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1 **Danger-associated metabolic modifications during bacterial**
2 **infection of macrophages**

3

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23 **Abstract**

24 In this review, we propose that certain modifications in cellular metabolism might function as
25 danger signals triggering inflammasome-mediated immune responses. We propose to call
26 them danger-associated metabolic modifications (DAMMs). As intracellular bacteria can
27 actively modulate macrophage metabolism for their benefit, infected host cells might sense
28 bacteria-induced metabolic alterations and activate immune reactions. Here we report the
29 known metabolic interactions that occur during infection of macrophages by intracellular
30 bacteria and discuss the possible emergence of DAMMs upon bacteria-induced alterations of
31 cellular metabolism.

32

33 **Introduction**

34 Macrophages are key immune cells that are at the frontline of defence against bacterial
35 infections. Reprogramming of macrophage cellular metabolism has been shown to be a
36 determinant for their activation [1,2]. The discovery that metabolic alterations in macrophages
37 precede the expression of their antimicrobial functions, such as the production of cytokines
38 and antibacterial compounds, nourished an emerging field of investigation named
39 immunometabolism — the study of the interplay between immunological and metabolic
40 processes [3]. Recently substantial advances have been made in the understanding of how
41 immune activators such as pathogen-associated molecular patterns (PAMPs) reprogram the
42 cellular metabolism of macrophages and how these metabolic changes benefit host cells as
43 they are needed to initiate antimicrobial responses [4-6].

44 Many intracellular bacterial pathogens specifically infect human macrophages (**Table 1**),
45 thereby attacking one of the most important phagocytic cell types that normally defends the
46 infected organism against bacterial infection [7]. Indeed, several intracellular bacteria invade
47 human macrophages and replicate either within a vacuole in their cytoplasm or within the
48 cytoplasm. Bacteria that establish a protected, replication-permissive vacuole within
49 macrophages include *Mycobacterium tuberculosis*, *Salmonella enterica*, *Legionella*
50 *pneumophila* and *Chlamydia pneumoniae*, causing the human diseases tuberculosis,
51 salmonellosis, Legionnaires' disease and pneumonia, respectively (**Table 1**). Bacteria that are
52 able to escape in the cytosol and primarily replicate therein include *Listeria monocytogenes*,
53 *Shigella flexneri* or *Francisella tularensis* causing listeriosis, shigellosis or tularaemia,
54 respectively (**Table 1**).

55 To replicate within human macrophages, intracellular bacteria subvert the cellular defences
56 of these immune cells. This is mainly achieved by the use of specialized molecular machines
57 called secretion systems that allow the injection of bacterial effectors into the cytoplasm of
58 infected macrophages to hijack host proteins, pathways and processes. Examples are bacterial
59 effectors that modify the phagolysosomal pathway, that allow the bacteria to evade immune
60 detection, that modulate autophagy or that manipulate cell death pathways [8-10].

61 As intracellular bacteria obtain the nutrients to grow only from the host cell they infect, the
62 determining factor for intracellular bacterial replication seems to be their capacity to sequester
63 metabolic pathways and metabolites from the host cell. Indeed, recent studies on how
64 bacteria-induced metabolic reprogramming of macrophages benefits their replication are

65 revealing a growing number of virulence strategies employed by intracellular bacteria to
66 cause infection [11-16].

67 Metabolic alterations that benefit host cells and bacteria during infection may occur in the
68 macrophage in parallel [17]. Thus the same phenomenon can be studied either by analysing
69 how metabolic pathways in macrophages are altered upon their exposure to bacterial
70 components, or by studying how bacteria induce changes in the metabolism of macrophages
71 to snatch nutrients, survive and grow inside host cells. As metabolic alterations that benefit
72 host cells and bacteria seem to happen simultaneously, both parts of the puzzle should be
73 analysed in parallel to gain a better understanding of how immunometabolism operates in
74 macrophages during bacterial infection. In this review, we will present both perspectives and
75 propose an integrated multi-step model to better understand immunometabolic host–pathogen
76 interactions during bacterial infection of human macrophages.

77 **Metabolic alterations that benefit host cells: macrophages produce** 78 **cytokines and antimicrobials**

79 Although metabolic alterations of human macrophages upon phagocytosis were already
80 described in the 1960s [18], there was evidence only in recent years that specific metabolic
81 alterations in macrophages are essential to exert their inflammatory and anti-bacterial
82 functions, such as cytokine and antimicrobial compound production [1,2].

83 Upon macrophage activation, a combination of metabolic changes benefits host cells
84 during bacterial infection. Several reports demonstrated that upregulation of glycolysis and
85 modifications in the mitochondrial metabolism occur upon activation of macrophages with
86 bacterial PAMPs such as lipopolysaccharide (LPS). Upon exposure to LPS, macrophages
87 show an altered tricarboxylic acid (TCA) cycle where some intermediates, such as succinate
88 or citrate, accumulate [4]. LPS challenge increases the levels of the glycolytic enzyme
89 pyruvate kinase M2 (PKM2) in dimers and tetramers. Although PKM2 dimers are
90 enzymatically inactive, they induce succinate accumulation and also migrate to the nucleus,
91 where they induce PKM2-mediated transcription of interleukin (IL)-1 β (**Fig. 1**) [19]. On the
92 other hand, PKM2 tetramers are enzymatically active and catalyze the rate-limiting step of
93 glycolysis; therefore LPS-induced upregulation increases the rate of glycolytic fluxes.

