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1 **Metabolic reprogramming of host cells upon bacterial infection:**
2 **Why shift to a *Warburg-like* metabolism?**

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13 **Key words**

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16 **Running title**

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29 **ABSTRACT**

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

39

40 **Abbreviations**

41 ATP: adenosin triphosphate; TCA: tricarboxilic acid; CO₂: carbon dioxide; CI - CV: Complex
42 I to Complex V; OXPHOS: oxidative phosphorylation; FDG-PET: ¹⁸F-DeoxiGlucose
43 Positron Emission Tomography; PI3K: Phosphoinositide 3-kinase; AKT: Ak transforming;
44 HIF1: Hypoxia-inducible factor 1; p53: 53-kilodalton protein; MYC: similar to
45 myelocytomatosis viral oncogene; AMPK: AMP-activated protein kinase; mTORC1:
46 mammalian target of rapamycin complex 1; CoA: Coenzyme A; NADH: reduced form of
47 nicotinamide adenine dinucleotide; NADPH: reduced form of nicotinamide adenine
48 dinucleotide phosphate; PPP: pentose phosphate pathway; DC: dendritic cell; PRR: pathogen
49 recognition receptor; TLR: Toll-like receptor; LPS: lipopolysaccharide; APC: antigen-
50 presenting cell; MHC: major histocompatibility complex; CD: cluster of differentiation; IL:
51 interleukin; TCR: T-cell receptor; IFN: interferon; T_H: T-helper; iNOS: inducible nitric oxide
52 synthase; NO: nitric oxide; 2DG: 2-DeoxiGlucose; BMDM: bone-marrow-derived
53 macrophages; TNF: tumor necrosis factor; ETC: electron transport chain; PKM2: pyruvate
54 kinase M2; ROS: reactive oxygen species; Pam3Cys: tri-palmitoyl cysteine; M1:
55 macrophages activated by T_H1 cytokines; M2: macrophages activated by T_H2 cytokines; M0:
56 non-activated macrophages; FAO: fatty-acid oxidation; UDP-GlcNAc: uridine diphosphate
57 N-acetylglucosamine; GM-CSF: granulocyte-macrophage colony-stimulating factor; M-CSF:
58 macrophage colony-stimulating factor; T4SS: type IV secretion system; LncP: Legionella
59 nucleotide carrier protein; MitF: mitochondrial fragmentation factor; HUVEC: human
60 umbilical vein endothelial cell.

61

62

63 **Introduction**

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

74 Most non-proliferating, differentiated mammalian cells (such as somatic cells in tissues)
75 have a quiescent metabolic state characterized by a low catabolic and anabolic activity and an
76 efficient generation of ATP by the catabolic process known as mitochondrial respiration.
77 Such cells convert glucose to pyruvate via anaerobic glycolysis most of which is then routed
78 to mitochondria, where pyruvate is completely oxidized to CO₂ via the tricarboxylic acid
79 (TCA) cycle (Fig. 1). Oxygen is the final acceptor of an electron transport chain (ETC)
80 composed of five assembled complexes (CI to CV) that generate an electrochemical gradient
81 in the mitochondria that facilitates ATP production by Complex V (the F₁-F₀-ATPase), a
82 process termed oxidative phosphorylation (OXPHOS) (Fig. 1). Under these conditions, a non-
83 proliferating, differentiated mammalian cell have a total energy gain of 36 ATP molecules per
84 molecule of absorbed glucose [6,18]. OXPHOS is therefore the most important metabolic
85 pathway used by differentiated, non-proliferating cells.

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

96 Several reports have claimed that mammalian cells infected by intracellular bacteria
97 such as *Mycobacterium tuberculosis* [12-16], *Legionella pneumophila* [11], *Brucella abortus*
98 [7], *Chlamydia trachomatis* [8,9] or *Chlamydia pneumoniae* [10] have an altered metabolism
99 consisting in an increment of glucose uptake and/or glycolysis, which resembles the
100 metabolic program widely seen in proliferating cancer cells known as the “Warburg effect”
101 (or aerobic glycolysis). Here, we review current knowledge on metabolic reprogramming and
102 discuss putative causes underlying the preferential remodelling of host cells to *Warburg-like*
103 metabolic programs during infection by intracellular bacteria.

