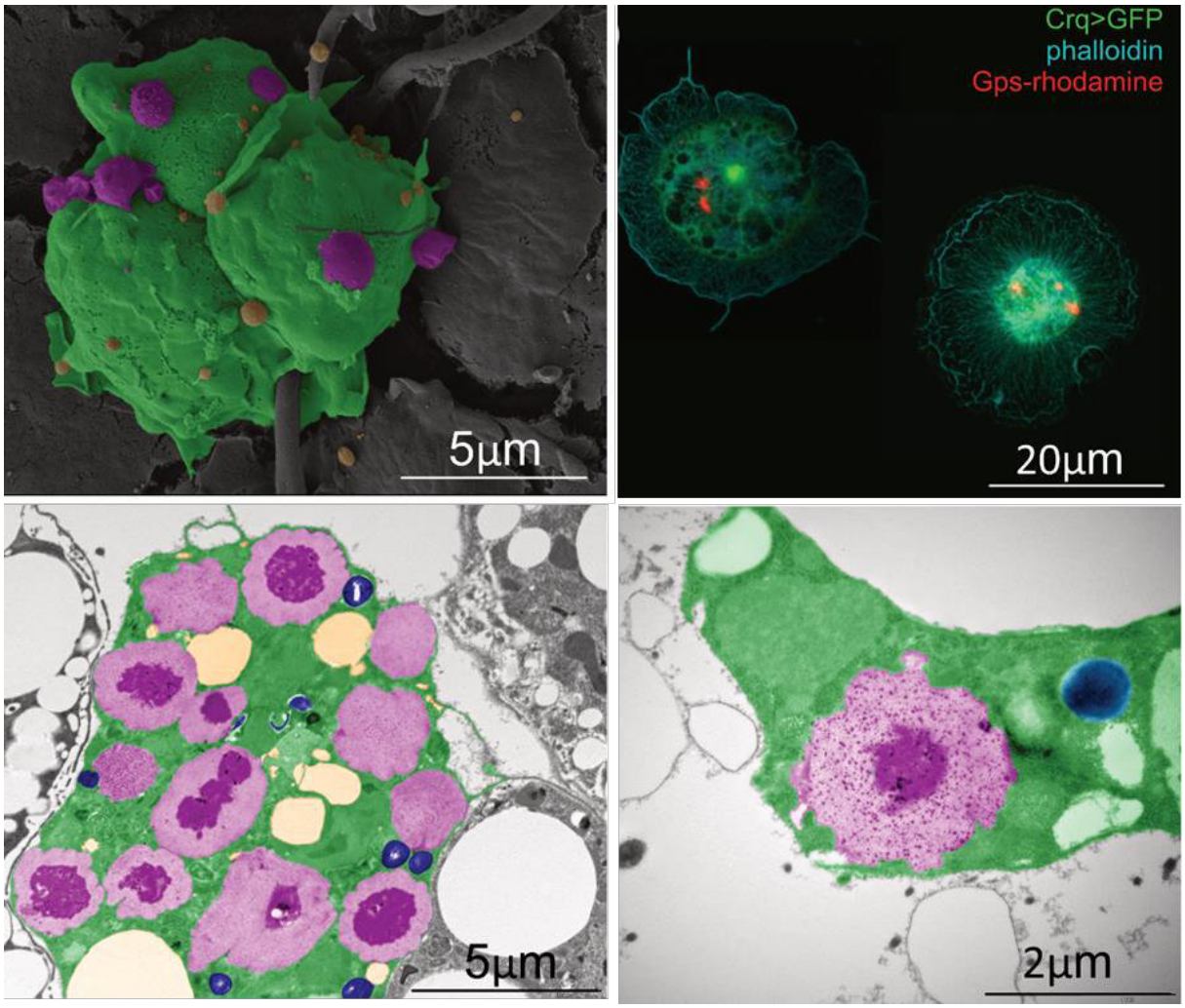

Figure 22. *Drosophila macrophages containing or engulfing glucan microparticles as documented by confocal and electron microscopy images. Macrophages are labeled in green, glucan microparticles in violet, phagocytosed streptococci are labeled in blue. Image credit: Krejčová G., Bajgar A.*

Conclusion

In my career so far, I have been dedicated to studying macrophage biology in Drosophila to understand their functioning in various stressful situations. The vast majority of my research consists of studies characterizing the metabolic changes of macrophages during bacterial infection and identifying factors that cause systemic metabolic changes. Drosophila represents an ideal model organism for such studies because we need to observe the coordinated action of several tissues in one organism and ideally influence individual players without completely disrupting the entire experimental system.