94 One of the effects of accumulated succinate is the stabilization of hypoxia-inducible factor
95 1- α (HIF1- α), which then is translocated to the nucleus and, together with PKM2 dimers,

96 drives IL-1 β gene expression. High levels of succinate in mitochondria lead to the reverse
97 flow of the electron transport chain (ETC), thus producing mitochondrial reactive oxygen
98 species (mROS) that also induce HIF1 α -mediated IL-1 β mRNA expression [20].

99 Therefore, several alterations in the TCA cycle upon LPS challenge finally lead to IL-1 β
100 production, which is essential for the anti-microbial functions of macrophages. In addition,
101 LPS-induced expression of the mitochondrial citrate carrier diverts citrate from the
102 mitochondrial TCA cycle to the cytoplasm. Cytoplasmic citrate is then used by the
103 macrophage to generate nitric oxide (NO), ROS and prostaglandins, as well as to produce the
104 antimicrobial metabolite itaconate [21-23].

105 The interaction of the aforementioned metabolic responses with other signalling pathways
106 should be also considered. For instance, as discussed above, succinate accumulation upon
107 exposure of TLR4 to LPS results in the expression of IL-1 β mRNA, and this pathway seems
108 synergistic with “classical” NF- κ B-dependent expression of IL-1 β mRNA upon LPS
109 challenge [20]. In this regard, it was shown that succinate alone did not activate the
110 transcription of the IL-1 β gene but a synergistic effect on IL-1 β expression could only be
111 observed in combination with LPS [20]. Therefore, metabolic and “classical” pathways may
112 work in parallel or at different time scales, thus adding a level of complexity that remains
113 largely unexplored for now and whose investigation might be important to fully understand
114 how immunological responses are regulated.

115 Specific metabolic programs are also installed in the macrophage during their polarization
116 [24]. The metabolism of M1-polarized macrophages, which secrete pro-inflammatory
117 cytokines and have anti-microbial functions, relies on glycolysis. In this case, glycolytic-
118 generated pyruvate is primarily fermented to lactate, instead of being routed to mitochondria
119 to feed OXPHOS. The metabolism of M2-polarized macrophages, which secrete anti-
120 inflammatory cytokines and have wound-healing functions, relies on fatty-acid oxidation and
121 oxidative phosphorylation (OXPHOS) [1,3,24]. This shows that macrophage plasticity
122 exceeds macrophage functions and also commits their metabolic capacities. Metabolic
123 reprogramming is thus at the core of macrophage responses upon encountering bacterial
124 compounds and is an essential response determining the display of their anti-microbial
125 functions [2].

126 However, metabolic changes in macrophages upon bacterial infection might be more
127 complex than those elicited upon exposure to a single PAMP such as LPS. It has been shown

128 that different PAMPs lead to different metabolic responses in human primary monocytes [25].
129 Therefore, the detection of different bacteria-derived PAMPs might induce mixed metabolic
130 responses. Moreover, bacteria are active players during infection and they can actively
131 manipulate host bioenergetics by using target-specific strategies (discussed below). The active
132 role of infectious bacteria on the metabolic changes induced in human cells was illustrated
133 when exposure to heat-killed or living bacteria was compared as it revealed that they showed
134 different metabolic phenotypes [25].

135 We should be cautious when discussing metabolic reprogramming in human macrophages,
136 as most of the results on metabolic alterations upon LPS challenge have been obtained from
137 murine macrophages but studies of metabolic reprogramming of human macrophages upon
138 activation or infection remain scarce. Indeed, a recent report suggested that these metabolic
139 alterations might be different in murine and human macrophages [26].

140 **Metabolic alterations that benefit bacterial pathogens: sequestering** 141 **nutrients**

142 Several reports suggest that metabolic reprogramming of macrophages during infection can
143 also benefit intracellular pathogens. Intracellular bacteria, which are metabolically diverse and
144 adaptable, can exploit the variety of metabolic environments they find within macrophages.
145 Indeed, the exploitation of host metabolites has been suggested as one of the explanations of
146 the so-called “macrophage paradox” — that is, why many pathogenic bacteria replicate in
147 macrophages [27] although macrophages are well-equipped to destroy bacteria. Thus it seems
148 paradoxical and counterintuitive that many intracellular bacteria causing human disease
149 (**Table 1**) can survive and replicate within these immune cells [27]. Indeed, it has been shown
150 that the intracellular environment of macrophages can be metabolically compatible with the
151 nutrient needs of certain intracellular bacteria, such as *S. enterica* [28], *Brucella abortus* [29],
152 *M. tuberculosis* [11] or *L. pneumophila* [12].