104 **The Warburg effect is a hallmark of cancer cells**

105 Cancer cells are the most prominent example of glycolytic-based proliferation. Tumour cells
106 convert the majority of their absorbed glucose to lactate, even under oxygen-rich conditions,
107 and this dependency on a high glycolytic rate and high glucose uptake was termed the
108 “Warburg effect” in honour of Otto Warburg, who discovered this altered metabolism that
109 tumour cells have compared to differentiated cells in tissue [20]. Elevated glucose uptake and
110 lactate secretion are, since then, considered as two general, metabolic hallmarks of solid
111 tumours as they have been observed in a wide variety of cancers. These seminal findings by
112 Otto Warburg have also led to improved diagnosis and planning of an appropriate cancer
113 therapy by using ¹⁸F-DeoxyGlucose Positron Emission Tomography (FDG-PET), an imaging
114 method that allows to measure the increased uptake of the marked glucose analogue FDG
115 prior to PET scanning of tumours [19]. Otto Warburg also suggested that this metabolic
116 alteration was an essential cause of cancer and that mitochondrial defects inhibiting the ability
117 of cancer cells to fully oxidize glucose to CO₂ were the cause of this metabolic switch to a
118 glycolytic metabolism in cancer cells [20-22].

119 Nowadays is widely accepted that a switch to a glycolytic metabolism (the Warburg
120 effect) is a core hallmark of cancer cells. However, contrary to what was suggested by
121 Warburg, we know today that mitochondria of tumours are not defective and therefore are not
122 the cause of the observed metabolic reprogramming. Compelling evidences indicate that the
123 metabolic reprogramming of cancer cells to aerobic glycolysis is a direct response to the
124 activation of growth factor signalling, even in the absence of extracellular growth-factors [23-
125 25]. The signalling hubs regulating the metabolic responses to growth factor signalling are
126 mainly i) the PI3K/AKT axis; ii) the transcription factors HIF1, p53 and MYC; iii) the
127 metabolic sensors AMPK and mTORC1; iv) the Ras proteins and v) the alternatively spliced

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

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

160

161 **Immunometabolism focuses on the metabolic reprogramming of immune cells**

162 Metabolic changes alter functions of immune cells, a field of investigation called
163 immunometabolism. Some interesting historical reports in this discipline [28-31] already
164 anticipated the renewed interest on immunometabolism. New tools to study cellular
165 metabolism, such as extracellular flux assays [32] or mass spectrometry-based metabolomics
166 [33], have recently boosted the field by allowing researchers to study metabolic dynamics of
167 immune cells upon activation.

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

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

206 The current model of immunometabolism proposes that innate or adaptive naïve (non-
207 activated) immune cells mainly rely in an OXPHOS metabolism that is shifted to Warburg
208 metabolism upon immune activation (Fig. 2), a metabolic switch that is essential for the
209 immune functions of these activated cells [6]. For instance, specific subsets of effector T-cells
210 switch to Warburg metabolism upon activation by APCs [35], including T_H1 and T_H17 cells
211 [36,37]. A switch to Warburg metabolism has also been observed upon immune activation of
212 other immune cells, such as macrophages [38,39], DCs [39,40], neutrophils [41], B-cells [42]
213 and natural killer cells [43]. Key examples of metabolic reprogramming from OXPHOS to
214 Warburg upon immune activation are i) macrophages activated by PRRs [38,39], ii) T-cells
215 activated by cytokine receptors [44] or iii) B-cells activated by antigen receptors [42]. This
216 metabolic remodelling seems essential for the exertion of immune functions by these
217 activated cells. Examples of immune functions directly depended on the switch to a Warburg
218 metabolism are i) phagocytosis and IL-1 β production by macrophages [39]; ii) acquisition of
219 co-stimulatory capacity by DCs [39]; iii) T_H17 polarization by activated T-cells [45] or iv)
220 formation of nets by neutrophils [41].

