

# Metabolic reprogramming in macrophages and dendritic cells in innate immunity

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**Activation of macrophages and dendritic cells (DCs) by pro-inflammatory stimuli causes them to undergo a metabolic switch towards glycolysis and away from oxidative phosphorylation (OXPHOS), similar to the Warburg effect in tumors. However, it is only recently that the mechanisms responsible for this metabolic reprogramming have been elucidated in more detail. The transcription factor hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) plays an important role under conditions of both hypoxia and normoxia. The withdrawal of citrate from the tricarboxylic acid (TCA) cycle has been shown to be critical for lipid biosynthesis in both macrophages and DCs. Interference with this process actually abolishes the ability of DCs to activate T cells. Another TCA cycle intermediate, succinate, activates HIF-1 $\alpha$  and promotes inflammatory gene expression. These new insights are providing us with a deeper understanding of the role of metabolic reprogramming in innate immunity.**

**Keywords:** macrophage; dendritic cell; metabolic reprogramming; glycolysis; oxidative phosphorylation  
*Cell Research* (2015) 25:771-784. doi:10.1038/cr.2015.68; published online 5 June 2015

## Introduction

Macrophages and dendritic cells (DCs) are the front-line cells of innate immunity. They sense and immediately respond to invading pathogens, thus providing an early defense against external attack. Pattern recognition receptors (PRRs) on the surfaces of these cells recognize pathogen-associated molecular patterns (PAMPs) present in the invaders and, as a result, activate intracellular signaling cascades, leading to the induction of a general pro-inflammatory response [1]. This includes the release of antimicrobial mediators, which target the invading pathogen, chemokines, which recruit more immune cells to the site of infection, and pro-inflammatory cytokines, which drive further inflammation, and induce the adaptive immune response, which is mediated by T and B lymphocytes and is more specific for the particular invading pathogen. PRRs are also capable of detecting endogenous danger-associated molecular patterns (DAMPs) [2], which indicate damage to host cells. There are several families of PRRs, the best characterized being the toll-like receptors (TLRs) and the NOD-like receptors (NLRs).

The key output of PRRs is increased gene expression. However, cells activated by PAMPs also undergo profound metabolic changes. These changes are required for biosynthesis and energy production, and in addition are involved in signaling processes. Here, we describe recent findings in the field of metabolic reprogramming in innate immunity and place them in the context of our current understanding of the complex events occurring during innate immune cell activation.

## Early work on metabolism in innate immune cells

The Warburg effect is an important concept for understanding metabolic changes occurring in innate immune cells upon activation [3]. Otto Warburg described a metabolic profile of tumors in normoxic conditions, in which glycolysis predominates even though there is oxygen available for oxidative metabolism to proceed. Pyruvate that is produced by the glycolytic pathway, instead of feeding into the tricarboxylic acid (TCA) cycle and subsequent oxidative phosphorylation (OXPHOS), is metabolized to lactate. The Warburg Effect is depicted in Figure 1.

This concept reappeared, but in the innate immune world, in the 1950s, with the discovery that neutrophils depend on aerobic glycolysis for ATP production, and have few mitochondria [4]. Glucose consumption in

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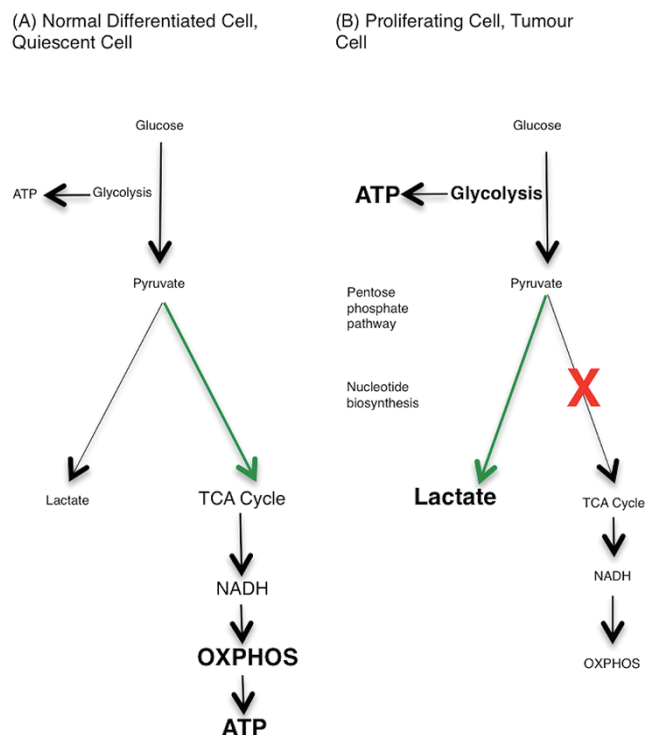
these cells was high, while oxygen consumption was low. Activation of TLR4 by the Gram-negative bacterial product lipopolysaccharide (LPS) in neutrophils increases glucose consumption. It also increases oxygen consumption, but this oxygen is used to produce the reactive oxygen species  $H_2O_2$ , which is important for neutrophil-mediated killing [5]. Increased flux through the pentose phosphate pathway (PPP), which branches off glycolysis, generates nicotinamide adenine dinucleotide phosphate (NADPH), which is used by NADPH oxidase to generate  $H_2O_2$  [6].

In 1969, Hard [7] observed that activated macrophages exhibited increased glycolysis, accompanied by decreased oxygen consumption. Almost 20 years later,

Newsholme *et al.* [8] investigated macrophage metabolism and showed that most of the glucose consumed by resting macrophages was converted to lactate, with little being used for OXPHOS. Activated macrophages were shown to have increased expression of the glycolytic enzymes hexokinase and glucose-6-phosphate dehydrogenase, indicating an increase in glycolytic activity in these cells [9].

However, despite these early observations about the metabolism of innate immune cells, it is only more recently that we have begun to elucidate the mechanisms behind these metabolic changes, and appreciate their impact on immune function.

### How does LPS promote Warburg metabolism in macrophages and DCs?



**Figure 1** The Warburg effect. **(A)** In resting cells, glucose is metabolized to pyruvate via glycolysis. Some pyruvate is converted to lactate, but most is directed to the TCA cycle via acetyl-CoA. The TCA cycle generates NADH, which donates electrons to the mitochondrial electron transport chain so that OXPHOS can progress. **(B)** In highly proliferative or tumour cells, the metabolic profile switches from OXPHOS to aerobic glycolysis, known as the Warburg effect. Mature innate immune cells also rely on glycolysis, although they do not proliferate after activation. The majority of the pyruvate generated by glycolysis is converted to lactate, and glycolytic intermediates build up, meeting the high energy demand of the cell. Glycolysis is the source of ATP in these cells, and also provides glucose-6-phosphate for nucleotide biosynthesis in the PPP.

