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Krebs Cycle Reborn in Macrophage Immunometabolism

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Abstract

A striking change has happened in the field of immunology whereby specific metabolic processes have been shown to be a critical determinant of immune cell activation. Multiple immune receptor types rewire metabolic pathways as a key part of how they promote effector functions. Perhaps surprisingly for immunologists, the Krebs cycle has emerged as the central immunometabolic hub of the macrophage. During proinflammatory macrophage activation, there is an accumulation of the Krebs cycle intermediates succinate and citrate, and the Krebs cycle–derived metabolite itaconate. These metabolites have distinct nonmetabolic signaling roles that influence inflammatory gene expression. A key bioenergetic target for the Krebs cycle, the electron transport chain, also becomes altered, generating reactive oxygen species from Complexes I and III. Similarly, alternatively activated macrophages require α-ketoglutarate-dependent epigenetic reprogramming to elicit anti-inflammatory gene expression. In this review, we discuss these advances and speculate on the possibility of targeting these events therapeutically for inflammatory diseases.

1. INTRODUCTION

The Krebs cycle: the second step of cellular respiration after glycolysis; a series of chemical reactions used by all aerobic organisms to release energy from nutrients, such as glucose

Electron transport chain (ETC): a series of complexes in the inner mitochondrial membrane that couple the transfer of electrons to the pumping of protons across the membrane

Over the last decade, immunologists have increasingly turned their attention to changes in intracellular metabolic pathways in immune cells that facilitate their effector functions. These metabolic processes go beyond a simple role in bioenergetics or biosynthesis to regulate immune cell responses during infection, autoimmunity and cancer. Excitingly, identification of the metabolic events that govern immune cell function is also presenting new therapeutic opportunities for the treatment of immune and inflammatory diseases.

Substantial advances have now been made in the elucidation of metabolic changes occurring during macrophage activation. Macrophages are myeloid-derived innate immune cells ubiquitously distributed throughout the human body and are essential for the maintenance of biological homeostasis in the face of infection or tissue damage. To ensure homeostasis, they possess an intricately evolved system of germline-encoded pattern recognition receptors (PRRs) that facilitate the sensing of specific microbial compounds, termed pattern-associated molecular patterns (PAMPs) or products of damaged tissue, termed danger-associated molecular patterns (DAMPs) (1, 2). The sensing of these products activates downstream signaling cascades that we now know reprogram cellular metabolism in a manner dependent on the nature of the stimulus. One such stimulus employed to mimic bacterial infection and activate macrophages in vitro is lipopolysaccharide (LPS), an important component of gram-negative bacteria outer membranes (3). LPS engages Toll-like receptor 4 (TLR4), a PRR belonging to the TLR family, to elicit the global rewiring of several major metabolic pathways to govern macrophage phagocytic capabilities, enhance bactericidal killing, and regulate the production of cytokines and chemokines. While engagement of PRRs induces a classical proinflammatory polarization state (M[LPS]), macrophages can also undergo alternative activation to a more anti-inflammatory state (4). This alternatively-activated state is usually induced by IL-4 stimulation (M[IL-4]) in vitro and is also essential in maintaining homeostasis in vivo through tissue remodeling, wound repair, and the resolution of inflammation (4). Similar to M[LPS], metabolic reprogramming of alternatively activated macrophages underlies their ability to execute their specific functions (4).

While there are a growing number of metabolic processes implicated in both pro- and anti-inflammatory macrophage activation, one key pathway has emerged as a central regulator and is largely responsible for directing intracellular metabolic adaptation and initiating signaling events that influence key effector outputs. Perhaps a surprise to immunologists, this pathway is the Krebs cycle, also known as the tricarboxylic acid (TCA) cycle or the citric acid cycle. The Krebs cycle was initially discovered as the final common pathway for the complete oxidation of fuel molecules. However, it is now evident that key Krebs cycle intermediates and their derivatives accumulate in macrophages and have distinct nonmetabolic signaling capacities to dictate immune cell activation. This review gives a detailed overview of how the Krebs cycle and oxidative phosphorylation (OXPHOS) are repurposed upon macrophage activation and explores how our preconceived notions of these ancient pathways as simply a means to generate energy and biological macromolecules must be revised. It is now clear that the Krebs cycle metabolites succinate, α-ketoglutarate, and citrate, and the Krebs cycle–derived metabolite itaconate regulate inflammatory gene expression. Equally, the electron transport chain (ETC), also known as the respiratory chain, has an important role in signaling processes during macrophage activation. Immunometabolism has therefore entered a new era whereby critical signaling functions can be assigned to metabolites whose precise role in bioenergetics, while important, has been eclipsed by a determining role in immunity and inflammation.