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

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

235 Macrophages are the best-studied examples of immune cells performing metabolic
236 reprogramming. As a first-line guard, macrophages have a key role in host defence during
237 bacterial infection. Macrophages phagocytose bacteria to clear infection and secrete
238 antimicrobial compounds to directly kill pathogenic bacteria. However, several intracellular
239 pathogens such as *M. tuberculosis* or *L. pneumophila* infect preferentially macrophages and
240 are able to replicate within them, albeit macrophages are one of the main immune cells to kill
241 pathogenic bacteria. Several lines of evidence suggest that intracellular bacteria trigger
242 specific metabolic programs during infection of macrophages (discussed below) and that this
243 metabolic remodelling might be key for bacterial replication within these immune cells
244 [49,50]. Interestingly, certain pathogenic bacteria induce metabolic programs that favour their
245 replication, while others induce metabolic programs that are restrictive for bacterial
246 replication [49,50]. Thus, the study of the macrophage metabolism during bacterial infection
247 should shed light on how pathogenic bacteria subvert macrophage functions and cause disease.

248 **The metabolism of macrophages and monocytes is reprogrammed upon stimulation** 249 **with microbial compounds**

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

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

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

278 However, by using human monocytes isolated from blood, a recent report showed that a
279 shift from OXPHOS to glycolysis was observed only in monocytes stimulated with LPS, and
280 not in monocytes stimulated with the TLR-2 ligand Pam3Cys or other whole-pathogen lysates.
281 In monocytes stimulated with Pam3Cys, increased glycolysis was accompanied by increased
282 OXPHOS that was needed for retention of their phagocytic capacity and cytokine production
283 [53]. Interestingly, elevated succinate, itaconate and citrate levels were only observed in LPS-
284 stimulated monocytes and redirection of these TCA intermediates was not observed in
285 Pam3Cys-stimulated monocytes. Moreover, the same study showed that stimulation of
286 human monocytes with whole-pathogen lysates of *Escherichia coli*, *Staphylococcus aureus* or
287 *M. tuberculosis* increased glycolysis, while OXPHOS only increased after exposure to *E. coli*
288 or *S. aureus*. Exposure of monocytes to *M. tuberculosis* whole-bacteria lysates did not affect
289 OXPHOS, which remained at the same level as unstimulated monocytes, in contrast to
290 OXPHOS reduction upon LPS exposure [53]. These findings challenge the notion that a shift

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

304 **The metabolic reprogramming of macrophages is more complicated than the M1/M2** 305 **model**

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

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

328 However, the binary model of M1/M2 macrophages has some limitations [62-64]. First,
329 M1/M2 is mostly a model for *in vitro* differentiation of macrophages. Most of the markers
330 observed for murine macrophages do not translate to human macrophages, probably because
331 murine macrophages are generally differentiated from bone marrow cells while human
332 macrophages are obtained by *in vitro* differentiation of blood monocytes [57]. An example of
333 the heterogeneity in the methods used to polarize human macrophages is the utilization of
334 GM-CSF or M-CSF to differentiate blood monocytes to M1 or M2 macrophages, respectively
335 [57,65]. As GM-CSF and M-CSF are growth factors instead of cytokines or microbial
336 products, it is very difficult to know whether the obtained cells are indeed the human
337 counterparts of murine M1/M2 macrophages. Thus, intuitively murine LPS+IFN- γ -activated
338 M1 macrophages and human GM-CSF-differentiated M1 macrophages might be quite
339 different cell types. An additional difficulty is that polarization seems to be reversible both *in*
340 *vivo* and *in vitro* [64]. Thus, these limitations in the M1/M2 model reveal the necessity to
341 improve the model of macrophage polarization. Such improvement should then be applied to
342 the study of the specific metabolic signatures of polarized macrophages.

