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Running title

Bacterial-induced *Warburg-like* metabolic programs

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ABSTRACT

The finding that the Warburg effect observed in proliferating cancer cells is also observed during immune responses renewed the interest in the study of metabolic reprogramming of immune cells, a field of investigation called immunometabolism. However, the specific mechanisms and processes underlying metabolic changes of host cells upon bacterial infection remain poorly understood. Several recent reports have reported that mammalian cells infected with intracellular bacteria have an altered metabolism that resembles the Warburg effect seen in cancer cells. In this Review, we will summarize current knowledge on metabolic reprogramming and discuss putative causes underlying the preferential remodelling of host cells to *Warburg-like* metabolic programs during infection by intracellular bacteria.

Abbreviations

ATP: adenosin triphosphate; TCA: tricarboxilic acid; CO_2: carbon dioxide; CI - CV: Complex I to Complex V; OXPHOS: oxidative phosphorylation; FDG-PET: ^{18}F-DeoxiGlucose Positron Emission Tomography; PI3K: Phosphoinositide 3-kinase; AKT: Ak transforming; HIF1: Hypoxia-inducible factor 1; p53: 53-kilodalton protein; MYC: similar to myelocytomatosis viral oncogene; AMPK: AMP-activated protein kinase; mTORC1: mammalian target of rapamycin complex 1; CoA: Coenzyme A; NADH: reduced form of nicotinamide adenine dinucleotide; NADPH: reduced form of nicotinamide adenine dinucleotide phosphate; PPP: pentose phosphate pathway; DC: dendritic cell; PRR: pathogen recognition receptor; TLR: Toll-like receptor; LPS: lipopolysaccharide; APC: antigen-presenting cell; MHC: major histocompatibility complex; CD: cluster of differentiation; IL: interleukin; TCR: T-cell receptor; IFN: interferon; T_H: T-helper; iNOS: inducible nitric oxide synthase; NO: nitric oxide; 2DG: 2-DeoxiGlucose; BMDM: bone-marrow-derived macrophages; TNF: tumor necrosis factor; ETC: electron transport chain; PKM2: pyruvate kinase M2; ROS: reactive oxygen species; Pam3Cys: tri-palmitoyl cysteine; M1: macrophages activated by T_H1 cytokines; M2: macrophages activated by T_H2 cytokines; M0: non-activated macrophages; FAO: fatty-acid oxidation; UDP-GlcNAc: uridine diphosphate N-acetylglucosamine; GM-CSF: granulocyte-macrophage colony-stimulating factor; M-CSF: macrophage colony-stimulating factor; T4SS: type IV secretion system; LncP: Legionella nucleotide carrier protein; MitF: mitochondrial fragmentation factor; HUVEC: human umbilical vein endothelial cell.

Introduction

Cellular metabolism is at the crossroads of diverse disciplines, such as immunity, cancer and aging [1-3]. It comprises i) the biochemical reactions leading to the generation of energy (in the form of ATP) by breaking down biomolecules (catabolism) and ii) the reactions leading to the biosynthesis of biomolecules to build up cellular components (anabolism) (Fig. 1). In mammals, a bi-directional relationship between the functions and the metabolic status of each single cell in the organism exists. Therefore, different metabolic programs are executed in non-differentiated stem cells *versus* differentiated cells or in proliferating *vs.* non-proliferating cells (Fig. 2). The metabolic program executed by a cell at a certain time is hence essential for its functional status [4-6]. Recent evidences suggest that mammalian cells infected by bacteria also develop different metabolic programs compared to their non-infected counterparts [7-17].

Most non-proliferating, differentiated mammalian cells (such as somatic cells in tissues) have a quiescent metabolic state characterized by a low catabolic and anabolic activity and an efficient generation of ATP by the catabolic process known as mitochondrial respiration. Such cells convert glucose to pyruvate via anaerobic glycolysis most of which is then routed to mitochondria, where pyruvate is completely oxidized to CO_2 via the tricarboxilic acid (TCA) cycle (Fig. 1). Oxygen is the final acceptor of an electron transport chain (ETC) composed of five assembled complexes (CI to CV) that generate an electrochemical gradient in the mitochondria that facilitates ATP production by Complex V (the F_1-F_O-ATPase), a process termed oxidative phosphorylation (OXPHOS) (Fig. 1). Under these conditions, a non-proliferating, differentiated mammalian cell have a total energy gain of 36 ATP molecules per molecule of absorbed glucose [6,18]. OXPHOS is therefore the most important metabolic pathway used by differentiated, non-proliferating cells.

On the other hand, proliferating mammalian cells (such as embryonic stem cells or activated lymphocytes) require a high catabolic and anabolic activity, which requires an increased glucose uptake and the rapid (although less efficient) generation of ATP driven by glycolysis coupled to fermentation. In the proliferating state, cells convert glucose to pyruvate through glycolysis, and then pyruvate is fermented to lactate, process taking place in the cytosol (Fig. 1). In this case, only 2 ATP molecules are generated per molecule of glucose. Proliferating cells thus rely on a high rate of glycolysis and a high glucose uptake to support the doubling of their components during cell division. An outstanding question is why proliferating cells use a glycolytic metabolism although this metabolic pathway is less efficient than OXPHOS (at least in terms of ATP production)[18].

Several reports have claimed that mammalian cells infected by intracellular bacteria such as *Mycobacterium tuberculosis* [12-16], *Legionella pneumophila* [11], *Brucella abortus* [7], *Chlamydia trachomatis* [8,9] or *Chlamydia pneumoniae* [10] have an altered metabolism consisting in an increment of glucose uptake and/or glycolysis, which resembles the metabolic program widely seen in proliferating cancer cells known as the "Warburg effect" (or aerobic glycolysis). Here, we review current knowledge on metabolic reprogramming and discuss putative causes underlying the preferential remodelling of host cells to *Warburg-like* metabolic programs during infection by intracellular bacteria.

The Warburg effect is a hallmark of cancer cells

Cancer cells are the most prominent example of glycolytic-based proliferation. Tumour cells convert the majority of their absorbed glucose to lactate, even under oxygen-rich conditions, and this dependency on a high glycolytic rate and high glucose uptake was termed the "Warburg effect" in honour of Otto Warburg, who discovered this altered metabolism that tumour cells have compared to differentiated cells in tissue [20]. Elevated glucose uptake and lactate secretion are, since then, considered as two general, metabolic hallmarks of solid tumours as they have been observed in a wide variety of cancers. These seminal findings by Otto Warburg have also led to improved diagnosis and planning of an appropriate cancer therapy by using ^{18}F-DeoxiGlucose Positron Emission Tomography (FDG-PET), an imaging method that allows to measure the increased uptake of the marked glucose analogue FDG prior to PET scanning of tumours [19]. Otto Warburg also suggested that this metabolic alteration was an essential cause of cancer and that mitochondrial defects inhibiting the ability of cancer cells to fully oxidize glucose to CO_2 were the cause of this metabolic switch to a glycolytic metabolism in cancer cells [20-22].