Activation of macrophages or DCs with a range of stimuli, including LPS [10], the TLR3 ligand poly(I:C) [11], and type I interferon (IFN) [11], induces a metabolic switch from OXPHOS to glycolysis, in a phenomenon similar to the Warburg effect [10]. TCA cycle activity is decreased [10, 12], while lactate production and flux through the PPP are increased [13, 14]. Enhanced PPP activity boosts production of purines and pyrimidines, which can then be used for biosynthesis in the activated cell. It also provides NADPH for the NADPH oxidase enzyme, which produces ROS [15]. This metabolic shift occurs in mature activated immune cells even though they are not highly proliferative. The increase in glycolysis could be a mechanism to rapidly generate ATP. It is an inefficient means of generating ATP when compared to the TCA cycle, but it can be quickly induced and could be a particularly useful means of synthesizing ATP when cellular glucose uptake capacity is high [16]. Furthermore, in activated macrophages, mitochondrial ROS production is increased, serving as a mechanism of bacterial killing [17]. Glycolytic ATP production may compensate for this shift in mitochondrial metabolism by the electron transport chain [18].

Substantial insights have been made into the underlying mechanisms of this metabolic switch. There are at least four main processes leading to Warburg metabolism in LPS-activated macrophages and DCs (Figure 2). As the majority of studies investigating this mechanism use LPS as a stimulus, this review focuses on the metabolic alterations induced by LPS in macrophages and DCs. Other innate immune cell stimuli also alter metabolic parameters in innate immune cells, including IFN $\gamma$  [11], poly(I:C) [11], and infection with bacterial species such as *Listeria monocytogenes*, *Mycobacterium bovis* BCG

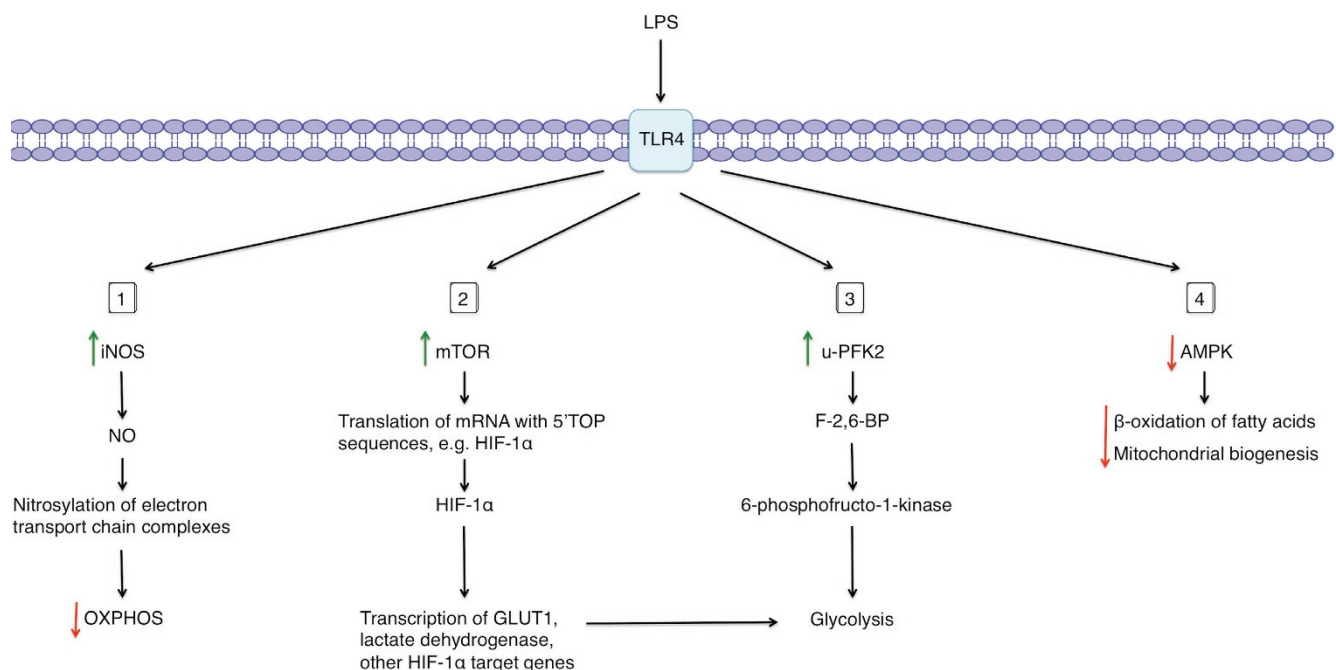
[19], and *Salmonella typhimurium* [17].

#### NO and metabolic changes in macrophages and DCs

Stimulation of macrophages [20] and DCs [21] with LPS and IFN- $\gamma$  increases the expression of inducible nitric oxide synthase (iNOS), which generates nitric oxide (NO), a reactive nitrogen species that can inhibit mitochondrial respiration. NO achieves this by nitrosylating iron-sulfur proteins present in electron transport chain complexes, e.g., Complex I [22, 23], as well as cytochrome c oxidase [24]. The metabolic switch in macrophages and DCs activated by LPS is therefore dependent on iNOS and NO-mediated inhibition of mitochondrial metabolism. Inhibition of iNOS after LPS stimulation restores normal mitochondrial respiration, and treatment of iNOS-deficient DCs with LPS fails to shut down mitochondrial metabolism. Simultaneously, the LPS-induced increase in glycolysis in these cells is blunted. Convincingly, re-addition of NO, using an exogenous NO donor, to DCs in which iNOS is still inhibited by a specific inhibitor again induces mitochondrial dysfunction and the switch towards glycolytic metabolism. This suggests that iNOS-generated NO is the mediator of the LPS-induced

mitochondrial functional collapse and increase in glycolysis [25]. Tumor necrosis factor (TNF)- $\alpha$ -induced protein 8-like 2 (TIPE2) is a recently described factor negatively regulating LPS-induced iNOS expression and NO production in macrophages [26].