153 Studies have found that *Salmonella* and *Brucella* prefer to infect and persist within M2
154 macrophages [28-30]. Although the reduced anti-microbial capacities of M2 macrophages
155 may be the reason, no direct evidence supporting this hypothesis has been reported yet. In
156 contrast, it seems that *Salmonella* and *Brucella* exploit the metabolic specificities of M2
157 macrophages to sustain chronic infections; these specificities include increased levels of
158 glucose, as found in experimentally infected mice. As M1 macrophages rely on glycolysis to
159 obtain energy, they consume high amounts of glucose. However, as M2 macrophages degrade

160 fatty acids to obtain energy, higher quantities of glucose are readily available within M2
161 macrophages, which can be easily used by intracellular bacteria [30,31].

162 The active role of the bacterium on these metabolic interactions with the host cell is
163 illustrated by the fact that *Salmonella* disrupts glycolytic fluxes in infected macrophages
164 when the bacterium reroutes glucose from the host cell to its vacuole to use macrophage
165 glucose as its carbon source [13,32]. Moreover, it has been suggested that, instead of
166 preferentially infecting M2-polarized macrophages, bacteria can directly induce the M2
167 phenotype in infected macrophages. Indeed, *Salmonella* and *Brucella* modulate the levels of
168 the nuclear peroxisome proliferator-activated receptors PPAR γ and PPAR δ , which are key
169 host factors in the polarization of macrophages to the M2 phenotype [28-30]. Moreover,
170 modulation of PPAR γ levels directly correlated with the capacity of *Salmonella* to replicate
171 inside macrophages, but not with the capacity of other intracellular bacteria such as
172 *Mycobacterium*, *Francisella*, or *Listeria*, supporting the idea of an active role of this
173 bacterium in driving macrophages to the M2 state [28].

174 *Mycobacterium* infection induces a metabolic program in macrophages that is
175 characterized by a decreased OXPHOS and the redirection of glycolytic intermediates to the
176 synthesis of large lipid bodies, which accumulate in the macrophage as shown by the
177 characteristic “foamy” phenotype of *M. tuberculosis*-infected macrophages [11]. These lipid
178 bodies and the lactate produced by glycolysis are used as nutrients by *M. tuberculosis*
179 [11,33,34], showing that *M. tuberculosis* also takes advantage of the metabolites of the host
180 cell. Moreover, it seems that *M. tuberculosis* actively induces a quiescent energy phenotype in
181 human macrophages, characterized by a decelerated flux through glycolysis and the TCA
182 cycle [35], which has recently also been linked to the subversion of IL-1 β production. By
183 upregulating microRNA-21, *M. tuberculosis* targets the glycolytic enzyme
184 phosphofructokinase muscle (PFK-M) isoform, reduces glycolysis and facilitates bacterial
185 growth by limiting pro-inflammatory cytokines such as IL-1 β [36].

186 Several intracellular bacteria, such as *L. monocytogenes*, *Chlamydia trachomatis*,
187 *M. tuberculosis* and *L. pneumophila*, modulate mitochondrial dynamics during infection of
188 human cells [12,15,37-40]. Mitochondrial dynamics govern the bioenergetics of eukaryotic
189 cells, as mitochondrial functions are critically influenced by the dynamic nature of these
190 organelles in terms of position, numbers or shape [41]. Thus, targeting mitochondria is a
191 powerful strategy of pathogenic bacteria to alter the bioenergetics of host cells [8].

192 *Legionella pneumophila* alters the mitochondrial shape in human macrophages through the
193 injection, via the type IV secretion system (T4SS), of the bacterial effector MitF/LegG1,
194 which leads to dynamin 1-like (DNM1L)-mediated fragmentation of the mitochondrial
195 networks of the host cell [12]. Mitochondrial fragmentation in infected macrophages is then
196 partially responsible for a T4SS-mediated decrease of OXPHOS, which might enforce the
197 glycolytic fluxes of *L. pneumophila*-infected macrophages that are induced in a T4SS-
198 independent manner. DNM1L-dependent mitochondrial fragmentation, as well as the
199 induction of glycolysis, was identified as necessary for optimal replication of the bacteria
200 within human primary macrophages.

201 As *L. pneumophila* preferentially oxidizes serine, and not glucose, to obtain energy for its
202 exponential growth, it can be hypothesized that the increment in glycolytic fluxes observed
203 during infection leads to rerouting of the metabolite pyruvate to the biosynthesis of serine, to
204 support intracellular replication of *Legionella* [12,17,42]. A recent report suggested that
205 *Legionella* might be able to use phosphorylated hexose sugars during intracellular growth in
206 murine macrophages. In these experimental settings, infection-induced glycolysis was
207 dispensable for intracellular replication of *L. pneumophila* [43]. Further studies are needed to
208 definitively elucidate whether a glycolytic metabolism in human macrophages promotes the
209 acquisition of serine or phosphorylated hexose sugars by *L. pneumophila*, and what the
210 mechanisms are by which fragmentation-dependent reduction of mitochondrial respiration
211 lead to increased bacterial replication.