Nowadays is widely accepted that a switch to a glycolytic metabolism (the Warburg effect) is a core hallmark of cancer cells. However, contrary to what was suggested by Warburg, we know today that mitochondria of tumours are not defective and therefore are not the cause of the observed metabolic reprogramming. Compelling evidences indicate that the metabolic reprogramming of cancer cells to aerobic glycolysis is a direct response to the activation of growth factor signalling, even in the absence of extracellular growth-factors [23-25]. The signalling hubs regulating the metabolic responses to growth factor signalling are mainly i) the PI3K/AKT axis; ii) the transcription factors HIF1, p53 and MYC; iii) the metabolic sensors AMPK and mTORC1; iv) the Ras proteins and v) the alternatively spliced

isoforms of pyruvate kinase (reviewed in [23,26]). Thus, activation of growth factor signalling even in the absence of extracellular growth factors is the main cause of the Warburg effect in cancer cells, and not defective mitochondria. In fact, mitochondria are not defective in most of the tumours. During the Warburg effect mitochondria of cancer cells are repurposed from the bioenergetic role of OXPHOS-mediated ATP generation (OXPHOS metabolism) to a biosynthetic role where mitochondrial enzymes are used in the synthesis of nucleotides, amino acids and lipids (FIG. 1). Therefore, although the question of why cancer cells rely on the Warburg effect is still a matter of debate [27], it has been suggested that the reprogramming of cellular metabolism towards macromolecular synthesis is critical for a proliferating cell and that aerobic glycolysis (the Warburg effect) is a well-suited metabolic program for this aim [18,26,27]. The Warburg metabolism is thus an anabolic program, essential for cell growth and proliferation, which is not focused on maximizing ATP production such as OXPHOS.

In order to fulfill the requirements of proliferation, the Warburg metabolism (Fig. 3A) leads to i) an increase of the reduced carbon uptake (glucose) to fuel glycolysis, which generates pyruvate and other glycolytic intermediates that are used in biosynthetic pathways; ii) an increase of the reduced nitrogen uptake (glutamine) for the biosynthesis of nucleotides and non-essential aminoacids, a process that involves mitochondria; iii) divert citrate from the TCA cycle towards the generation of acetyl-CoA in the mitochondria, which is exported to the cytosol and used in lipid synthesis; and iv) generate enough NADH and NADPH in the cytosol through glycolysis and the pentose phosphate pathway (PPP), respectively, to be used in these reductive biosynthetic reactions [18,26,27]. Aerobic glycolysis coupled to fermentation fulfils all these requirements by a fast process that is easy to regulate. Thus, the metabolic program known as aerobic glycolysis (Warburg metabolism) maximizes biosynthetic pathways by redirecting cytosolic glycolysis and mitochondrial TCA cycle to the biosynthesis of nucleotides, aminoacids and lipids. One outcome of the switch to this program is the fast withdrawal of pyruvate and TCA intermediates in the mitochondria that were previously dedicated to the OXPHOS program. This explains the reduction of oxygen consumption observed during the Warburg metabolism. Moreover, OXPHOS seems not completely shut down in cancer cells. It has been suggested in tumours that, although the Warburg metabolism uses 85% of pyruvate, 5% of pyruvate is still normally routed to OXPHOS [18].

Immunometabolism focuses on the metabolic reprogramming of immune cells

Metabolic changes alter functions of immune cells, a field of investigation called immunometabolism. Some interesting historical reports in this discipline [28-31] already anticipated the renewed interest on immunometabolism. New tools to study cellular metabolism, such as extracellular flux assays [32] or mass spectrometry-based metabolomics [33], have recently boosted the field by allowing researchers to study metabolic dynamics of immune cells upon activation.

In the following paragraphs we will briefly delineate how the immune system works upon bacterial infection, and then we will present the current working models on immunometabolism. The theory of immunity during infection states that the first line of the immune defence against bacteria is the innate immune system, composed of immune cells such as monocytes, macrophages, dendritic cells (DCs) and neutrophils. As they reside in tissues or patrol the blood, these cells are the main immune cells encountering bacteria. Moreover, once bacteria are detected, dozens of these cells are recruited to the site of infection. They can be activated by microbial-derived compounds that are sensed by pathogen recognition receptors (PRRs), which are located either on the cell surface, in phagosomal membranes or in the cytosol of innate immune cells. The most studied example is the activation of monocytes and macrophages by the PRR called Toll-like receptor 4 (TLR-4). In this case, the binding of the outer membrane component of gram negative bacteria called lipopolysaccharide (LPS) to TLR-4 in innate immune cells mainly leads to i) an increase of phagocytosis; ii) the secretion of bactericidal compounds; iii) the secretion of immune mediators (i.e. interleukins and other cytokines) that in turn activate and recruit other immune cell types; and iv) the upregulation of antigen presentation. In this way, bacterial attachment or phagocytosis leads to PRR signalling activation of the so-called professional antigen-presenting cells (APCs), mainly macrophages and DCs, which trigger the secretion of specific cytokines such as interleukin-8 (IL-8) that recruits neutrophils to the site of infection. Bacterial phagocytosis by these APCs also stimulates the loading of lysosome-derived antigens from ingested bacteria into the antigen-presenting adaptors that are the MHC class II molecules, which are expressed on APC cell surface. Upon activation, APCs migrate to nearby lymph nodes for antigen presentation to $CD4^+$ T-helper cells, the main effectors of the adaptive immune system during bacterial infection. In the lymph node, the specific matching of antigen-loaded MHC molecules expressed on APC to antigen-specific T-Cell receptors (TCRs) expressed on T-cell surfaces, create an immune synapse that triggers the activation of

antigen-specific $CD4^+$ helper T-cells. Then, activated $CD4^+$ helper T-cells start to i) secrete cytokines such as IL-2 and interferon gamma (IFN-γ) and ii) proliferate (clonal expansion). The specific context of APC-secreted cytokines, such as the presence of IL-12, also modulates (polarizes) the immune response of T-helper cells to specific subtypes of responses, where the T_H1 and the T_H17 responses are the most important during bacterial infection. T-cells also orchestrate the production of antigen-specific antibodies by B-cells. IFN-γ secreted by T_H1-polarized T-cells reinforces the activation of macrophages to phagocytose and digest intracellular bacteria, and to activate iNOS to produce nitric oxide (NO) from L-arginine to directly kill intracellular bacteria. Although the role of adaptive immunity during bacterial infection is well established, most of the non-symptomatic cases of bacterial infection are successfully resolved by the sole action of innate immune cells such as macrophages and neutrophils [34].