2. THE KREBS CYCLE AND OXIDATIVE PHOSPHORYLATION

Most of the energy-rich adenosine triphosphate (ATP) generated in metabolism is provided by the aerobic processing of key fuel molecules and starts with the complete oxidation of their derivatives to CO_2 (5–7). This oxidation takes place in a series of chemical reactions first described by Hans Adolf Krebs in 1937, a metabolic cycle that now bears his name (5). In eukaryotic organisms, the reactions of the Krebs cycle take place inside the mitochondrial matrix, whereby its primary function is the harvesting of high-energy electrons from carbon fuel sources (6). It is a truly remarkable process that begins with the energy in sunlight in the form of photons being captured in carbohydrates in plants during photosynthesis (8). Electrons from carbohydrates are then captured in the Krebs cycle, which then creates a proton gradient used to drive ATP production: photons to electrons to protons to ATP. Indeed, the Krebs cycle is the final common pathway for the oxidation of not only carbohydrates but also fatty acids and amino acids, with most entering the cycle as acetyl coenzyme A (acetyl-CoA) (6). As such, it is the gateway to the aerobic metabolism of any molecule that can be transformed into an acetyl group or an intermediate of the cycle itself. Importantly, the Krebs cycle is also a source of precursors for the building blocks of many other biological molecules, such as nonessential amino acids, nucleotide bases, and porphyrin (6). For example, oxaloacetate is an intermediate for the synthesis of glucose from noncarbohydrate precursors, a process termed gluconeogenesis (6). Likewise, citrate provides essential carbon units for the synthesis of fatty acids, a process termed lipogenesis (6). This orchestrated removal of Krebs cycle intermediates to feed biosynthetic pathways is termed cataplerosis, whereas the diversion of nutrients to replenish Krebs cycle intermediates, such as glutaminolysis, is termed anaplerosis. As such, the Krebs cycle is the central metabolic hub of the cell acting as an important nexus for the integration of many anabolic (gluconeogenesis and lipid synthesis) and catabolic (glycolysis and β-oxidation) pathways. It is in fact the central biochemical process in eukaryotic life.

The oxidation of acetyl-CoA to CO_2 by the Krebs cycle is the key process in energy metabolism because it is essential for the generation of the high-transfer-potential electron carriers nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) (6). These redox metabolites once generated then act as shuttles for the transfer of high-energy electrons to O_2, the terminal electron acceptor in the oxidation of carbon, via a series of electron carriers collectively termed the ETC (9, 10). The famous Albert Szent-Gyorgyi, who is credited with co-discovering the Krebs cycle, once defined life as being "nothing but an electron looking for a place to rest" (11, p. 23). He would be amazed that this process has been co-opted for signaling by the immune system. In one turn, the Krebs cycle oxidizes two-carbon units transferring three hydride ions to three molecules of NAD^+ and one pair of hydrogen atoms to one molecule of FAD, thus producing two molecules of CO_2 and one molecule of guanosine triphosphate (GTP) or ATP. In total, eight reactions are required (**Figure 1**).

The Krebs cycle begins with the condensation of oxaloacetate and acetyl-CoA to form citryl-CoA, which is then hydrolyzed to yield citrate and CoA. This first reaction is catalyzed by citrate synthase (12). The next reaction involves the isomerization of citrate to isocitrate and is catalyzed by aconitase (13). This requires two reactions; the dehydration of citrate to *cis*-aconitate and the rehydration of *cis*-aconitate to isocitrate (13). The third step of the cycle is catalyzed by isocitrate dehydrogenase (IDH) and is the first of four oxidation-reduction reactions (14). Here, IDH converts isocitrate to α-ketoglutarate via an oxalosuccinate intermediate, which yields the first molecule of NADH while one carbon unit is lost in the form of CO_2 (14). The formation of α-ketoglutarate is an important rate-determining step of the cycle as IDH can be allosterically activated (citrate, ADP) or inhibited (ATP), a form of regulation determined by the energy status of the cell (14). In the fourth step of the cycle, the α-ketoglutarate/oxoglutarate dehydrogenase

Gluconeogenesis: metabolic pathway that synthesizes glucose from noncarbohydrate precursors

Lipogenesis: metabolic process by which acetyl-CoA is converted to triglycerides for fat storage

Cataplerosis: removal of intermediary metabolites to prevent their accumulation in the mitochondrial matrix and for biosynthetic and other purposes

Glutaminolysis: metabolic pathway by which glutamine is converted to glutamate and subsequently transformed into α-ketoglutarate, an intermediate of the TCA cycle