The amino acid arginine is the substrate of iNOS. In macrophages activated by LPS and IFN- $\gamma$  or bacterial infection (e.g., with *M. bovis* BCG), extracellular arginine is imported into the cell and is metabolized to NO and citrulline by iNOS. The produced citrulline is then exported from the cell. When extracellular arginine is depleted, citrulline can be imported into the cell and recycled to arginine via argininosuccinate synthase (Ass1) and argininosuccinate lyase (Asl) [19]. This optimum NO production sustained by citrulline recycling via Ass1 and Asl may be important for controlling mycobacterial infections, as Ass1-deficient mice were less well able to control *M. tuberculosis* infection [19]. In fact, some pathogens themselves have mechanisms for depleting arginine. For example, *Helicobacter pylori* expresses an arginase that inhibits NO production by activated macrophages, leading to less effective bacterial killing by these macrophages [27].



**Figure 2** Mechanisms of LPS-induced Warburg metabolism in macrophages or DCs. Upon LPS stimulation of TLR4, a range of metabolic changes occur in macrophages or DCs. **(1)** LPS activation upregulates iNOS expression, increasing the production of NO, which nitrosylates and thus inhibits target proteins in the mitochondrial electron transport chain, thereby dampening OXPHOS. **(2)** LPS activates mTOR, thereby increasing the translation of mRNA with 5'-TOP sequences, including HIF-1 $\alpha$  mRNA. HIF-1 $\alpha$  then increases expression of its target genes. **(3)** LPS increases expression of u-PFK2, an isoform of PFK2, thereby increasing levels of the metabolite F-2,6-BP. F-2,6-BP activates the glycolytic enzyme 6-phosphofructo-1-kinase. **(4)** Finally, LPS inhibits AMPK, resulting in decreased  $\beta$ -oxidation of fatty acids and mitochondrial biogenesis.

A previous study used mass spectrometry to identify nitrosylated proteins in various mouse tissues and revealed that many metabolic enzymes are S-nitrosylated on cysteine residues by NO, including enzymes involved in glycolysis, the TCA cycle and fatty acid metabolism [28]. It is likely that cysteine nitrosylation could affect the activity of these enzymes. Indeed, nitrosylation of the liver enzyme very long-chain acyl-CoA dehydrogenase (VLCAD) was reported to increase its activity, thereby boosting fatty acid metabolism, as VLCAD catalyzes the first step in  $\beta$ -oxidation of fatty acids [28]. It remains to be investigated whether nitrosylation of these metabolic enzymes is involved in the metabolic switch in activated innate immune cells.

In summary, NO is a key intermediate in the metabolic switch of activated immune cells, and nitrosylation of cellular targets is an important mechanism by which NO exerts its effects.

#### *Hypoxia-inducible factor-1 $\alpha$ (HIF-1 $\alpha$ ) and glycolysis*

In tumors, many cells are often exposed to hypoxic microenvironments where they cannot rely on OXPHOS and must modify their metabolism to survive in these conditions of reduced oxygen tension. The transcription factor HIF-1 $\alpha$  [29] promotes the switch to glycolysis so that these cells can continue to produce ATP when oxygen is limited, as oxygen is not required for glycolysis. In such situations of anaerobic glycolysis, pyruvate, the end product of glycolysis, does not feed into the TCA cycle to boost subsequent OXPHOS, but is instead metabolized to lactate. Thus, a hallmark of anaerobic glycolysis is increased lactate production. HIF-1 $\alpha$  facilitates this metabolic switch by binding to hypoxia response elements in target genes [30, 31], such as the glucose transporter GLUT1 [32] and glycolytic enzymes. HIF-1 $\alpha$  induces expression of lactate dehydrogenase (LDH) [33], which catalyzes lactate production from pyruvate, thereby limiting the production of acetyl-CoA for the TCA cycle. HIF-1 $\alpha$  also increases the expression of pyruvate dehydrogenase kinase [34, 35], which inhibits pyruvate dehydrogenase, an enzyme that catalyzes the formation of acetyl-CoA from pyruvate. HIF-1 $\alpha$  promotes the glycolytic switch in hypoxic macrophages, and ATP levels are greatly reduced in HIF-1 $\alpha$ -deficient macrophages [36].

A similar process occurs in LPS-activated macrophages under normoxic conditions [12, 37]. Furthermore, macrophage activation by LPS is dependent on glycolysis, as inhibition of glycolysis with 2-deoxyglucose (2DG) decreases the inflammatory response, whereas inhibitors of mitochondrial respiration have no such effect, consistent with the fact that oxidative metabolism is already

shut down under such conditions [38]. 2DG decreases LPS-induced production of the inflammatory cytokine interleukin (IL)-1 $\beta$  and inhibits HIF-1 $\alpha$  activation [12]. HIF-1 $\alpha$ -knockout macrophages also have decreased expression of iNOS after IFN $\gamma$  stimulation [39]. Bacterial infection induces HIF-1 $\alpha$  expression in macrophages, and HIF-1 $\alpha$ -null macrophages are less well able to kill bacteria [40].

A key mechanism for HIF-1 $\alpha$  activation by LPS involves the mammalian target of rapamycin (mTOR). This serine/threonine protein kinase is active when nutrients are in abundance, and so is highly active in proliferating cells and metabolically demanding situations, e.g., after TLR stimulation [41]. mTOR helps cells meet this high metabolic demand by increasing HIF-1 $\alpha$  expression, which in turn increases the expression of glycolytic genes, as well as inflammatory genes. mTOR mediates the increase in HIF-1 $\alpha$  expression by promoting translation of mRNAs containing 5'-terminal oligopyrimidine (5'-TOP) signals, motifs that are present in HIF-1 $\alpha$  mRNA [42]. Furthermore, the mTOR complex component Raptor interacts with HIF-1 $\alpha$  via an mTOR signaling motif located in HIF-1 $\alpha$ , thereby promoting HIF-1 $\alpha$  activity [43]. The LPS-induced increase in HIF-1 $\alpha$  expression level and activity therefore provides a further parallel with tumour cell metabolism. The mTOR-HIF-1 $\alpha$  axis also has a role in the memory function of the innate immune system, known as trained immunity. This memory component is based on epigenetic reprogramming of innate immune cells, as demonstrated by the epigenetic changes observed during the monocyte-to-macrophage differentiation induced by  $\beta$ -glucan training [44]. Trained monocytes rely on aerobic glycolysis, a metabolic programme orchestrated by an Akt-mTOR-HIF-1 $\alpha$  pathway. Mice with myeloid cell-specific HIF-1 $\alpha$  deficiency lack the ability to induce a trained innate immune response to sepsis [45].