The current model of immunometabolism proposes that innate or adaptive näive (non-activated) immune cells mainly rely in an OXPHOS metabolism that is shifted to Warburg metabolism upon immune activation (Fig. 2), a metabolic switch that is essential for the immune functions of these activated cells [6]. For instance, specific subsets of effector T-cells switch to Warburg metabolism upon activation by APCs [35], including T_H1 and $T_H17$cells [36,37]. A switch to Warburg metabolism has also been observed upon immune activation of other immune cells, such as macrophages [38,39], DCs [39,40], neutrophils [41], B-cells [42] and natural killer cells [43]. Key examples of metabolic reprogramming from OXPHOS to Warburg upon immune activation are i) macrophages activated by PRRs [38,39], ii) T-cells activated by cytokine receptors [44] or iii) B-cells activated by antigen receptors [42]. This metabolic remodelling seems essential for the exertion of immune functions by these activated cells. Examples of immune functions directly depended on the switch to a Warburg metabolism are i) phagocytosis and IL-1β production by macrophages [39]; ii) acquisition of co-stimulatory capacity by DCs [39]; iii) T_H17 polarization by activated T-cells [45] or iv) formation of nets by neutrophils [41].

An important advance made during the last years has been the delineation of precise metabolic programs that specific immune cells develop upon activation [6,41]. Although the term “Warburg metabolism” has been widely used to describe the prominent use of aerobic glycolysis by activated immune cells, cell type-specific metabolic programs seem to exist among cells of the immune system performing aerobic glycolysis upon activation. For instance, despite the clear glycolytic program displayed by macrophages upon activation, a

broken TCA cycle was also observed in murine macrophages activated by bacterial LPS, where TCA intermediates such as succinate and citrate accumulates in the cell (discussed below, Fig. 3B, [46-48]). This broken TCA cycle, however, has not been observed in other immune cells such as DCs, which also shift to aerobic glycolysis upon activation. This indicates that cell-type and context-specific particularities might exist and, therefore, it might be more appropriate to consider that several *Warburg-like* metabolic programs can be activated in immune cells depending on the cell type concerned and the specific context of activation.

Macrophages are the best-studied examples of immune cells performing metabolic reprogramming. As a first-line guard, macrophages have a key role in host defence during bacterial infection. Macrophages phagocytose bacteria to clear infection and secrete antimicrobial compounds to directly kill pathogenic bacteria. However, several intracellular pathogens such as *M. tuberculosis* or *L. pneumophila* infect preferentially macrophages and are able to replicate within them, albeit macrophages are one of the main immune cells to kill pathogenic bacteria. Several lines of evidence suggest that intracellular bacteria trigger specific metabolic programs during infection of macrophages (discussed below) and that this metabolic remodelling might be key for bacterial replication within these immune cells [49,50]. Interestingly, certain pathogenic bacteria induce metabolic programs that favour their replication, while others induce metabolic programs that are restrictive for bacterial replication [49,50]. Thus, the study of the macrophage metabolism during bacterial infection should shed light on how pathogenic bacteria subvert macrophage functions and cause disease.

The metabolism of macrophages and monocytes is reprogrammed upon stimulation with microbial compounds

The specific metabolism of macrophages and of their precursor cells, monocytes, studied in several early reports showed an increased glycolytic activity and decreased OXPHOS upon exposure to microbial compounds [28,30,31,51,52]. Recent reports confirmed these early observations and show that specific metabolic programs are activated in monocytes and macrophages upon exposure to microbial products or to whole-bacteria lysates [38,46,47,53].

Tannahill *et al.* showed that 2-deoxyglucose (2DG)-mediated inhibition of glycolysis in mouse bone-marrow-derived macrophages (BMDMs) specifically inhibits LPS- and *Bordetella pertussis*-induced transcription of IL-1β, while other cytokines such as TNF-α and

IL-6 remain transcriptionally unaffected. This suggests that metabolic reprogramming to a glycolytic Warburg program is necessary for the transcription of IL-1β by LPS-activated BMDMs, a process depended on LPS-induced HIF-1α expression [46]. A key observation in this study was that, although an overall decrease in TCA cycle activity and mitochondrial respiration occurred at 24 h post-LPS-treatment, there was an accumulation of succinate, a key TCA cycle intermediate, that was derived from glutamine. Therefore LPS-induced succinate can act as a signal to increase IL-1β expression through HIF-1α [46]. Later studies from this and other groups revealed further clues about the metabolic reprogramming upon exposure of BMDM to LPS (Fig. 3B), specifically that i) LPS-induced succinate oxidation by succinate deshydrogenase (Complex II of the electron transport chain, ETC) leads to an elevated mitochondrial membrane potential that seems sustained by reverse flow of the ETC, which produces mitochondrial reactive oxygen species (mROS) that drive HIF-1α-mediated IL-1β expression [54]; ii) pyruvate kinase M2 (PKM2) is also determinant for HIF-1α-mediated IL-1β expression [38]; and iii) LPS-induced expression of the mitochondrial citrate carrier diverts citrate from mitochondrial TCA cycle to the cytoplasm, which accumulates in the cytoplasm and seems then to be used to generate NO, ROS and prostaglandins by macrophages, as well as to produce the antimicrobial metabolite itaconate [47,55,56]. Thus, exposure of mouse macrophages to the TLR-4 agonist LPS reprograms their metabolism from OXPHOS to glycolysis and rewires TCA cycle intermediates such as citrate and succinate to biosynthetic pathways.

However, by using human monocytes isolated from blood, a recent report showed that a shift from OXPHOS to glycolysis was observed only in monocytes stimulated with LPS, and not in monocytes stimulated with the TLR-2 ligand Pam3Cys or other whole-pathogen lysates. In monocytes stimulated with Pam3Cys, increased glycolysis was accompanied by increased OXPHOS that was needed for retention of their phagocytic capacity and cytokine production [53]. Interestingly, elevated succinate, itaconate and citrate levels were only observed in LPS-stimulated monocytes and redirection of these TCA intermediates was not observed in Pam3Cys-stimulated monocytes. Moreover, the same study showed that stimulation of human monocytes with whole-pathogen lysates of *Escherichia coli*, *Staphylococcus aureus* or *M. tuberculosis* increased glycolysis, while OXPHOS only increased after exposure to *E. coli* or *S. aureus*. Exposure of monocytes to *M. tuberculosis* whole-bacteria lysates did not affect OXPHOS, which remained at the same level as unstimulated monocytes, in contrast to OXPHOS reduction upon LPS exposure [53]. These findings challenge the notion that a shift

from OXPHOS to glycolysis is a general response of all immune cells activated upon stimulation with bacterial components, suggesting that induction of specific metabolic programmes depends on individual stimuli. In addition, this study opens the question of whether these immunometabolic responses of host cells to bacterial components are cell-type specific (e.g. monocytes vs. macrophages), host-species specific (e.g. mouse vs. human) and/or bacterial-species specific (e.g. bacterial components from *S. aureus* vs. those from *M. tuberculosis*). Clarification of these pending questions is crucial. Furthermore, the fact that exposure of human monocytes to whole-bacterial lysates from *M. tuberculosis* did not affect OXPHOS [53] but infection of the same cell type with living *M. tuberculosis* clearly reduced OXPHOS [14] raised the questions whether the immunometabolic responses elicited by bacterial components can be translated into an understanding of metabolic reprogramming of host cells during infection. Indeed, metabolic responses to living bacteria seem more complex than the sum of metabolic responses to individual bacterial components.