In my work, I have characterized the metabolism of infection-activated macrophages and found that macrophages adopt aerobic glycolysis as an essential metabolic program for their bactericidal function. Since the process is analogous to that observed in mammals, it indicates the evolutional conservation of this process between insect and mammalian immune systems.

However, this metabolic program is highly inefficient and its activation is associated with an increase in nutritional demands as macrophages rapidly consume their intracellular reserves. Their metabolic demands must be therefore satisfied from an external source. Macrophages for that purpose produce factors governing the systemic metabolism of the individual, thus inducing the mobilization of nutrients from the central metabolic tissues and limiting their consumption in immune-unrelated anabolic processes. In our work, we experimentally demonstrated the influence of two such factors, one reflecting the metabolic state of the cell, while the other coupled to the activation of the expression program leading to the metabolic switch in cellular metabolism. However, we may expect that more factors will be involved in the complex rewiring of systemic metabolism. The most likely mechanism underlying the metabolic changes is cytokine-induced insulin resistance, which is most often associated with maladaptive states of cachexia and chronic inflammation. Our work suggests that cytokine-induced insulin resistance may under certain conditions represent an adaptive metabolic program that allows for the mobilization of sufficient resources. Our observations thus link the change in cellular metabolism of activated immune cells with the change in systemic metabolism.

Our work suggests that this phenomenon could be conserved in mammals as well. This could have a significant impact on understanding cytokine-induced insulin resistance as not merely a pathological phenomenon. Furthermore, our work could provide a new perspective on a range of diseases where pro-inflammatory polarization of macrophages and subsequent production of pro-inflammatory cytokines cause significant metabolic problems, such as during obesity.

Our subsequent project revealed that macrophages play a crucial role in recycling nutrients from the larval fat body during the metamorphosis of holometabolous insects. Material from histolyzing cells is not simply eliminated from the body as waste. Macrophages phagocytize cellular fragments and, through a combination of properties characteristic of macrophages and adipocytes, produce large amounts of lipoproteins and storage peptides, in which they recycle

components of dying cells. This project revealed how metabolically plastic macrophages could be and that they can under certain conditions stand for the function of another tissue. The remaining question is whether this phenomenon is present only in insects. We have observed it in many holometobolous insect species indicating its importance in insect metamorphosis. Nevertheless, it may be essential also for metamorphosis in amphibians as well as for any substantial remodeling of tissue such as in embryonic development. Moreover, an analogous mechanism may theoretically be involved in the daily turnover of billions of cells in the human body.

In a more technical aspect of our projects, we focused on developing tools that would allow for macrophage-specific delivery of various active substances. We have developed glucan micro-particles, which serve as a very effective tool for influencing the physiology of these cells, by delivering transcription factors, metabolic inhibitors, and interfering RNA.

The ultimate goal of this project is to pave the way for regulating the metabolic polarization of macrophages in Drosophila and to demonstrate whether this contemplated strategy is meaningful for future medical treatment. In parallel, we are developing tools that facilitate further research of immune cells even from non-model insect species. That can broaden our scope to other insect species such as honey bees, mosquitoes, and many others whose immune systems remain broadly unexplored.

In future projects, we aim to focus on infiltrating adipose tissue macrophages. We want to fully establish Drosophila as a model for the research of adipose tissue macrophages, which we believe represents a valuable knowledge gap and would certainly be worth exploring further.

List of author's publications attached to this habilitation thesis

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Responsible for *Drosophila* part of the project

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