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

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

365 **Metabolic reprogramming of host cells upon infection with intracellular bacteria seems** 366 **pathogen-specific**

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

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

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

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

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

423 Another bacterium that has been shown to induce a *Warburg-like* phenotype in infected
424 cells is *L. pneumophila* [11]. This intracellular bacterium is the etiologic agent of
425 Legionnaires' disease, a serious pulmonary infection during which the pathogen replicates
426 within human lung macrophages. By injecting more than 300 bacterial effectors in the host
427 cell through a type IV secretion system (T4SS), *L. pneumophila* subverts cellular functions in
428 order to replicate within eukaryotic cells [74,75]. Some of these *L. pneumophila* effectors
429 target mitochondria or mitochondrial functions, such as the bacterial effector LncP that is
430 targeted to the mitochondrial inner membrane and might function as an ATP transporter, or
431 the bacterial effector MitF, which promotes the fragmentation of the mitochondrial network
432 during infection of human primary macrophages [11,76,77]. We recently showed that
433 *L. pneumophila* induces a biphasic alteration of the macrophage metabolism at very early
434 times post-infection [11]. Upon infection, a first phase of increased glycolysis and OXPHOS
435 that peaks at 1h post-infection is followed by a second phase where glycolysis remains high
436 while OXPHOS is severely reduced. This second phase is extended, at least, until 5h post-
437 infection, which is prior to bacterial replication and to the activation of macrophage cell
438 death pathways. By using mutants deficient in the T4SS that cannot inject bacterial effectors
439 and therefore cannot replicate within infected cells, we determined that this first phase is
440 T4SS-independent, while the second phase is T4SS-dependent [11]. As *L. pneumophila*
441 infection mainly activates TLR-2 [78-80] and activation of TLR-2 leads to increased
442 glycolysis and OXPHOS in human primary cells [53], it is possible that the induction of the
443 first phase, i.e. increased glycolysis and increased OXPHOS, is T4SS-independent but TLR-
444 2-dependent . On the other hand, as T4SS-deficient mutants are phagocytosed in the same
445 way as *L. pneumophila* wild type (wt), and both wt and mutants express LPS and other TLR
446 ligands, the induction of the T4SS-dependent *Warburg-like* program observed in the second
447 phase, i.e. increased glycolysis and reduced OXPHOS (Fig. 4B), cannot be a macrophage
448 response to bacterial LPS or TLR ligands (present in both wt and mutants). Thus this
449 *Warburg-like* program is specifically induced by the pathogen through the injection of T4SS
450 bacterial effectors. Moreover, T4SS-dependent alteration of mitochondrial dynamics is, at
451 least partially, responsible for the *Warburg-like* effect observed in the second phase, thus it is
452 linking bacterial-induced alteration of mitochondrial dynamics and altered bioenergetics
453 during infection. Finally, inhibition of glycolysis reduced bacterial replication in human

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

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

484 **Concluding remarks and future perspectives**

485 Although research on metabolic reprogramming has gained new interest, the mechanisms and
486 processes underlying metabolic changes of host cells upon bacterial infection remain still
487 poorly understood. The finding that the Warburg effect observed in proliferating cancer cells
488 is also observed in immune cells also renewed the interest for the field of immunometabolism
489 [6], and several studies analysing metabolic changes of host cells, such as macrophages, upon
490 microbial stimulation have been conducted.

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

499 The data available suggest that metabolic reprogramming of primary host cells upon
500 infection with intracellular bacteria is pathogen-specific and that each pathogen induces a
501 specific metabolic program that fits its respective metabolic needs (Fig. 4). Pathogens such as
502 *M. tuberculosis* or *L. pneumophila* induce a Warburg metabolism in macrophages where
503 increased glycolysis is accompanied by decreased OXPHOS [11,16], whereas *C. trachomatis*,
504 induces a Warburg metabolism with increased glucose uptake that is accompanied by a
505 routing of glycolytic intermediates to PPP to increase the biosynthesis of nucleotides, to
506 enhance bacterial replication [8]. However, the specific metabolic programs elicited by each
507 pathogen are far from being completely uncovered, thus the name “bacterial-induced
508 *Warburg-like* programs” instead of induction of the “Warburg effect” might describe the
509 actual situation the best. For instance, although *M. tuberculosis* infection induces glycolysis
510 and reduces OXPHOS, glycolytic intermediates are specifically routed to lipid body synthesis
511 for the benefit of the pathogen [72,73]. In contrast, *L. pneumophila* infection increases both
512 glycolysis and OXPHOS shortly upon infection but OXPHOS activity is reduced later during
513 infection due to a T4SS-dependent disruption of the mitochondrial network [11]. Thus
514 glycolysis seems to be the preferred host metabolism for intracellular bacteria, possibly
515 because glycolysis can create nutrients for bacterial growth. Moreover, intracellular
516 pathogenic bacteria such as *M. tuberculosis*, *Chlamydia* or *Legionella* use as major energy
517 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* or *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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- 793

794 **FIGURE LEGENDS**

795

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

805

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

811 .

