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Abstract

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

Introduction

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

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

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

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

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

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

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

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

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

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

drives IL-1β gene expression. High levels of succinate in mitochondria lead to the reverse flow of the electron transport chain (ETC), thus producing mitochondrial reactive oxygen species (mROS) that also induce HIF1α-mediated IL-1β mRNA expression [20].

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

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

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

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

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

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

Metabolic alterations that benefit bacterial pathogens: sequestering nutrients

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Concluding remarks and future perspectives

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

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

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

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

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

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

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675 # Tables

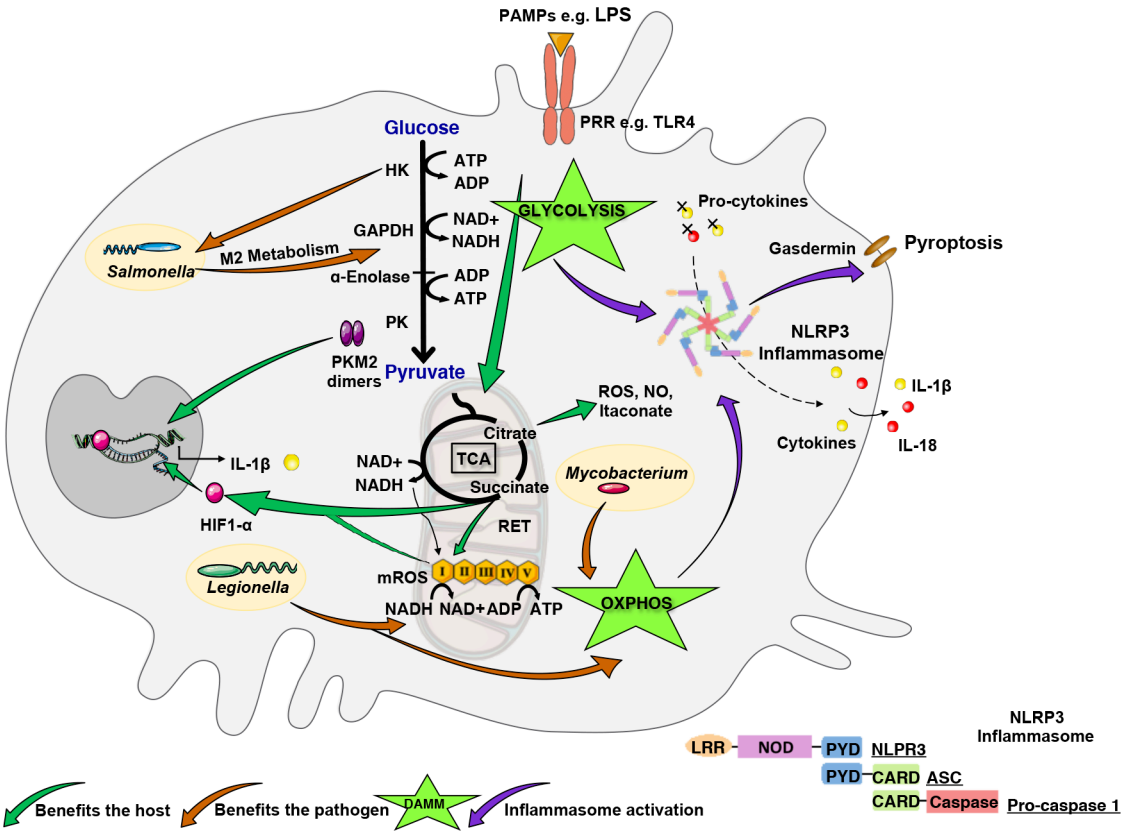
676 **Table 1.** Examples of intracellular bacteria that infect human macrophage**s** (adapted from [7])

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

677
678

Figure legends

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


PAMPs e.g. LPS
PRR e.g. TLR4
Glucose
HK
ATP
ADP
GLYCOLYSIS
GAPDH
NAD+
NADH
Salmonella
M2 Metabolism
α-Enolase
ADP
ATP
PK
PKM2
dimers
Pyruvate
Pro-cytokines
Gasdermin
Pyroptosis
NLRP3
Inflammasome
IL-1β
IL-18
Cytokines
ROS, NO,
Itaconate
Citrate
TCA
Succinate
NAD+
NADH
IL-1β
HIF1-α
Mycobacterium
RET
mROS
I
II
III
IV
V
NADH
NAD+
ADP
ATP
OXPHOS
Legionella
Benefits the host
Benefits the pathogen
DAMM
Inflammasome activation
NLRP3
Inflammasome
LRR
NOD
PYD
NLPR3
PYD
CARD
ASC
CARD
Caspase
Pro-caspase 1