Inhibition of glycolysis also blocks DC maturation in response to LPS, as evidenced by reduced expression of the costimulatory molecules CD80 and CD86 in the presence of 2DG [46]. Similarly to macrophages, HIF-1 $\alpha$  is a key factor in the metabolic switch and immune function in DCs. Compared with hypoxic treatment, a classical inducer of HIF-1 $\alpha$ , LPS stimulation in normoxia induces a greater amount of HIF-1 $\alpha$  protein and mRNA in DCs [46]. This is accompanied by increased induction of the HIF-1 $\alpha$  target genes GLUT1 and phosphoglycerate kinase (PGK). HIF-1 $\alpha$  deficiency in LPS-stimulated DCs reduces the levels of the costimulatory molecules CD80 and CD86 on the cell surface [46, 47], and these DCs are less well able to drive T-cell proliferation [46, 47]. DCs activated by TLR2 ligation also have increased

levels of HIF-1 $\alpha$  [48]. This metabolic shift is evident in DCs *in vivo*, in response to poly(I:C)-stimulated type I IFN signaling [11]. DCs activated in this manner exhibit increased glycolysis and reduced mitochondrial respiration, as well as increased *Hif1a* transcription. This response was impaired in mice lacking IFNAR, the type I IFN receptor [11]. In contrast to macrophages [12], however, HIF-1 $\alpha$  may not have a role in cytokine release from LPS-activated DCs [47].

LPS also promotes mTOR activation in murine DCs [49], though this may not translate to human DCs [50]. Rapamycin, an inhibitor of mTOR, abolishes LPS-induced IL-10 induction in murine DCs, which leads to increased IL-12 levels, as IL-10 inhibits the production of IL-12 in an autocrine manner [49]. DCs lacking tuberous sclerosis complex 1 (TSC1), an upstream negative regulator of mTOR, exhibit increased glycolysis and expression of glycolytic enzymes, including hexokinase-2 (*HK2*) and *LDHA* [51]. Surprisingly, DCs lacking TSC1 have increased mitochondrial respiration (as well as increased glycolysis), and are developmentally impaired [51]. Furthermore, TSC1-deficient DCs are less capable of inducing T<sub>h</sub>1 responses [51]. In another study, mTOR inhibition in DCs promotes their long-term survival and their ability to stimulate T-cell responses [50]. DCs activated in the presence of rapamycin are better able to induce the primary expansion of CD8<sup>+</sup> T cells in response to LPS plus OVA antigen, as well as the later establishment of a memory CD8<sup>+</sup> T-cell population, compared to DCs without rapamycin treatment [50]. The downstream effects of mTOR inhibition in DCs may depend on the particular population of DCs under investigation, as well as the experimental context, and further work is needed to clarify the role of mTOR in DC function.

HIF-1 $\alpha$  and mTOR are central regulators of the metabolic switch in both macrophages and DCs, with a wide range of downstream targets that facilitate this process. The mTOR-HIF-1 $\alpha$  pathway is complex, and more work is needed to dissect the effects of mTOR-HIF-1 $\alpha$  signaling in various inflammatory situations.

#### *u-PFK2 and glycolysis in macrophages*

Another LPS-regulated target involved in the switch to glycolysis is the *pfkfb3* gene encoding u-PFK2 (PFKFB3), an isoform of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2) [52, 53]. PFK-2 regulates intracellular levels of the glycolytic intermediate fructose-2,6-bisphosphate (F-2,6-BP), as it has dual kinase:bisphosphatase activities and thus catalyzes both the synthesis and degradation of this intermediate. F-2,6-BP allosterically activates 6-phosphofructo-1-kinase, thereby increasing flux through the glycolytic pathway

[54]. u-PFK2 is the isoform of this enzyme that has the highest kinase:bisphosphatase activity ratio, and therefore maintains the highest levels of the biphosphorylated form of the metabolite, F-2,6-BP [55, 56]. Interestingly, *pfkfb3* is a HIF-1 $\alpha$  target gene in response to hypoxia in human glioblastoma cell lines and mouse embryonic fibroblasts [57], and thereby provides another mechanism by which HIF-1 $\alpha$  promotes glycolysis.

The purine nucleoside adenosine, a metabolite of ATP, can synergize with LPS to boost glycolysis in macrophages [58]. LPS stimulation of macrophages increases the expression of the A<sub>2A</sub> and A<sub>2B</sub> cell surface receptors (A<sub>2A</sub>R and A<sub>2B</sub>R) for adenosine [58]. Adenosine binds to these receptors, which further increases LPS-induced expression of *pfkfb3*. This synergistic induction of *pfkfb3* is not mediated by HIF-1 $\alpha$ , but by the transcription factor Sp1 [58]. Adenosine also synergizes with LPS to boost lactate production, indicating that adenosine enhances the LPS-induced increase in glycolysis [58]. In general, however, adenosine signaling often acts in an anti-inflammatory manner. For example, following LPS stimulation, A<sub>2B</sub>R-deficient macrophages showed increased production of NO and pro-inflammatory cytokines [59]. Adenosine boosts *E. coli*-induced production of the anti-inflammatory cytokine IL-10 in peritoneal macrophages, and *E. coli* is unable to induce IL-10 production in macrophages lacking A<sub>2A</sub>R [60]. Interestingly, inosine, a breakdown product of adenosine, exerts anti-inflammatory effects by decreasing the LPS-induced production of TNF- $\alpha$  and IL-1 in mouse peritoneal macrophages [61].

Taken together, increasing u-PFK2 expression level is a critical step in the induction of glycolysis in activated innate immune cells.

#### *AMPK and activation of macrophages and DCs*

Finally, LPS also decreases activation of the energy-sensing enzyme AMP-activated protein kinase (AMPK) in macrophages [62]. AMPK activity opposes the metabolic switch towards glycolysis in DCs [10]. AMPK is active when the cellular energy charge is low, as binding of either AMP or ADP to this kinase renders it more susceptible to stimulatory phosphorylation by its upstream kinases, liver kinase B1 (LKB1) and calmodulin-dependent protein kinase kinase 2 (CaMKK2). As the function of AMPK is to conserve energy when it is limited, this enzyme inhibits anabolic pathways, such as gluconeogenesis, and drives catabolic pathways, including  $\beta$ -oxidation of fatty acids. AMPK induces the expression of proteins involved in OXPHOS, such as peroxisome proliferator-activated receptor (PPAR)- $\gamma$  coactivator (PGC) 1 $\beta$ , a transcriptional coactivator that promotes mitochondrial biogenesis [63], and the activities of mito-

chondrial enzymes, including succinate dehydrogenase (SDH) [64]. AMPK also upregulates enzymes involved in fatty acid metabolism [64], such as carnitine palmitoyltransferase 1 $\alpha$  (CPT1 $\alpha$ ) that promotes fatty acid uptake by mitochondria for  $\beta$ -oxidation. LPS-induced inhibition of AMPK dampens the activity of these pathways, which is useful to highly activated cells as they need to boost biosynthetic pathways to produce inflammatory mediators.