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

821

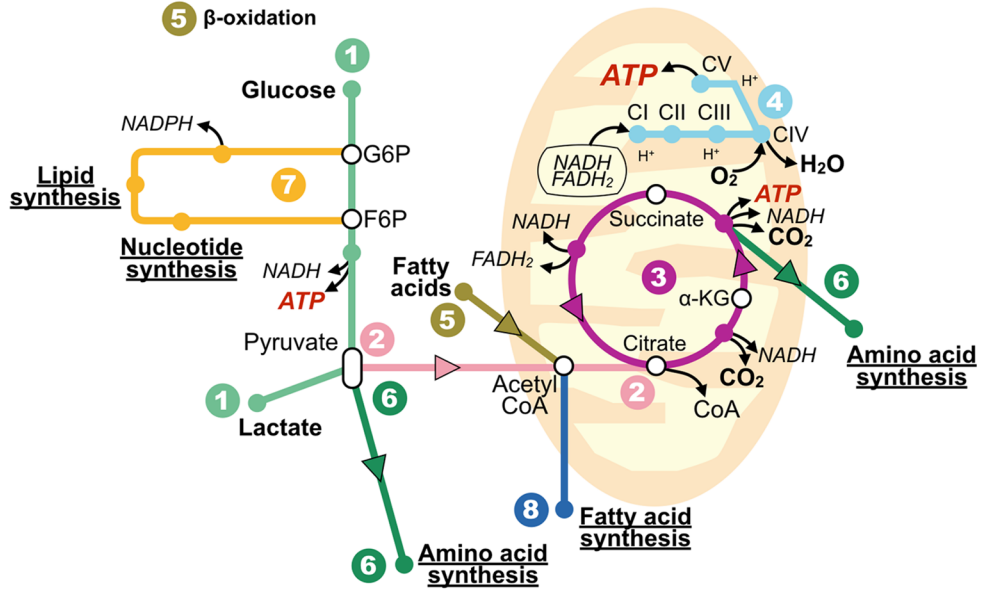
822 **Figure 4. Warburg-like metabolic programs activated upon infection with intracellular**
823 **bacteria.** Known metabolic routes activated upon infection of primary human cells with (A)
824 *Mycobacterium tuberculosis*, (B) *Legionella pneumophila*, and (C) *Chlamydia trachomatis*.