The metabolic reprogramming of macrophages is more complicated than the M1/M2 model

Another level of complexity in the study of macrophage immunometabolism is the metabolic rearrangements that occur during macrophage polarization. Similarly to T-cells, polarization of macrophages is driven by the presence of different cytokines in the microenvironment, and it is thought that this process determines macrophage immune functions. *In vivo*, the source of these polarizing cytokines could be surrounding activated cells. As different experimental models exist for *in vitro* macrophage polarization, the nomenclature of polarized macrophages is confusing [57]. The traditional nomenclature is a binary classification of polarized macrophages into two distinct phenotypes: M1 or "classically-activated" by T_H1 cytokines, and M2 or "alternatively-activated" by T_H2 cytokines. For instance, a widely used model for *in vitro* polarization of murine macrophages is to expose them to the combination of IFN-γ (T_H1 cytokine) and LPS to polarize macrophages from M0 "resting macrophages" to M1 "classically-activated" macrophages, or to expose them to IL-4 (T_H2 cytokine) to polarize macrophages from M0 to M2 "alternatively-activated" macrophages. *In vitro* polarized M1 and M2 macrophages have different functional characteristics: whereas M1 macrophages have pro-inflammatory microbicidal and tumoricidal properties, M2 macrophages promote tissue homeostasis, wound healing and anti-helminth immunity [58]. Importantly, *in vitro* polarized M1 and M2 macrophages have also different metabolic programs. M1 macrophages have increased glycolytic flux and reduced OXPHOS compared to M0 cells [59], as well as a

broken TCA cycle as explained above [6,55,60]. On the other hand, M2 macrophages have higher OXPHOS than M0 cells, an intact TCA cycle, increased mitochondrial fatty acid oxidation (FAO), a glutamine-depended production of α-ketoglutarate and the UDP-GlcNAc pathway is activated [6,55,59-61].

However, the binary model of M1/M2 macrophages has some limitations [62-64]. First, M1/M2 is mostly a model for *in vitro* differentiation of macrophages. Most of the markers observed for murine macrophages do not translate to human macrophages, probably because murine macrophages are generally differentiated from bone marrow cells while human macrophages are obtained by *in vitro* differentiation of blood monocytes [57]. An example of the heterogeneity in the methods used to polarize human macrophages is the utilization of GM-CSF or M-CSF to differentiate blood monocytes to M1 or M2 macrophages, respectively [57,65]. As GM-CSF and M-CSF are growth factors instead of cytokines or microbial products, it is very difficult to know whether the obtained cells are indeed the human counterparts of murine M1/M2 macrophages. Thus, intuitively murine LPS+IFN-γ-activated M1 macrophages and human GM-CSF-differentiated M1 macrophages might be quite different cell types. An additional difficulty is that polarization seems to be reversible both *in vivo* and *in vitro* [64]. Thus, these limitations in the M1/M2 model reveal the necessity to improve the model of macrophage polarization. Such improvement should then be applied to the study of the specific metabolic signatures of polarized macrophages.

A first approach for improving the M1/M2 model has been the suggestion to use a new, more appropriate nomenclature for *in vitro* polarized macrophages, which implies naming macrophages according to the specific polarization stimulus used, e.g. M (LPS), M(IFN-γ), M(LPS+IFN-γ), M(IL-13), M(IL-4), etc [57]. It was also recommended to abandon the use of M-CSF or GM-CSF as polarization factors and only use them as differentiation reagents [57]. By using this new nomenclature, the metabolic reprogramming delineated before for M1 and M2 macrophages now corresponds to M(LPS+IFN-γ) and M(IL-4) murine macrophages, respectively, highlighting the lack of information on the metabolic reprogramming of other macrophage subtypes or on human macrophages [46,61]. Moreover, recent reports indicate that the polarization process, both *in vitro* and *in vivo*, results in a spectrum of macrophage phenotypes rather than a few discrete and separate subsets [58,64,66]. This suggests that a continuum of polarized macrophages can be found in any specific situation and might mean that the dynamic plasticity of macrophage functions exceeds our current tools for phenotyping macrophages.

Therefore, similarly to the study of metabolic reprogramming of macrophages upon stimulation with single bacterial components, the adscription of specific metabolic programs to macrophage subtypes might not be very helpful to understand the metabolic reprogramming of macrophages during bacterial infection. It seems that the complexity of host-pathogen interactions, and particularly bacteria-macrophage interactions, cannot be mimicked by stimulation or polarization using single or combined stimuli and, consequently, caution should be taken when studying the metabolic responses of macrophages during infection with living bacteria.

Metabolic reprogramming of host cells upon infection with intracellular bacteria seems pathogen-specific

Infection of host cells with living bacteria is instrumental to understand metabolic reprogramming upon bacterial infection and to study metabolism during host-pathogen interactions. As intracellular bacteria, such as *Mycobacterium*, *Chlamydia* or *Legionella*, infect and replicate inside host cells, the energy and nutrients needed to support their replication can only be obtained from the infected host cell. Thus the investigation of the metabolism of cells infected by intracellular bacteria is an exciting "closed system" of host-pathogen interactions that can be, for instance, isolated after *in vivo* infection or studied at the single cell level while maintaining the biological and pathological relevance.

Importantly, to study the host metabolism during infection it is key to choose relevant host cells as model. Given the fact, that most of the extensively used cell lines are derived from cancer cells that have already an altered metabolism and an enhanced Warburg metabolism due to their tumour nature, it seems instrumental to use primary cells as host cells for such analyses [17]. Gillmaier *et al.* nicely showed this, by studying the infection of primary murine BMDM and of murine macrophage-like J774A.1 cells with the intracellular bacteria *Listeria monocytogenes*, the causative agent of a serious food-borne and invasive disease called listeriosis [67]. They show that a high induction of glucose uptake and of glycolysis was only observed upon infection of primary BMDMs, but not in the J774A.1 cells, as they already exhibit altered glucose transport and glycolysis due to their cancer origin [67]. Importantly, many pathogenic bacteria are able to replicate within cancer cell lines such as THP-1, A549, U937 or RAW 264.7 cells [17,68], suggesting that the Warburg metabolism exhibited by these cell lines does not inhibit bacterial replication. Indeed, when intracellular growth of *L. monocytogenes* in primary BMDM and J774A.1 cells was compared in parallel

experiments, intracellular bacteria replicated to five fold higher numbers within the cancer cell line, suggesting that the Warburg metabolism exhibited by cancer cells may even be beneficial for the pathogen [67]. Thus, in the following paragraphs we will summarize only metabolic data, obtained during *in vivo* and *in vitro* infections of primary cells with intracellular bacteria, to avoid confusion with results obtained during *in vitro* infections of cancer cell lines.