Consistent with its role in decreasing anabolism, AMPK can inhibit mTOR in various cell types to decrease protein synthesis, a downstream effect of mTOR [65, 66]. Inhibition of AMPK by LPS may therefore represent another means by which LPS promotes mTOR activity and consequently HIF-1 $\alpha$  signaling, which could be another mechanism by which LPS induces the metabolic switch in innate immune cells.

Genetic manipulation of AMPK provides evidence for its role in inflammatory signaling. Macrophages and DCs derived from AMPK $\alpha$ 1<sup>-/-</sup> mice show increased production of TNF- $\alpha$  and IL-6, and decreased production of IL-10 in response to LPS [67]. Macrophages from mice lacking the AMPK $\beta$ 1 subunit have reduced rates of palmitate oxidation, and as a consequence show increased production of the pro-inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  [68]. LPS activation of DCs decreases stimulatory phosphorylation of AMPK, and shRNA targeting of AMPK results in increased IL-12p40 and CD86 expression [10].

AMPK can also modulate inflammatory signaling downstream of LPS by affecting transcription factors involved in the inflammatory response. AMPK antagonizes nuclear factor  $\kappa$ B (NF $\kappa$ B) signaling, a pathway responsible for the transcription of pro-inflammatory cytokines such as IL-1 $\beta$  and TNF- $\alpha$ . AMPK stabilizes inhibitor of  $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ ) and p53, both of which inhibit NF $\kappa$ B activity in various cell types [69]. Furthermore, AMPK can activate Sirtuin 1 (SIRT1), an NAD-dependent deacetylase that has been shown to deacetylate and thus inactivate p65, a subunit of NF $\kappa$ B, in HEK 293T cells expressing p65 [70].

Overall, AMPK counters many metabolic changes induced in pro-inflammatory macrophages and DCs, and its inhibition by LPS allows these changes to proceed.

### Citrate in macrophages and DCs

A key functional outcome for the switch to glycolysis is increased ATP production and PPP activity for biosynthesis. However, it has also recently been found that another metabolic event occurs in response to LPS earlier in the time course of macrophage activation (be-

fore NO accumulation limits OXPHOS), which involves citrate. Citrate is a TCA cycle intermediate formed from oxaloacetate and acetyl-CoA by citrate synthase in the mitochondria. LPS increases expression of the mitochondrial citrate carrier, solute carrier family 25 member 1 (Slc25a1), via NF $\kappa$ B in macrophages [71]. This carrier transports citrate out of the mitochondrion in exchange for malate, causing cytosolic citrate accumulation. When ATP is present, the enzyme ATP-citrate lyase (ACLY) in the cytosol converts citrate back to oxaloacetate and acetyl-CoA. Acetyl-CoA is a source of acetyl groups for acetylation of histones [72]. Interestingly, the transcription of genes encoding glycolytic enzymes *HK2*, phosphofructokinase-1 (PFK1) and LDHA is regulated downstream of ACLY. Silencing of ACLY suppressed expression of these genes, thereby decreasing glucose consumption. This effect was rescued by the addition of acetate, which can provide the acetyl groups needed for histone acetylation on these genes. Citrate metabolism can therefore influence the induction of glycolysis [72]. The increase in acetyl-CoA levels after TLR stimulation has another function in activated macrophages and DCs. Acetyl-CoA has been shown to help activated macrophages meet their increased biosynthetic demands, particularly in lipid biosynthesis. For example, ACLY is needed for the production of the eicosanoid prostaglandin E2 (PGE<sub>2</sub>) in response to LPS or TNF- $\alpha$  stimulation in phorbol-12-myristate-13-acetate (PMA)-differentiated U937 cells, human monocytic cells from histiocytoma [73]. The function of acetyl-CoA in promoting lipid biosynthesis depends on acetyl-CoA carboxylase (ACC), which converts acetyl-CoA to malonyl-CoA, a metabolite that is used for fatty acid synthesis. In LPS-activated DCs, citrate is preferentially removed from the TCA cycle and used for *de novo* synthesis of fatty acids, and increased glycolytic flux is needed to support this metabolic shift [74]. This increase in glycolysis is mediated by the kinases TANK-binding kinase 1 (TBK1), inhibitor of  $\kappa$ B kinase  $\epsilon$  (IKK $\epsilon$ ) and Akt, which drive the association of the glycolytic enzyme *HK2* with the mitochondria. Genetic silencing of Slc25a1 or inhibition of fatty acid synthesis by blocking the activity of ACC both result in the impairment of LPS-induced DC activation. The decreased fatty acid biosynthesis limits membrane production needed for the expansion of endoplasmic reticulum and Golgi, which then decreases protein production, including the generation of cytokines needed for T-cell activation by DCs [74]. Consistent with this, liver DCs with a high lipid content are immunogenic, inducing T-cell and NK-cell activation, while those with lower lipid concentrations are more tolerogenic and induce regulatory T cells [75].

ACLY-catalyzed citrate metabolism also contributes to the production of inflammatory mediators such as NO and ROS. Oxaloacetate, the other product of citrate metabolism, is further metabolized to pyruvate with the concomitant generation of NADPH from NADP<sup>+</sup>. NADPH and molecular oxygen can be used by NADPH oxidase to generate ROS. NADPH is also required as a substrate for the conversion of L-arginine to NO and citrulline. NO could then participate in the LPS-induced metabolic switch by inhibiting mitochondrial OXPHOS, as could ROS, as they stabilize HIF-1 $\alpha$  [76]. Activation of macrophages by either LPS, TNF- $\alpha$ , or IFN $\gamma$  alone, or a combination of TNF- $\alpha$  and IFN $\gamma$  induces ACLY expression, and inhibition of ACLY activity decreases the production of NO and ROS [73]. Inhibition of Slc25a1 or its silencing also results in decreased production of NO, ROS, and PGE<sub>2</sub> in U937 cells [71].