212 Together, these reports demonstrate that, in addition to their protective role and roles in
213 immune responses, metabolic alterations of infected macrophages also benefit intracellular
214 bacteria. Moreover, intracellular bacteria are active players during infection as they can
215 manipulate the bioenergetics of infected cells to obtain nutrients and create a replication-
216 permissive intracellular environment. Furthermore, immune reactions in response to these
217 bacteria-induced metabolic alterations might also occur in the infected cells, which would add
218 an additional layer of complexity to the study of metabolic host–pathogen interactions.

219 **Sensing molecular patterns and metabolic processes: activation of** 220 **inflammasomes during host–microbe interactions**

221 Macrophages undergo metabolic modifications upon sensing bacterial components to adapt
222 their metabolism to the needs linked to their role during infection — that is, the production of
223 cytokines and anti-microbial compounds [1,2].

224 Macrophages, as professional phagocytes, ingest bacteria to clear infection but, although
225 most of ingested bacteria are degraded by xenophagy, some pathogenic bacteria are able to
226 modulate autophagy and escape from phagosome–lysosome fusion [9,44]. Once bacterial
227 degradation is avoided, intracellular bacteria need to obtain nutrients to replicate within host
228 cells. Indeed, they evolved mechanisms that directly subvert, modulate and reroute metabolic
229 pathways to nourish nascent intracellular microbes [8]. Therefore, the metabolic alterations
230 that occur in the same infected macrophage are those that equip the cell to respond to
231 infection, and those that are induced by the bacteria to obtain nutrients. However, the
232 immunometabolic interactions between bacteria and host cells during infection do not end
233 here because there are additional steps that can follow, where macrophages can also sense and
234 react to bacteria-induced alterations of their metabolic pathways.

235 Indeed, recently it was shown that macrophages sense the alteration of the cellular
236 metabolism and trigger defensive responses to recover cellular homeostasis. These regulatory
237 mechanisms have been named homeostasis-altering molecular processes (HAMPs, [45]) and
238 danger-associated metabolic modifications (DAMMs [17]), describing how cells react against
239 indirect evidence of danger by sensing homeostatic alterations. DAMMs and HAMPs trigger
240 inflammasome activation as a unique and robust response after sensing these alterations
241 [17,45]. Although it can be hypothesized that, in addition to inflammasome activation, other
242 immunometabolic reactions might be triggered in cells upon sensing these homeostatic
243 perturbations, such as regulation of autophagy, it seems that most of the activation
244 mechanisms triggered upon sensing DAMMs/HAMPs converge exclusively on
245 inflammasome activation.

246 Inflammasomes are protein complexes that are involved in inflammatory immune
247 responses. Their activation is elicited upon the intracellular recognition of PAMPs or danger-
248 associated molecular patterns (DAMPs), implying either an undergoing infection or a
249 disturbance of cell homeostasis [46].

250 NLRPs [nucleotide-binding oligomerization domain (NOD)-like receptors containing pyrin
251 domains (PYDs)] are the pattern-recognition receptors (PRRs) at the centre of inflammasome
252 formation. Upon activation, NLRP monomers oligomerize *via* their NOD domains and recruit
253 ASC [apoptosis-associated speck-like proteins containing caspase recruitment domains
254 (CARDs)]. These ASC proteins contain a PYD as well as a CARD, and act hence as adapters
255 in the recruitment and cleavage of pro-caspases (**Fig. 1**) [46]. Subsequent cleavage and
256 activation of caspases leads to the secretion of pro-inflammatory cytokines such as IL-18 or

257 IL-1 β by the caspase-mediated cleavage of their pro-proteins. Gasdermin-D, a pore-forming
258 protein, is also cleaved by inflammasomes and its cleavage and activation leads to a pro-
259 inflammatory form of programmed cell death called pyroptosis, causing the release of
260 cytokines [47].

261 Fourteen NLRP inflammasomes are known today and, although they have been largely
262 studied in macrophages and other immune cells, they can also be found in epithelial cells [46].
263 In any cell type, inflammasomes are key molecular players against bacterial infection because
264 they respond to several intracellular bacterial PAMPs, such as components of T3SS [48],
265 components of bacterial flagellum (flagellin) or components of the peptidoglycan of gram
266 positive and gram negative bacteria (muramyl dipeptides) [46].

267 Some interesting PAMPs are microbe-derived metabolites that trigger inflammasome
268 activation. Metabolites derived from colonic bacteria, such as taurine, pinitol, sebacate and
269 undecanedioate, trigger NLRP6-mediated caspase-1 activation and subsequent IL-18
270 secretion [49]. NLRP6-mediated release of IL-18 leads to the production of anti-microbial
271 peptides that influence the composition of the colonic microbial community [49]. As these
272 bacteria-derived metabolites are chemically divergent, it seems that NLRP6 activation by
273 these compounds occurs by means other than direct binding and recognition of molecular
274 patterns [46]. How these diverse metabolites activate NLRP6 remains unknown, but it can be
275 hypothesized that indirect detection of alterations in the metabolic interactions between
276 colonic bacteria and host cells might be involved.