The best-studied example of bacterial-induced metabolic reprogramming of host cells is the infection by *Mycobacterium tuberculosis*, the causative agent of tuberculosis. Several reports indicate that *M. tuberculosis* induces a Warburg metabolism in the infected cells, both *in vivo* and *in vitro* [12,13,15,16,53,69,70]. One of the main features of the immune response to *M. tuberculosis* is the formation of an organized structure called granuloma through the recruitment of different cell types to the site of infection, mainly i) macrophages; ii) highly differentiated cells such as multinucleated giant cells; iii) epithelial cells; iv) "foamy" macrophages; and v) surrounding lymphocytes. Similarly to the clinical use of FDG-PET scanning of tumours as a diagnostic tool of cancer and disease progression, the *in vivo* imaging of granulomas through FDG-PET scanning of host lungs (human or animal) has been used to judge the severity of the disease and assess the efficacy of antimicrobial therapy [71]. This demonstrates that the increased incorporation of the glucose analogue FDG into granulomas is a surrogate marker for a Warburg metabolism induced by *M. tuberculosis* infection. Specifically, the metabolic reprogramming of macrophages exerted by *M. tuberculosis* infection includes (Fig. 4A): i) the upregulation of key glycolytic enzymes and transporters for glucose uptake [12,69]; ii) the downregulation of enzymes participating in the TCA cycle and OXPHOS [12,69]; iii) the redirection of the glycolytic pathway towards ketone body and lipid synthesis, which accounts for the classical "foamy phenotype" of *M. tuberculosis*-infected macrophages [72,73]; iv) the dependency of the observed Warburg effect on the recruitment of IFN-γ-activated macrophages to lung granulomas [13]; and v) the utilization of the Warburg-produced lactate for *M. tuberculosis* growth [70]. In summary, the *Warburg-like* program induced by *M. tuberculosis* in the infected cells is characterized by the classical upregulation of glucose uptake and glycolysis coupled to the deviation of glycolytic intermediates to the synthesis of large lipid bodies, which are accumulated in the macrophage and feed intracellular bacteria in the form of nutritional fatty acids [72,73]. The lactate produced by glycolysis can also be used by the pathogen [70], which suggests that the

benefits of the *Warburg-like* phenotype for *M. tuberculosis* might be, like in tumour cells, the biosynthetic role of aerobic glycolysis.

Another bacterium that has been shown to induce a *Warburg-like* phenotype in infected cells is *L. pneumophila* [11]. This intracellular bacterium is the etiologic agent of Legionnaires' disease, a serious pulmonary infection during which the pathogen replicates within human lung macrophages. By injecting more than 300 bacterial effectors in the host cell through a type IV secretion system (T4SS), *L. pneumophila* subverts cellular functions in order to replicate within eukaryotic cells [74,75]. Some of these *L. pneumophila* effectors target mitochondria or mitochondrial functions, such as the bacterial effector LncP that is targeted to the mitochondrial inner membrane and might function as an ATP transporter, or the bacterial effector MitF, which promotes the fragmentation of the mitochondrial network during infection of human primary macrophages [11,76,77]. We recently showed that *L. pneumophila* induces a biphasic alteration of the macrophage metabolism at very early times post-infection [11]. Upon infection, a first phase of increased glycolysis and OXPHOS that peaks at 1h post-infection is followed by a second phase where glycolysis remains high while OXPHOS is severely reduced. This second phase is extended, at least, until 5h post-infection, which is prioir to bacterial replication and to the activation of macrophage cell death pathways. By using mutants deficient in the T4SS that cannot inject bacterial effectors and therefore cannot replicate within infected cells, we determined that this first phase is T4SS-independent, while the second phase is T4SS-dependent [11]. As *L. pneumophila* infection mainly activates TLR-2 [78-80] and activation of TLR-2 leads to increased glycolysis and OXPHOS in human primary cells [53], it is possible that the induction of the first phase, i.e. increased glycolysis and increased OXPHOS, is T4SS-independent but TLR-2-dependent . On the other hand, as T4SS-deficient mutants are phagocytosed in the same way as *L. pneumophila* wild type (wt), and both wt and mutants express LPS and other TLR ligands, the induction of the T4SS-dependent *Warburg-like* program observed in the second phase, i.e. increased glycolysis and reduced OXPHOS (Fig. 4B), cannot be a macrophage response to bacterial LPS or TLR ligands (present in both wt and mutants). Thus this *Warburg-like* program is specifically induced by the pathogen through the injection of T4SS bacterial effectors. Moreover, T4SS-dependent alteration of mitochondrial dynamics is, at least partially, responsible for the *Warburg-like* effect observed in the second phase, thus it is linking bacterial-induced alteration of mitochondrial dynamics and altered bioenergetics during infection. Finally, inhibition of glycolysis reduced bacterial replication in human

primary macrophages, while inhibition of OXPHOS had no effect over bacterial replication, highlighting the key importance of the induction of glycolysis and reduction of OXPHOS for the pathogen [11]. However, what may be the benefit of a *Warburg-like* metabolism for *L. pneumophila* replication? We have recently shown that *L. pneumophila* cannot use glucose for its own respiration during growth and that the pathogen uses, instead, diverse amino acids for bacterial respiration, such as serine or alanine, some TCA intermediates, such as pyruvate or α-ketoglutarate, and fatty acids [81]. This suggests that the induction of glycolysis by *L. pneumophila* during infection of human cells is not beneficial for the pathogen due to an increased availability of glucose. Instead, an increased glycolysis in the infected macrophage might be used in the synthesis of glycolytic serine, similarly to the pathway used in proliferating cancer cells [82], therefore providing the main amino acid necessary for growth of *L. pneumophila*. Thus the *Warburg-like* metabolism induced by *L. pneumophila* in the host cell might benefit the pathogen due to the biosynthetic role of glycolysis.

Chlamydia trachomatis, an intracellular bacterium that can cause genital or ocular infections, and that is the leading cause for infectious blinding disease worldwide has undergone genome reduction and lacks several biosynthetic pathways. Thus, to replicate *C. trachomatis* needs to take up nutrients from the infected host cells such as nucleotides, amino acids, and lipids [83]. It has been shown recently that *Chlamydia* infection of primary human umbilical vein endothelial cells (HUVECs) induces a marked downregulation of p53 [8]. As *C. trachomatis* depends on the uptake of glucose from the host cells [84] and p53 regulates cellular metabolism, including the downregulation of glucose transport and glycolysis [85,86], it was analysed whether glycolysis of *C. trachomatis* infected cells was regulated by p53. The results show that, albeit glucose uptake is increased upon infection, glycolysis is not a major pathway regulated by p53, but p53 downregulation in the host cell leads to the activation of the pentose phosphate pathway (PPP) (Fig. 4C), a nucleotide biosynthetic pathway [8]. As the induction of PPP is also part of the Warburg metabolism of cancer cells to provide NADPH for reductive reactions, it can be considered that also *C. trachomatis* induces a *Warburg-like* metabolism during infection. However, the characteristics are different, as *C. trachomatis* induces an increased glucose uptake coupled to an increased PPP that provides nucleotides for intracellular replication of the pathogen.