These changes in citrate transport and metabolism (Figure 3) would therefore appear to be critical for the activation of macrophages and DCs by LPS, the latter in turn triggers T-cell activation and adaptive immunity.

### Succinate as a signal in macrophages and DCs

Succinate, another TCA cycle intermediate, has been found to be involved in macrophage activation by LPS [12]. Succinate levels are elevated in macrophages after LPS stimulation [12], and are also increased in mouse models of obesity and diabetes [77], metabolic disorders associated with inflammation. This LPS-stimulated increase in succinate levels does not originate from the TCA cycle, as the activity of this system is dampened in response to LPS [10, 12, 25]. Glutamine metabolism provides the source for this LPS-induced increase in succinate generation, via anaplerosis [52, 78] proceeding through  $\alpha$ -ketoglutarate ( $\alpha$ -KG), as well as via the  $\gamma$ -aminobutyric acid (GABA) shunt [12]. Succinate can also be generated via the glyoxylate shunt, a system that operates in bacteria, fungi, protists, and plants [79-81]. The first enzyme of this shunt, isocitrate lyase (ICL), converts isocitrate to succinate and glyoxylate. ICL has been shown to have a positive role in the persistence of *M. tuberculosis* in LPS-activated macrophages [82]. However, it is unclear whether LPS increases succinate production via the ICL pathway in mammals upon microbial infection. Interestingly, mammalian macrophages highly express *immunoresponsive gene 1* (*Irg1*) during inflammation, which catalyzes the generation of itaconic acid from the TCA cycle intermediate *cis*-aconitate [83]. Itaconic acid is a metabolite thought to inhibit ICL and thereby decrease microbial growth [83, 84]. The role of itaconic acid in the metabolism of innate immune cells

warrants further studies.

Succinate stabilizes HIF-1 $\alpha$ , thereby promoting the switch to glycolysis and driving inflammation. It accomplishes this by inhibiting the activity of prolyl hydroxylase (PHD) enzymes, so that they can no longer hydroxylate and destabilize HIF-1 $\alpha$ . HIF-1 $\alpha$  can then interact with coactivators to induce the glycolytic metabolic program, and drive inflammation by increasing transcription of IL-1 $\beta$  [12]. Vigabatrin, an inhibitor of the key GABA shunt enzyme GABA transaminase, is protective in a mouse model of LPS-induced sepsis [12].

Extracellular succinate can signal via the G protein-coupled receptor (GPR) 91. GPR91 is highly expressed by DCs, and can synergize with TLR signaling to boost the induction of the pro-inflammatory cytokine TNF- $\alpha$  by the TLR3 ligand poly(I:C) or the TLR7 ligand imiquimod [85].

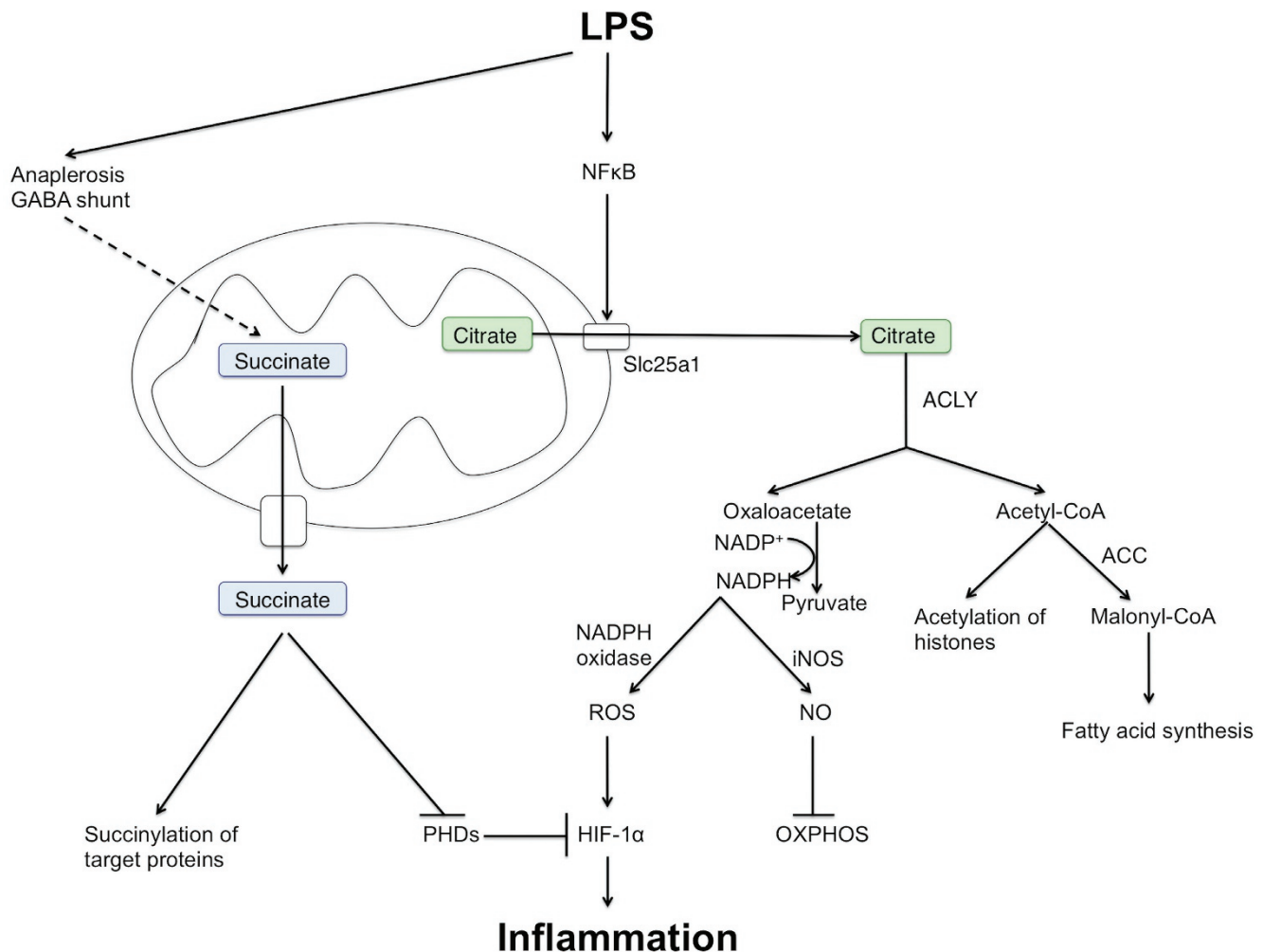
Succinate can also act as a substrate for succinylation, a post-translational modification of proteins in which a succinyl group is added to a lysine residue. LPS increases succinylation of multiple proteins [12]. Succinylated targets include the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as well as malate dehydrogenase (MDH), LDH, and glutamate carrier 1 [12]. It is not yet known whether and how succinylation affects the activity of these proteins.