277 In addition to PAMPs, and independently of infection, several DAMPs also activate
278 inflammasomes. For instance, extracellular ATP is one of the most used DAMPs to activate
279 NLRP3 inflammasomes [50]. It has also been shown that particular crystals and aggregates,
280 such as gout-associated crystals (uric acid and calcium pyrophosphate dehydrate crystals),
281 cholesterol crystals or environmental crystalline structures (including silica, asbestos,
282 aluminum salt crystals and aluminum hydroxide) also trigger NLRP3 inflammasome
283 activation [47,50]. DAMPs related to the detection of metabolic and homeostatic alterations
284 are oxidized mitochondrial DNA (mtDNA), which is released in the cytoplasm upon
285 mitochondrial damage, as well as phosphatidylinositol-4-phosphate, which is dispersed in the
286 cytoplasm upon *trans*-Golgi disassembly [51]. These examples show that some DAMPs can
287 emerge during metabolic alterations.

288 In summary, inflammasomes are activated by pathogen-associated PAMPs and danger-
289 associated DAMPs. However, it can be assumed that PAMPs are included among DAMPs, as
290 sensing pathogens is also a danger signal for the cell.

291 Beyond sensing molecular patterns, inflammasomes might also sense disturbances in
292 metabolic processes in the cell. In this case, alterations of metabolic pathways in the cell are
293 the triggers of the immune responses, instead of the direct detection of molecular patterns [46].
294 This is the core of the concept of DAMMs — metabolic modifications sensed by the cell as
295 danger signals. As these metabolic modifications should be sensed, we cannot discard the idea
296 that these metabolic alterations lead to the unmasking of molecular patterns that are then
297 sensed by the cell. We propose the concept of DAMMs to better identify these metabolic
298 pathways that trigger immune responses when altered. This would allow the integration of
299 diverse metabolic host–pathogen interactions in a unique model (**Fig. 1**). In the next section,
300 we will present two DAMMs we identified in the literature (glycolytic flux and perturbed
301 mitochondrial OXPHOS) and we will discuss how these DAMMs might emerge during
302 infection, thus representing a new level of complexity in the immunometabolic responses to
303 bacterial infection.

304 **DAMMs: an integrated view of different steps in metabolic host–pathogen** 305 **interactions**

306 We propose that certain modifications in cellular metabolism — that is, DAMMs — might
307 function as danger signals triggering inflammasome activation. We have identified in the
308 literature DAMMs that emerge upon alterations of the cellular metabolism. As intracellular
309 bacteria actively modulate the macrophage metabolism for their benefit, we propose that
310 infected host cells might sense bacteria-induced metabolic alterations and then activate
311 immune reactions such as the inflammasome.

312 Some recent reports showed that, independently of infection, inhibition of glycolytic fluxes
313 activates the NLRP3 inflammasome [32,52,53]. Interestingly, inflammasome activation has
314 been observed upon disruption of the glycolytic pathway at different steps. Inhibition of the
315 glycolytic enzyme hexokinase (HK) by different means, which is the enzyme that binds and
316 phosphorylates glucose to glucose 6-phosphate (G6P) at early steps in the glycolytic pathway,
317 leads to a robust activation of NLRP3 and subsequent IL-1 β secretion in macrophages [52].
318 The association of HK with the outer membrane protein VDAC (voltage-dependent anion
319 channel) at mitochondrial surface is known to increase its enzymatic activity [54]. By using a

320 peptide that competes with HK for binding to VDAC and induces its dissociation from the
321 mitochondria, it was shown that HK dissociation alone was sufficient to induce NLRP3
322 inflammasome activation [52]. How HK dissociation leads to inflammasome activation
323 remains unknown, but several metabolic perturbations that inhibit HK function, such as
324 treatment with G6P, 2-deoxyglucose or citrate, all lead to inflammasome activation [52].

325 Later in the pathway, inhibition of the glycolytic enzymes glyceraldehyde 3-phosphate
326 dehydrogenase (GAPDH) and α -enolase (**Fig. 1**) by different compounds also activates
327 NLRP3 inflammasome in macrophages, which triggers pyroptosis and secretion of IL-1 β [32].
328 Disruption of glycolytic fluxes at these steps appears to increase the NAD⁺/NADH ratio and
329 mROS accumulation, which were suggested to be the signals for NLRP3 inflammasome
330 formation downstream of glycolytic disruption [32]. On the contrary, inhibition of PKM2,
331 which catalyses the final and also rate-limiting reaction in the glycolytic pathway, reduces
332 NLRP3 inflammasome activation in macrophages, which might indicate that an increased
333 glycolytic flux at this step could contribute to inflammasome activation. It was also suggested
334 that mROS are responsible for this PKM2-dependent glycolytic pathway that is required for
335 inflammasome activation [53].