Concluding remarks and future perspectives

Although research on metabolic reprogramming has gained new interest, the mechanisms and processes underlying metabolic changes of host cells upon bacterial infection remain still poorly understood. The finding that the Warburg effect observed in proliferating cancer cells is also observed in immune cells also renewed the interest for the field of immunometabolism [6], and several studies analysing metabolic changes of host cells, such as macrophages, upon microbial stimulation have been conducted.

However, we might be facing a scenario where metabolic data obtained using i) single or combined microbial compounds such as TLR ligands; ii) whole-bacteria lysates; iii) polarizing cytokines; iv) extracellular bacteria; or v) cancer cell lines as host cells, may represent confusing models poorly relevant to understand metabolic changes of host cells upon bacterial infection. To date, the unique relevant *in vitro* model to study metabolic changes of host cells upon bacterial infection that may correctly be translated to the *in vivo* infection, is probably the study of primary host cells. Unfortunately, there are only few metabolic studies using living, intracellular bacteria in primary host cells.

The data available suggest that metabolic reprogramming of primary host cells upon infection with intracellular bacteria is pathogen-specific and that each pathogen induces a specific metabolic program that fits its respective metabolic needs (Fig. 4). Pathogens such as *M. tuberculosis* or *L. pneumophila* induce a Warburg metabolism in macrophages where increased glycolysis is accompanied by decreased OXPHOS [11,16], whereas *C. trachomatis*, induces a Warburg metabolism with increased glucose uptake that is accompanied by a routing of glycolytic intermediates to PPP to increase the biosynthesis of nucleotides, to enhance bacterial replication [8]. However, the specific metabolic programs elicited by each pathogen are far from being completely uncovered, thus the name "bacterial-induced *Warburg-like* programs" instead of induction of the "Warburg effect" might describe the actual situation the best. For instance, although *M. tuberculosis* infection induces glycolysis and reduces OXPHOS, glycolytic intermediates are specifically routed to lipid body synthesis for the benefit of the pathogen [72,73]. In contrast, *L. pneumophila* infection increases both glycolysis and OXPHOS shortly upon infection but OXPHOS activity is reduced later during infection due to a T4SS-dependent disruption of the mitochondrial network [11]. Thus glycolysis seems to be the preferred host metabolism for intracellular bacteria, possibly because glycolysis can create nutrients for bacterial growth. Moreover, intracellular pathogenic bacteria such as *M. tuberculosis, Chlamydia* or *Legionella* use as major energy sources some host-cell-derived energy-rich carbon substrates such as fatty acids, TCA

518 intermediates (malate) or amino acids (serine), respectively, which are less critical carbon
519 sources than glucose for the host cell. This has been has been called “bipartite metabolism”
520 and allows the pathogen to avoid unnecessarily depleting of glucose from the host cell to keep
521 the anabolic reactions running while reducing metabolic stress [50]. Infection-induced
522 Warburg-like metabolism thus serves the biosynthesis of these metabolites, which are
523 essential for the survival of the pathogen.

524 Taken together, we propose a model where specific bacterial-induced *Warburg-like*
525 programs within host cells support the growth of intracellular bacteria by providing their
526 specific nutritional needs. Additional work analysing other intracellular bacteria such as
527 *Salmonella* of *Brucella* that have different metabolic needs may allow to further support this
528 model.

529 Why to shift to a *Warburg-like* metabolism? In other words, what is the benefit for
530 intracellular bacteria of shifting the infected cell to a *Warburg-like* metabolism? The answer
531 might be given when asking, “*What is the benefit for cancer cells to shift to a Warburg*
532 *metabolism?”* Compelling data suggest that, cancer and bacterial-infection have in common,
533 that enormous biosynthetic requirements are necessary to double a eukaryotic cell (cancer) or
534 to exponentially multiply a bacterial cell (infection). This can only be provided by metabolic
535 programs that are based on glycolysis coupled to biosynthetic pathways, where the additional
536 redirection of TCA intermediates to biosynthetic pathways, shuts down OXPHOS. Thus, in
537 the coming years, it will be important to use more *in vivo* assays to study metabolic changes
538 upon infection. Indeed, *in vivo* bacterial infection of mice and other animal models has been
539 useful to show relevant metabolic features during infection [12,15,69], however, we should
540 keep in mind Robert Koch’s words: “Gentlemen, never forget that mice are not humans” [87].
541 In particular, as many important differences have been reported between murine and human
542 macrophages with respect to the behaviour of these immune cells [88,89].

543 The only method available today to study metabolic reprogramming to *Warburg-like*
544 programs *in vivo*, both in humans and animals is FDG-PET, but it is still rarely affordable for
545 research labs. Thus, the study of metabolic reprogramming during *in vitro* infection will still
546 remain a key approach in the next years. However, we should consider using primary host
547 cells and living, intracellular bacteria, which will be instrumental to acquire relevant
548 knowledge and to open new avenues to understand how pathogenic bacteria subvert host cells
549 to cause infection, and probably also to uncover new targets to fight bacterial infection.

550

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FIGURE LEGENDS

Figure 1. Overview of cellular metabolism. The major catabolic routes that break down molecules to generate energy in the form of ATP are indicated whereby oxidative phosphorylation (OXPHOS) is the most effective in ATP generation (4). Anabolic routes lead to the biosynthesis of biomolecules (underlined, routes 6, 7, 8). Catabolic and anabolic routes cooperate to adjust the optimal flow of metabolites to fulfil the metabolic requirements for cellular functions. Amino acids are synthesized mainly from pyruvate and TCA intermediates (6). TCA cycle: tricarboxylic acid cycle; PPP: pentose phosphate pathway; CI to CV: Complex I to Complex V; F6P: fructose 6 phosphate; G6P: glucose 6 phosphate; α-KG: α-ketoglutarate; CoA: coenzyme A.

Figure 2. Glycolysis and OXPHOS are regulated during proliferation, differentiation and activation of cells. OXPHOS is relatively inactive in proliferating or differentiating cells, such as stem cells, cancer cells, activated T-cells, or LPS-stimulated macrophages, where metabolism relies on aerobic glycolysis (the Warburg effect). OXPHOS is the preferred metabolism of differentiated cells in tissues. LPS: lipopolysaccharide.