Succinate accumulates during ischaemia *in vivo* in a mouse model of ischaemia-reperfusion (IR), suggesting that it may have a pathological role in IR. Succinate drives the production of mitochondrial ROS after reperfusion, as demonstrated in a primary cardiomyocyte model of IR injury. Decreasing succinate levels reduces IR injury in mouse models of stroke and heart attack [86]. The effect of succinate on mitochondrial ROS production may also occur in innate immune cells, representing another possible mechanism underlying the metabolic switch in these cells.

Clearly, succinate is an important signaling molecule in innate immune cells, and may also represent a therapeutic target in a variety of pathologies.

### M2 macrophages have a different metabolic profile compared to M1 macrophages

All of the above events have been shown to be critical in macrophages and DCs activated by classical stimuli such as LPS, IFN $\gamma$  and bacteria including *E. coli*. Such classically activated macrophages have been termed “M1 macrophages” and are positively associated with inflammation. Intriguingly, the so-called M2 or alternatively activated macrophages have a different metabolic profile and inflammatory phenotype [87, 88]. These two



**Figure 3** Roles of citrate and succinate in macrophage and DC activation by LPS. Metabolic intermediates of the TCA cycle, such as citrate and succinate, can act as signaling molecules in macrophages and DCs, even when TCA cycle activity is decreased. LPS increases expression of the citrate carrier, Slc25a1, which could lead to increased transport of citrate out of the mitochondria. Citrate is then metabolized to acetyl-CoA and oxaloacetate by ACLY. Acetyl-CoA is used in fatty acid synthesis and also provides acetyl groups for acetylation of histone proteins. The conversion of oxaloacetate to pyruvate generates NADPH, which serves as a substrate in both iNOS-catalyzed NO production and NADPH oxidase-catalyzed ROS generation. NO nitrosylates and inhibits components of the mitochondrial electron transport chain and thus inhibits OXPHOS. ROS can stabilize HIF1 $\alpha$ , and thus promote glycolysis and sustain transcription of the pro-inflammatory cytokine IL-1 $\beta$ . Succinate also promotes HIF-1 $\alpha$  stabilization by inhibiting PHD enzymes, which, when active, hydroxylate and increase the degradation of HIF-1 $\alpha$ . Succinate is also used to succinylate proteins, a post-translational modification with as yet unknown consequences. Sources of succinate in an LPS-activated macrophage include glutamine metabolism (anaplerosis and the GABA shunt), and possibly the glyoxylate shunt.

phenotypes are not rigid and there may in fact exist a “spectrum” of macrophage activation, involving macrophages with different metabolic and inflammatory signatures, and with different roles in host defense against various pathogens, as well as in wound healing and the resolution of inflammation [89, 90]. M2 macrophages exhibit increased flux through OXPHOS and higher expression of anti-inflammatory cytokines such as IL-10,

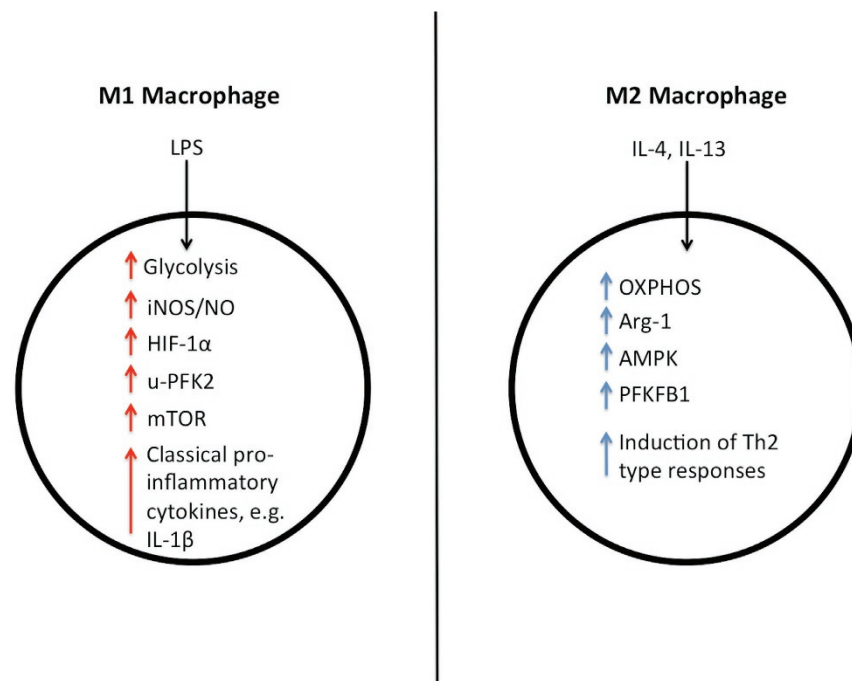
but have decreased production of NO and TNF- $\alpha$  [88]. They are involved in immune responses to parasites [91], as evidenced by their role in the induction of T<sub>H</sub>2-type responses in disease models of parasitic infection [92]. Why glycolysis is needed for M1 macrophage activation, whilst OXPHOS promotes the M2 macrophage phenotype, is not known. The cytokines IL-4 and IL-13 are key inducers of M2 macrophage polarization.



Many of the processes that drive the glycolytic switch in M1 macrophages are downregulated in M2 macrophages (Figure 4). First, arginase 1 (Arg-1) is highly expressed in M2 macrophages, while iNOS expression is decreased [93]. M1 macrophages have increased levels of citrulline and the inflammatory mediator NO, while in M2 macrophages, arginine is preferentially metabolized to urea and ornithine [94]. Ornithine can be further metabolized to polyamines and proline. Schistosome eggs induce Arg-1 expression in infected mice, and the combination of IL-4 and IL-13 increases proline production by macrophages [93]. This increased proline production could contribute to granuloma formation and liver fibrosis in infected mice, as both of these are enhanced when proline synthesis is boosted [93]. However, there is also conflicting evidence suggesting that Arg-1-expressing macrophages suppress schistosome-induced fibrosis during chronic infection [95]. Interestingly, Arg-1 can be induced in classically activated macrophages by intracellular infection with mycobacteria, and functions in this context to suppress NO production [96]. *M. tuberculosis*-infected mice lacking Arg-1 expression in macrophages are better able to clear the bacteria [96].