336 All together, these studies showed that perturbations of glycolytic fluxes at different levels
337 in the pathway could activate the NLRP3 inflammasome and induce IL-1 β secretion, pointing
338 to the alterations of these glycolytic fluxes as prominent DAMMs in macrophages.

339 In parallel, it has been shown that the glycolytic-dependent activation of the NLRP3
340 inflammasome can also be induced during infection. In this case, the metabolic modifications
341 occurring in the cells are triggered by intracellular bacteria. As explained before, *Salmonella*
342 reroute glucose from the host cell to its vacuole and therefore uses macrophage glucose as its
343 carbon source [13,32]. The bacteria-driven sequestering of host glucose has direct effects on
344 glycolytic fluxes, and is thought that this alteration in the metabolism of the host cell causes
345 NLRP3 activation upon *Salmonella* infection [32].

346 Similarly to non-infectious conditions, the reduction of glucose availability during
347 *Salmonella* infection diminishes the production of NADH, impacts the NAD⁺/NADH ratio
348 and leads to inflammasome formation, IL-1 β secretion and pyroptosis [32]. Moreover, Wolf
349 *et al.* showed that, in addition to binding glucose, HK can also bind N-acetylglucosamines
350 (NAGs) derived from the peptidoglycan of infecting bacteria. The binding of bacteria-derived
351 NAGs interferes with its interactions with VDAC, leading to the dissociation of HK from the

352 mitochondria and the activation of the NLRP3 inflammasome [52]. Therefore, the release of
353 bacteria-derived sugars leads to the inhibition of glycolytic fluxes at the level of HK, which
354 activates the NLRP3 inflammasome by a yet unknown mechanism. Taken together, these data
355 illustrate how a DAMM elicited by bacterial metabolic activities (i.e. perturbations of
356 glycolytic fluxes) triggers inflammasome activation.

357 As an example, when *Salmonella* infect a macrophage, the infected cell polarizes to a
358 glycolytic-based anti-bacterial M1 metabolism. In order to counteract this, the bacteria
359 actively induce an M2 state to exploit the increased levels of glucose typically present in M2
360 macrophages [28]. Subsequently, additional steps of immunometabolic interactions emerge
361 when macrophages sense a DAMM, in this case the piracy of glucose by *Salmonella* and the
362 subsequent perturbation of glycolytic fluxes. This ultimately leads to inflammasome
363 activation, IL-1 β secretion and pyroptosis [32].

364 It has been shown that *Salmonella* causes pyroptosis in macrophages during infection [55],
365 and the piracy of glucose seems partly involved [32]. However, as a certain number of
366 bacteria finally survive and replicate within macrophages, *Salmonella* must have evolved
367 some mechanisms to avoid the pyroptotic cell death of the macrophage in order to maintain
368 the niche for bacterial survival and replication. Indeed, it was shown that *Salmonella* regulates
369 the expression of its flagellum in order to reduce NLRC4 inflammasome activation in infected
370 macrophages [56]. Another study elegantly showed that *Salmonella* avoids the activation of
371 NLRP3 and pyroptosis by reducing the secretion of bacteria-derived citrate, which somehow
372 triggers mROS production at mitochondria [57]. It would be interesting to ascertain whether
373 *Samonella* also possesses mechanisms to specifically evade the emergence of DAMMs such
374 as the alteration of glycolysis.

375 Therefore, the concept of DAMMs — the example here being glycolytic perturbations —
376 allows integration of the diverse metabolic alterations in macrophages observed during
377 *Salmonella* infection in one model. A first step includes immunometabolic alterations that
378 benefit the host (M1, anti-bacterial); a second step includes those that benefit the bacteria (M2
379 induction, available glucose); a third step is derived from the second step and includes
380 DAMM-sensing and inflammasome activation in macrophages. Additional events, such as
381 bacterial evasion of DAMM-induced inflammasome-mediated pyroptosis, might be thus
382 expected in a subset of infected macrophages where bacterial replication successfully takes
383 place.

384 Perturbations of mitochondrial OXPHOS also seem to be a DAMM. Altered operation of
385 the ETC at mitochondrial cristae might lead to mROS production, a well-known activator of
386 the NLRP3 inflammasome [58,59]. It has been shown that small molecules inhibiting
387 mitochondrial Complex I (the largest enzyme complex of the respiratory chain) increased
388 mROS generation at the ETC and caused NLRP3 inflammasome activation that depended on
389 NEK7 (Never in mitosis A-related protein kinase 7) in murine dendritic cells [60]; moreover
390 rotenone, which is a classical Complex I inhibitor, also caused activation of NLRP3 in murine
391 macrophages [58]. In this case, aberrant high quantities (but not low quantities) of mROS and
392 mitochondrial hyperpolarization seem to be needed to activate NLRP3 [61].