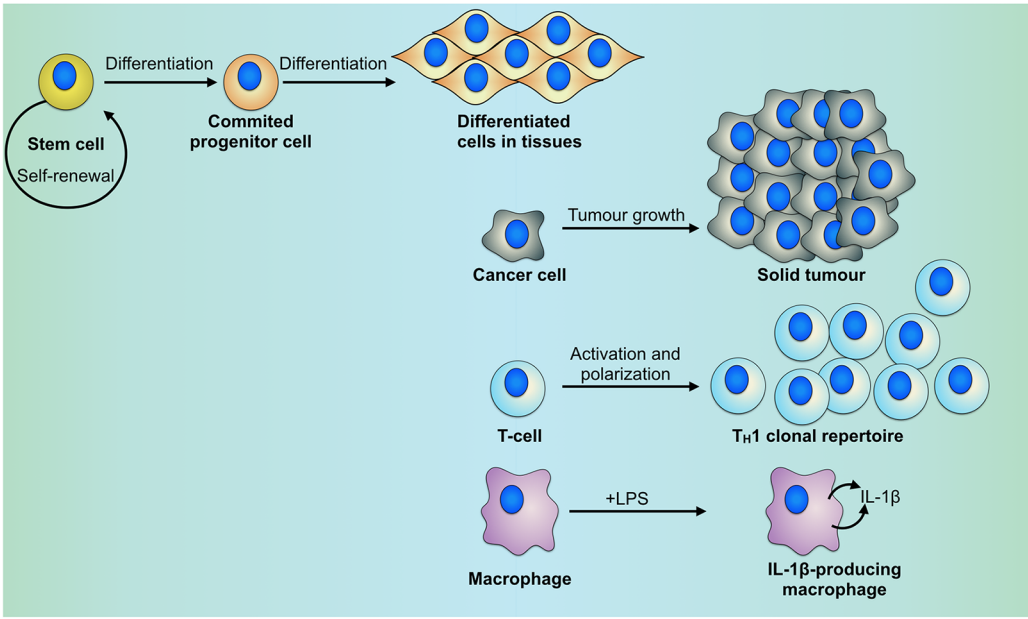
Catabolic Routes

- 1 Glycolysis
- 2 Pyruvate processing
- 3 TCA cycle
- 4 OXPHOS
- 5 β -oxidation

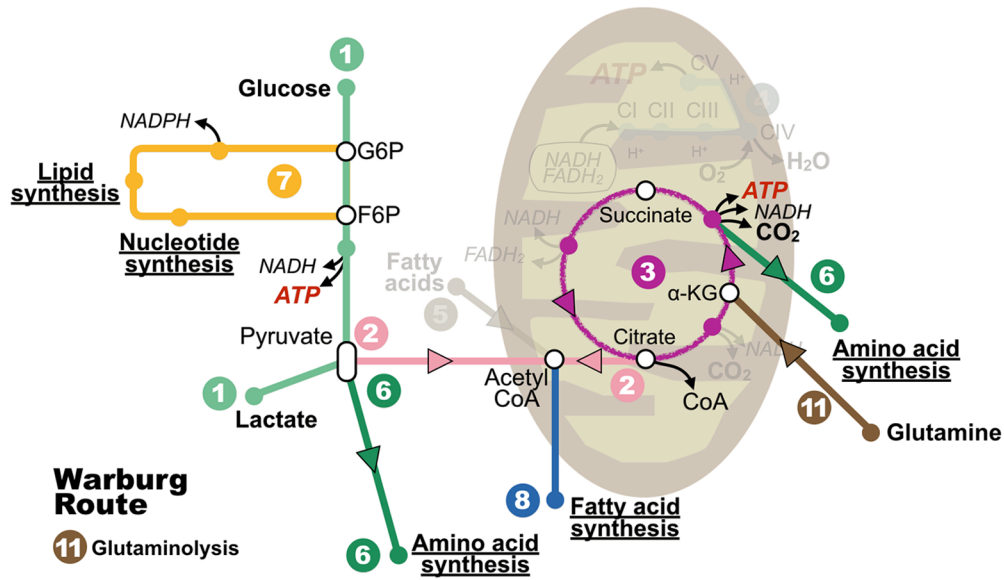
Anabolic Routes

- 6 Amino acid synthesis
- 7 PPP
- 8 Fatty acid synthesis

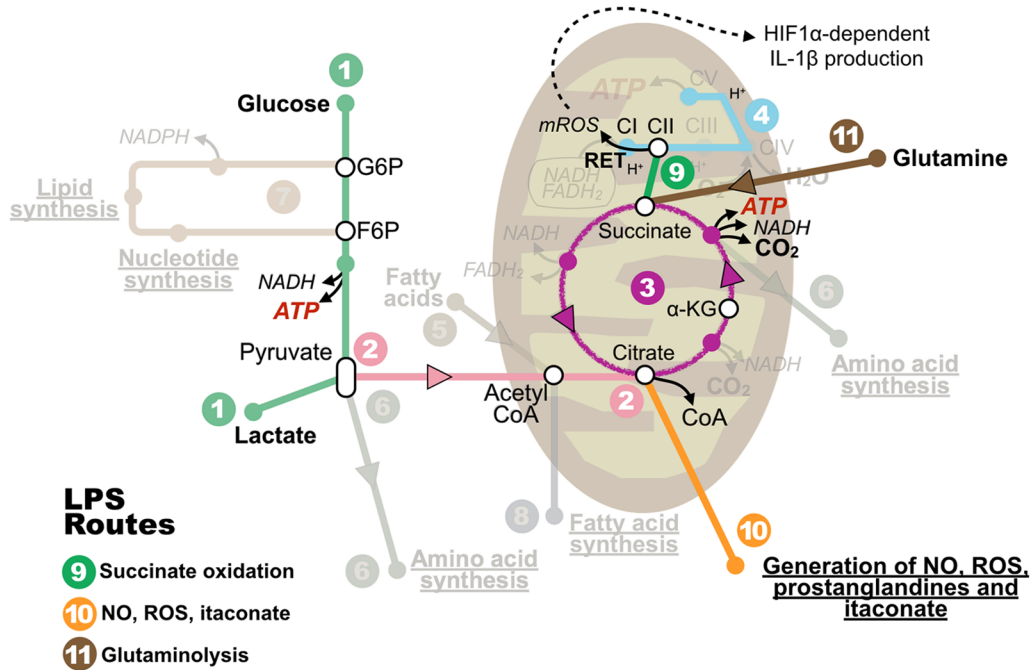




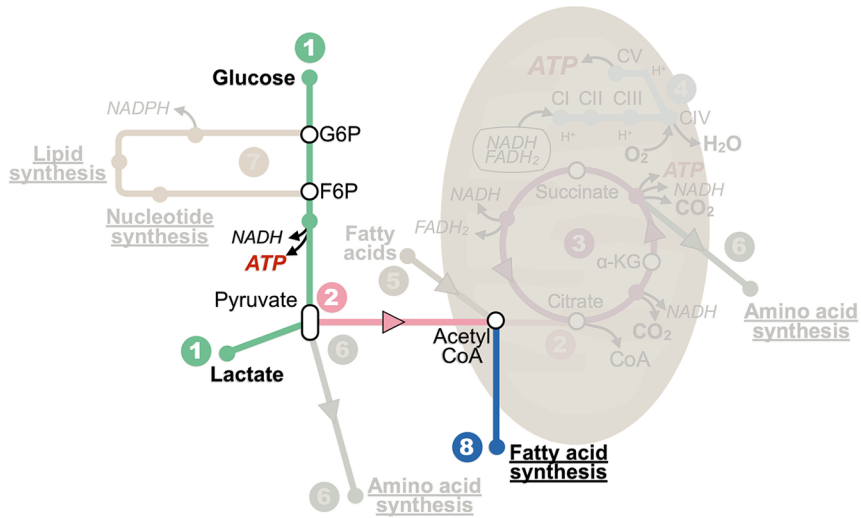