Secondly, IL-4 and IL-13 induce oxidative metabolism by inhibiting mTOR via activation of its upstream negative regulators TSC1 and TSC2. Macrophages lacking these negative regulators cannot switch towards OXPHOS, and *Arg-1* expression is reduced [41]. Inhibition of mTOR by rapamycin treatment or transient TSC2 expression [43] can also lead to a decrease in HIF-1 $\alpha$  levels, and therefore could result in reduced HIF-1 $\alpha$ -dependent glycolytic and inflammatory gene expression [97]. In contrast to M1 macrophages, expression of the HIF-2 $\alpha$  isoform of HIF is induced in IL-4- and IL-13-stimulated macrophages, while its levels are reduced in macrophages treated with LPS and IFN $\gamma$  [39]. HIF-2 $\alpha$ -knockout macrophages have decreased levels of Arg-1, while iNOS is unaffected [39]. Interestingly, HIF-2 $\alpha$  may still have some role in M1-like macrophages, particularly those in hypoxic conditions [98]. IL-1 $\beta$ , IL-12, and TNF- $\alpha$  levels are decreased in LPS-stimulated mice with macrophages lacking HIF-2 $\alpha$ , while IL-10 levels are increased. However, HIF-2 $\alpha$  may not have a role in induction of glycolysis and maintenance of ATP levels [98].

Thirdly, M2 macrophages predominantly express



**Figure 4** Metabolic differences between M1 and M2 macrophages. M1 macrophages rely on glycolysis for ATP production and have increased levels of iNOS, HIF-1 $\alpha$  and u-PFK2, while M2 macrophages are fueled by OXPHOS and have increased levels of Arg-1, AMPK and PFKFB1. M1 macrophages release pro-inflammatory cytokines such as IL-1 $\beta$ , while M2 macrophages are involved in the response to parasite infection, as well as in wound healing, and they release the anti-inflammatory cytokine IL-10. In fact, it is thought that a spectrum of macrophage activation exists, with different populations of macrophages exhibiting different inflammatory and metabolic phenotypes.

PFKFB1, an isoform of PFK2 [52]. PFKFB1 has higher bisphosphatase activity than u-PFK2 [55, 56], and therefore can more readily break down F-2,6-BP into fructose-6-phosphate, decreasing glycolytic activity.

Furthermore, AMPK activity is high in M2 macrophages, and drives production of the anti-inflammatory cytokine IL-10 [62]. M2 macrophages are fueled by fatty acid uptake and oxidation in the mitochondria, a metabolic program controlled by AMPK and its downstream targets, which include PPARs. IL-4 and IL-13 increase the expression of PPARs and their coactivator protein PGC1 $\beta$ , leading to increased expression of proteins of the mitochondrial respiratory chain. Knockdown of PGC1 $\beta$  antagonizes this anti-inflammatory skewing of macrophage metabolism [63]. IL-4 also enhances PPAR $\gamma$  activity via signal transducer and activator of transcription 6 (STAT6) in macrophages and DCs [99]. PPAR $\gamma$  boosts the ability of IL-4 to induce arginase expression in macrophages, and also has a role in the IL-4-stimulated increase in fatty acid  $\beta$ -oxidation in these macrophages [100]. AMPK also inhibits mTOR activity by activating TSC2 [65], as well as by directly phosphorylating the mTOR complex subunit Raptor [66]. Pharmacological activation of AMPK supports the view that it has anti-inflammatory activity. Treatment with the specific AMPK activator A769662 increases fatty acid oxidation and decreases activation of JNK, which is involved in inflammatory signaling [68]. In addition metformin, an AMPK activator and anti-diabetic drug, decreases inflammation in experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis [101].

There are other factors that contribute to a more M2-like phenotype in macrophages. Peritoneal macrophages lacking the transcription factor Gata6 are more like alternatively activated macrophages, expressing high levels of Arg-1 [102]. Gata6 is also involved in acetyl-CoA metabolism. Gata6-deficient macrophages have drastically reduced expression of aspartoacylase (AspA), an enzyme involved in acetyl-CoA synthesis. The decreased AspA expression does not seem to affect macrophage polarization, but may render peritoneal macrophages more susceptible to death.

Another intriguing enzyme recently implicated in macrophage polarization is the carbohydrate kinase-like protein (CARKL), a sedoheptulose kinase that catalyzes the production of sedoheptulose-7-phosphate. Its activity limits the flux into PPP, thereby decreasing levels of NADPH and glutathione [14]. Knockdown of CARKL potentiates LPS-induced cytokine production and M1 macrophage polarization, while CARKL overexpression decreases IL-6 secretion in response to LPS. Consistent with this, LPS treatment decreases CARKL levels,

while M2 macrophage polarization conditions increase CARKL levels [14].

Metabolic differences therefore appear to lie at the heart of the differing functions of M1 and M2 macrophages.

## Final remarks

Despite early observations about the metabolism of innate immune cells, it has taken some time to begin to mechanistically explain these observations and appreciate their impact on immune function. Moreover, there is vast scope for further discovery. There are many metabolites that are much less well characterized than those described here, and new ones are constantly being discovered, such as itaconic acid, discussed above [83].

Furthermore, insight into the interface between metabolism and innate immunity could provide novel tools to manipulate either of these cellular activities. This could be particularly relevant in the context of disease. For example, inflammation has a role in promoting insulin resistance and pancreatic  $\beta$ -cell dysfunction [103, 104]. The pro-inflammatory cytokine IL-1 $\beta$  induces apoptosis of  $\beta$ -cells [103] and therefore participates in the pathogenesis of type 2 diabetes mellitus [105, 106]. Conversely, metabolic pathways influence the course of immune diseases, as illustrated by the fact that the metabolic drugs metformin and AICAR, both of which can activate AMPK, can ameliorate inflammation [101, 107]. Metabolite screening could also become important in clinical diagnosis, as metabolic intermediates are often present at abnormal levels in diseased states [108].

Finally, improvements in metabolomics and other metabolism-based technologies should aid greatly in advancing the current knowledge of immunometabolism. There are wide areas of this field still to be explored, including epigenetic regulation of immune cell metabolism and inflammation [45], post-translational modifications utilizing metabolites [109-111], and the discovery of as yet unknown immunometabolic signaling pathways.

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