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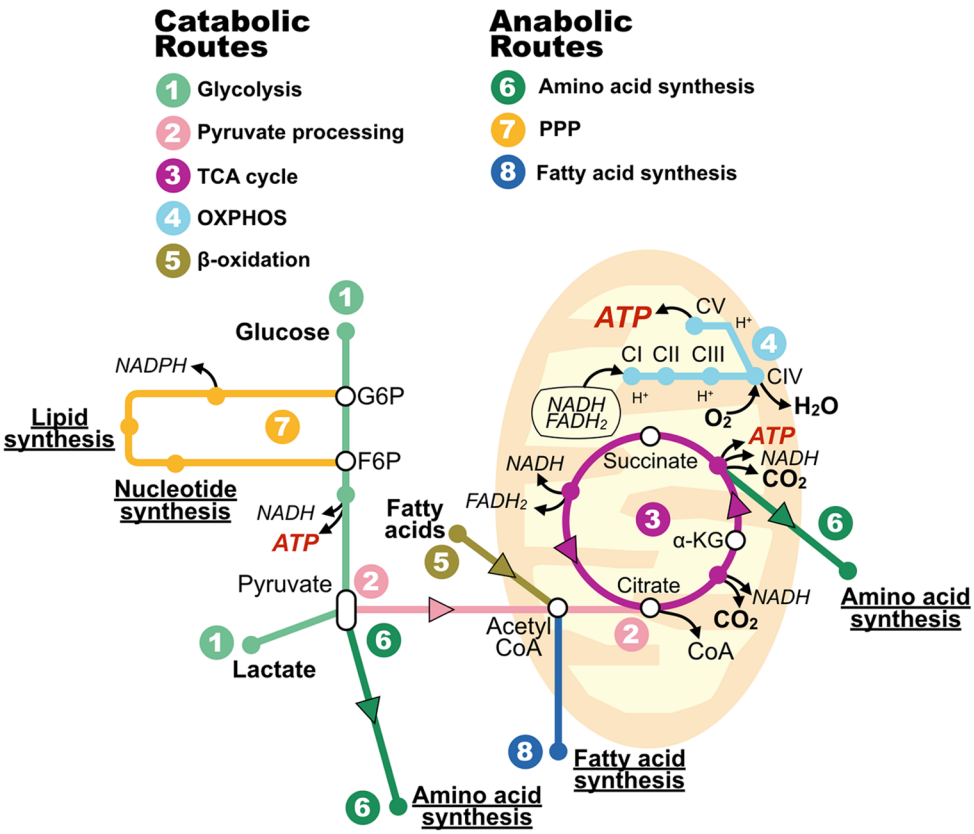
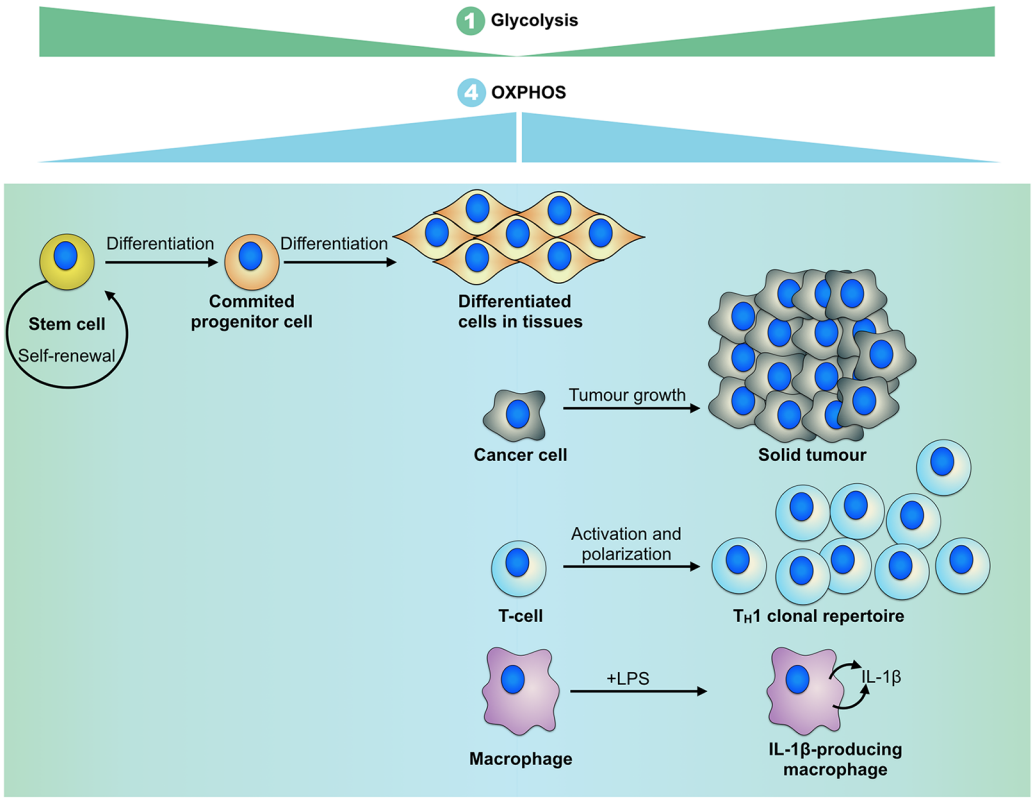
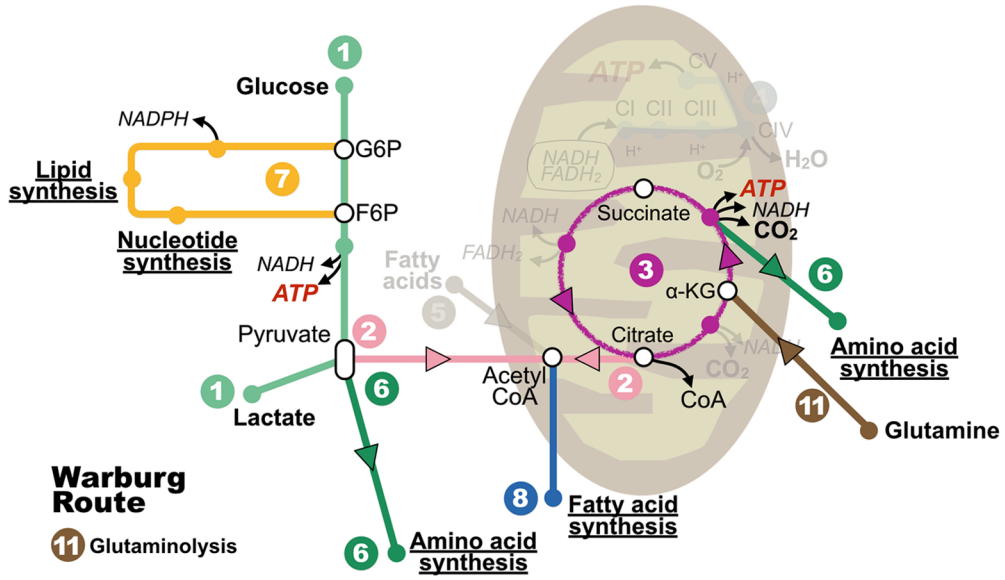
Figure 3. Warburg and *Warburg-like* metabolic programs. (**A**) Routes activated in the Warburg metabolic program performed by cancer cells. (**B**) Routes activated in the *Warburg-like* metabolic program performed by murine bone-marrow derived macrophages (BMDM) upon stimulation with bacterial lipopolysaccharide (LPS). LPS challenge generates mitochondrial reactive oxygen species (mROS) by reverse electron transport (RET) following the oxidation of succinate [54]. Warburg-induced and LPS-induced additional metabolic routes are indicated. The anapletoric route of glutaminolysis refills the TCA cycle with α-KG (A) or succinate (B) derived from the uptake and transformation of the amino acid glutamine. α-KG: α-ketoglutarate.