A Warburg metabolism of proliferating cancer cells



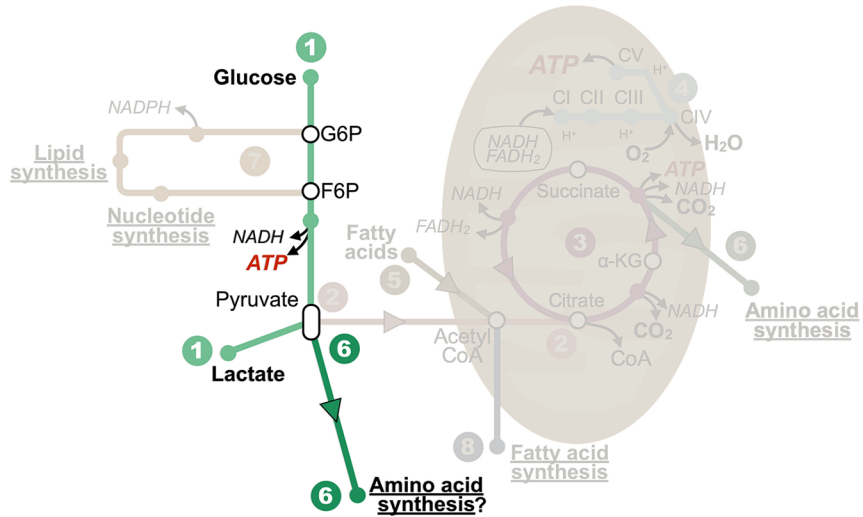
B LPS-induced Warburg-like metabolism in BMDM



A *Mycobacterium tuberculosis*-induced Warburg-like metabolism



B *Legionella pneumophila*-induced Warburg-like metabolism



C *Chlamydia trachomatis*-induced Warburg-like metabolism