393 Inhibition of Complex I during bacterial infection has been observed in diverse
394 experimental settings. Activation of Toll-like receptors (TLRs) by bacterial LPS leads to the
395 translocation of TNF receptor associated factor 6 (TRAF6) to mitochondria in murine
396 macrophages, where it binds to evolutionarily conserved signalling intermediate in Toll
397 pathway (ECSIT). As ECSIT is a protein involved in the assembly of Complex I at the ETC,
398 and TRAF6-mediated ubiquitination of ECSIT leads to increased mROS production in murine
399 macrophages, activation of the TLR–TRAF6–ECSIT axis might compromise the flow of
400 electrons through Complex I, leading to electron leakage and mROS production [62,63].

401 Interestingly, *Salmonella* replicates intracellularly to higher numbers within ECSIT-
402 deficient macrophages [63], suggesting that bacterial activation of the TLR–TRAF6–ECSIT
403 axis is needed in macrophages to mount effective anti-bacterial responses. As mROS is a
404 well-known NLRP3 activator, it is possible that TLR–TRAF6–ECSIT mediates mROS
405 generation and activates the inflammasome; however, NLRP3 activation or IL-1 β secretion
406 was not determined in the study [63].

407 Another report showed that infection of murine macrophages with extracellular
408 *Escherichia coli* or with *S. enterica* induced a reorganization of ETC complexes and super-
409 complexes by decreasing Complex I levels at mitochondrial membranes [6]. Although
410 changes in mROS production were not observed, rearrangement of the ETC during infection
411 was needed for IL-1 β secretion that depended on NLRP3 [6], suggesting that inflammasome
412 activation also has effects on ETC structure and activity.

413 Alterations in the OXPHOS machinery have also been observed during infection of murine
414 and human macrophages with *Porphyromonas gingivalis* (**Table 1**), a bacterium causing
415 periodontitis and that sheds large numbers of outer membrane vesicles (OMVs) during

416 infection. While bacterial infection and stimulation with these OMVs decreased OXPHOS
417 performance and generated mROS in macrophages, only OMVs activated the NLRP3
418 inflammasome [64], suggesting that *P. gingivalis* might have evolved mechanisms to avoid
419 NLRP3 activation. Therefore, alterations in the functioning of the OXPHOS machinery
420 during infection might also act as DAMM that activates inflammsomes; however, bacterial
421 strategies to avoid the emergence of DAMMs during infection might also exist. Indeed, it has
422 been shown that activation of the absent in melanoma 2 (AIM2) inflammasome, which is a
423 non-NLRP inflammasome, during infection of macrophages with avirulent *Francisella*
424 *novicida* is dependent on mROS, whereas highly virulent *F. tularensis* successfully avoids
425 triggering inflammasome activation [65], which might happen by a yet unknown mechanism
426 that avoids mROS production during infection.

427 **Concluding remarks and future perspectives**

428 The “immune surveillance hypothesis” proposes that pathogen-mediated disruption of host
429 physiology leads to immune activation [66]. We propose that identification of DAMMs may
430 help to recognize the existence of these specific checkpoints along cellular metabolic
431 pathways that, when altered, trigger immune responses such as inflammasome activation. It
432 seems that alterations of glycolytic fluxes and OXPHOS performance during infection are
433 prototypic examples of bacteria-induced DAMMs.

434 Is it possible to find additional DAMMs emerging during infection by intracellular
435 bacteria? Interestingly, several metabolites derived from colonic bacteria activate the NLRP6
436 inflammasome [49]. It would be interesting to investigate whether some of these metabolites
437 are also generated during intracellular growth of pathogenic bacteria and whether they can
438 disrupt host metabolic pathways or enzymes leading to immune activation. Moreover, fatty
439 acids such as palmitate have been shown to activate the NLRP3 inflammasome [67], whereas
440 the ketone metabolite β -hydroxybutyrate was shown to block NLRP3 [68]. This suggests that
441 alterations of the lipid metabolism in the cell might also act as DAMMs. As *M. tuberculosis*
442 metabolizes fatty acids, including palmitate, and also ketone bodies during infection within
443 human macrophages [11], we speculate that alterations in the equilibrium of these lipid
444 metabolites in host cells during *M. tuberculosis* infection might act as bacteria-induced
445 DAMMs triggering inflammasome activation.

446 There is a lack of tools to study DAMMs in the context of bacterial infection. Indeed, it
447 was only few years ago when it was possible to precisely measure metabolic fluxes in host

448 cells during infection [69]. However, in order to correlate metabolic alterations to
449 inflammasome activation in infected cells, it would be important to develop new methods to
450 simultaneously quantify metabolic alterations and inflammasome assembly in single, infected
451 cells. By using microfluidics combined with recent technological advances, such experiments
452 might be undertaken. As example, a recent report showed that by using a microfluidic droplet
453 platform it was possible to precisely measure pyruvate, ATP and lactate secretion by breast
454 cancer MCF-7 cells, in a single-cell and non-destructive manner [70]. Another study recently
455 managed to quantify 20 amino acids and 40 metabolites in single HeLa cells [71].