Figure 4. *Warburg-like* metabolic programs activated upon infection with intracellular bacteria. Known metabolic routes activated upon infection of primary human cells with (**A**) *Mycobacterium tuberculosis*, (**B**) *Legionella pneumophila*, and (**C**) *Chlamydia trachomatis*.

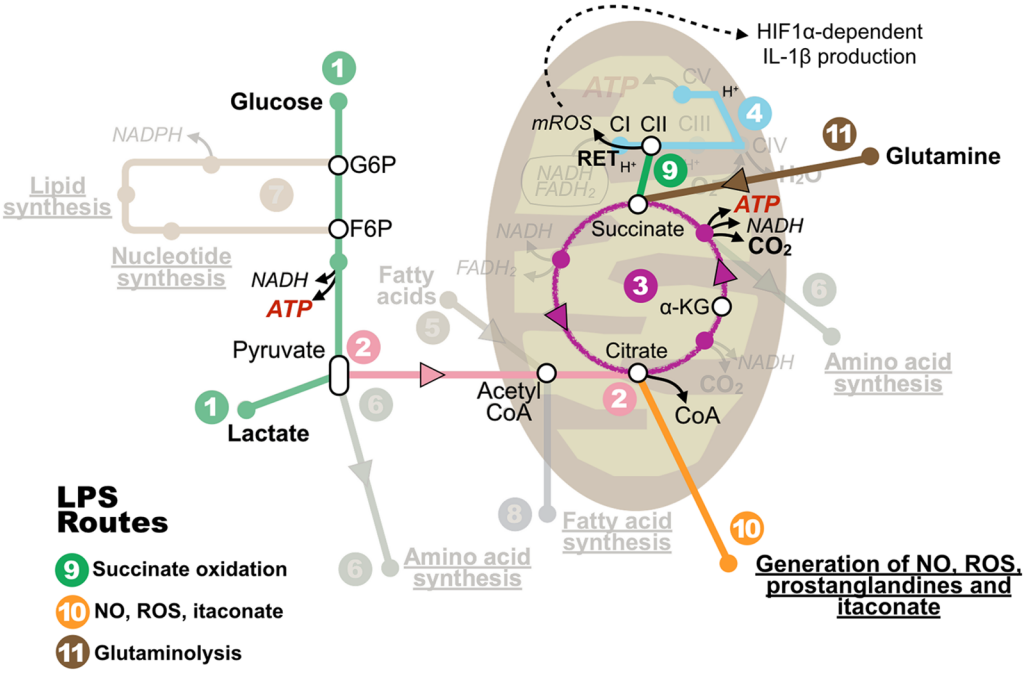

Catabolic Routes
1 Glycolysis
2 Pyruvate processing
3 TCA cycle
4 OXPHOS
5 β-oxidation
Anabolic Routes
6 Amino acid synthesis
7 PPP
8 Fatty acid synthesis
Glucose
NADPH
G6P
Lipid synthesis
7
F6P
Nucleotide synthesis
NADH
ATP
Pyruvate
Lactate
Amino acid synthesis
Fatty acids
Acetyl CoA
Fatty acid synthesis
ATP
CV
H+
CI CII CIII
CIV
NADH FADH2
H2O
O2
Succinate
NADH
FADH2
α-KG
Citrate
CO2
CoA
Amino acid synthesis


1 Glycolysis
4 OXPHOS
Differentiation
Differentiation
Stem cell
Self-renewal
Commited progenitor cell
Differentiated cells in tissues
Tumour growth
Cancer cell
Solid tumour
Activation and polarization
T-cell
TH1 clonal repertoire
+LPS
IL-1β
Macrophage
IL-1β-producing macrophage

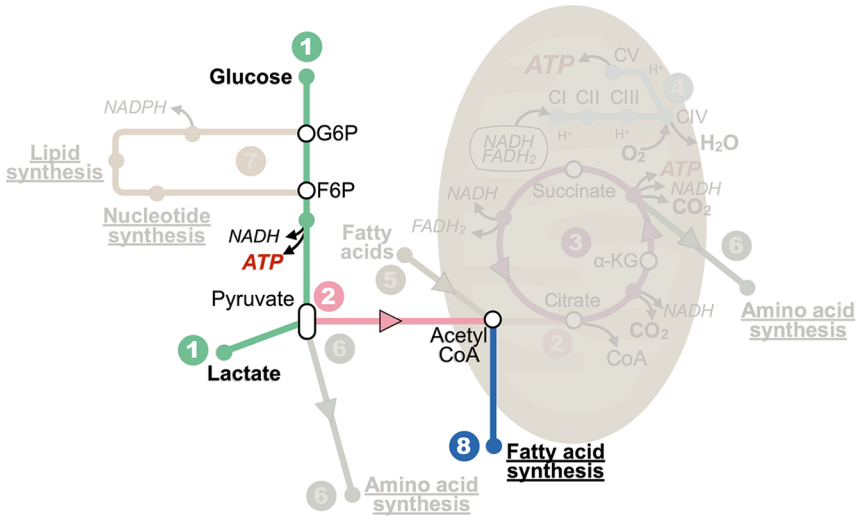
A ***Warburg*** **metabolism of proliferating cancer cells**



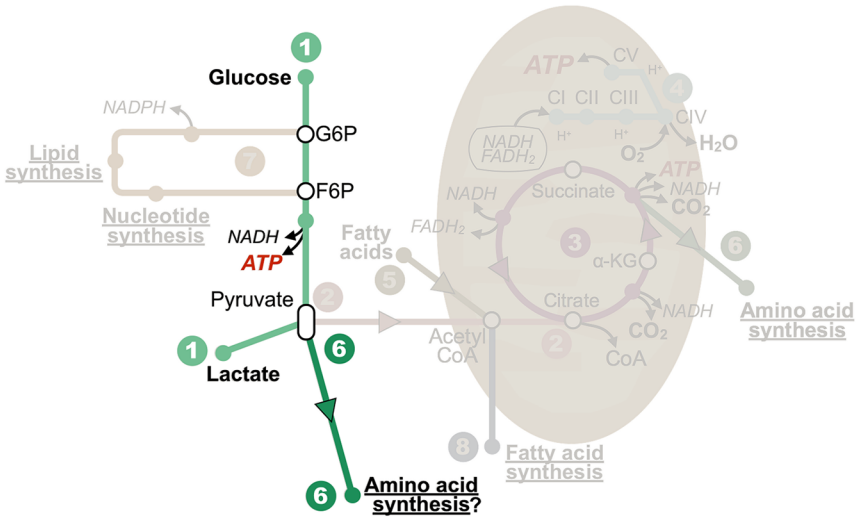
B **LPS-induced** ***Warburg-like*** **metabolism in BMDM**



A ***Mycobacterium tuberculosis*-induced *Warburg-like* metabolism



B ***Legionella pneumophila*-induced *Warburg-like* metabolism



C ***Chlamydia trachomatis*-induced *Warburg-like* metabolism