456 With respect to tools to monitor inflammasome activation in single cells, a recent article
457 described a method to monitor NLRP3 activation by imaging ASC speck formation in single
458 human peripheral blood mononuclear cells (PBMCs) by using imaging flow cytometry [72],
459 suggesting that similar methods might be implemented using microfluidic platforms.
460 Therefore, by combining single-cell metabolomics and inflammasome assembly in a
461 microfluidic platform loaded with host cells infected by bacterial mutants (lacking specific
462 enzymes or bacterial effectors) or treated with specific inhibitors of host metabolic pathways,
463 we might be able to definitely dissect the complex mechanisms operating at different levels of
464 metabolic host–pathogen interactions, also including the investigation and determination of
465 yet unidentified DAMMs.

466 As metabolic interactions between intracellular bacteria and human cells are an intimate
467 exchange that might determine the course of infection, all the efforts directed to identify new
468 nodes of this complex interplay seem challenging, but are very exciting.

469

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471

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675 **Tables**676 **Table 1.** Examples of intracellular bacteria that infect human macrophages (adapted from [7])

Pathogen	Secretion system	Replication niche	Human disease	Reference
<i>Brucella spp.</i>	T4SS	Vacuole	Brucellosis	[29]
<i>Burkholderia pseudomallei</i>	T3SS, T6SS	Cytosol	Melioidosis	[73]
<i>Chlamydia pneumoniae</i>	T3SS	Vacuole	Pneumonia, bronchitis	[74]
<i>Citrobacter koseri</i>	T3SS	Vacuole	Meningitis	[75]
<i>Coxiella burnetii</i>	T4SS	Vacuole	Q fever	[76]
<i>Ehrlichia chaffeensis</i>	T4SS	Vacuole	Ehrlichiosis	[77]
<i>Francisella tularensis</i>	T6SS	Cytosol	Tularemia	[78]
<i>Legionella pneumophila</i>	T4SS	Vacuole	Legionnaires' disease	[79]
<i>Legionella longbeachae</i>	T4SS	Vacuole	Legionnaires' disease	[85]
<i>Listeria monocytogenes</i>	T2SS	Cytosol	Listeriosis	[80]
<i>Mycobacterium tuberculosis</i>	T7SS	Vacuole	Tuberculosis	[11]
<i>Porphyromonas gingivalis</i>	T9SS	Vacuole	Periodontitis	[64,81]
<i>Rhodococcus equi</i>	T2SS	Vacuole	Pneumonia	[82]
<i>Rickettsia rickettsii</i>	T4SS	Cytosol	Rocky Mountain spotted fever	[83]
<i>Salmonella enterica</i>	T3SS, T6SS	Vacuole	Salmonellosis	[84]
<i>Shigella spp</i>	T3SS	Cytosol	Shigellosis	[86]

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680 **Figure legends**

681

682 **Fig. 1.** Multi-step metabolic macrophage–bacteria interactions during infection. Metabolic
683 changes that benefit host cells (green arrows) include several actions triggered by pathogen-
684 associated molecular pattern (PAMP)-mediated activation of a pattern-recognition receptor
685 (PRR), such as lipopolysaccharide (LPS) engagement of Toll-like receptor 4 (TLR4). LPS
686 increases dimer and tetramer levels of pyruvate-kinase 2 (PKM2), which leads to succinate
687 accumulation. Dimers can translocate into the nucleus, where they induce IL-1 β transcription.
688 LPS-induction of PKM2 tetramers increases the rate of glycolysis. LPS also alters the
689 tricarboxylic acid (TCA) cycle leading to a reduced level of oxidative phosphorylation
690 (OXPHOS) and subsequent succinate and citrate accumulation. Accumulated succinate
691 induces hypoxia-inducible factor 1- α (HIF1- α) translocation to drive IL-1 β gene expression.
692 In mitochondria, succinate oxidation leads to mitochondrial reactive oxygen species (mROS)
693 production and subsequent HIF1- α -mediated IL-1 β mRNA expression. Accumulated citrate
694 is used to generate nitric oxide (NO), ROS and the antimicrobial metabolite itaconate.
695 Metabolic changes that benefit pathogenic bacteria (brown arrows) include the induction of
696 M2 metabolism by *Salmonella enterica* to have glucose available, a metabolite that is then
697 sequestered inside the bacterial vacuole. *Legionella pneumophila*-induced alterations of
698 mitochondrial dynamics reduce OXPHOS. *Mycobacterium tuberculosis* also reduces
699 OXPHOS. Bacteria-induced alterations of glycolytic fluxes and OXPHOS are danger-
700 associated metabolic modifications (DAMMs). DAMMs are detected by inflammasomes such
701 as NOD-like receptor family, pyrin domain containing 3 (NLRP3) (violet arrows), which in
702 turn activates caspases and leads to the production of IL-1 β and IL-18, and to the
703 inflammatory gasdermin-mediated cell death called pyroptosis.