Anaplerosis: the act of replenishing intermediary metabolites (especially Krebs cycle intermediates) that have been extracted for biosynthetic or other purposes

Glycolysis: metabolic pathway that oxidizes glucose to pyruvate and ATP

β-Oxidation: metabolic pathway by which stored fatty acids are broken down to form acetyl-CoA

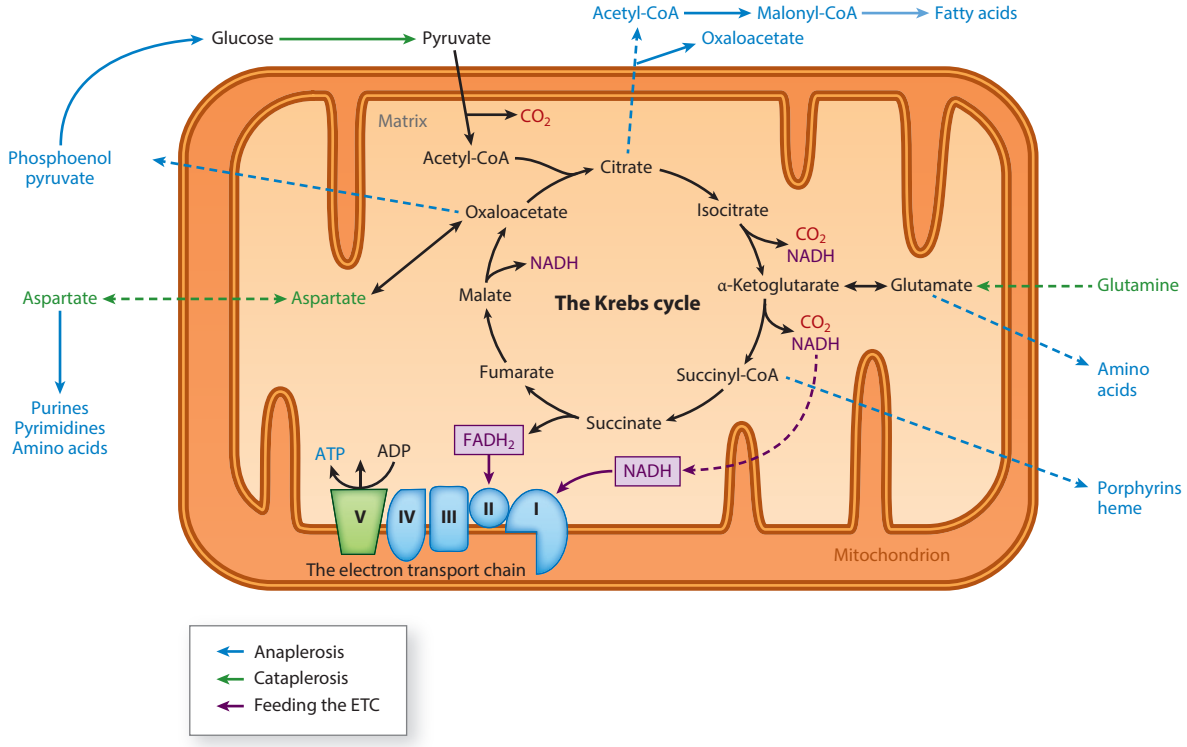

Figure 1

The Krebs cycle and oxidative phosphorylation. The Krebs cycle is presented demonstrating the eight reactions required for one full turn and all five complexes required for oxidative phosphorylation. Some anaplerotic (*blue*) and cataplerotic (*green*) sequences feeding the Krebs cycle are also shown. Key anaplerotic pathways indicated include the generation of phosphoenol pyruvate from oxaloacetate, which can then feed gluconeogenesis to synthesize glucose and citrate export from the mitochondria, where it can be converted into acetyl-CoA and malonyl-CoA to feed fatty acid synthesis (de novo lipogenesis). The synthesis of porphyrins and heme from succinyl-CoA, purines, and pyrimidines required for DNA synthesis from oxaloacetate and finally the synthesis of nonessential amino acids from oxaloacetate and α-ketoglutarate are also shown. Key cataplerotic pathways indicated include glucose feeding pyruvate (glycolysis), which can then enter the Krebs cycle as acetyl-CoA, aspartate feeding oxaloacetate, and glutamine feeding α-ketoglutarate. One full turn of the Krebs cycle generates two molecules of CO_2 (*red*) and three molecules of nicotinamide adenine dinucleotide (NADH), which are used directly by complex I, and one molecule of flavin adenine dinucleotide ($FADH_2$) (*purple*), which is used by complex II. Other abbreviation: ETC, electron transport chain.

complex (OGDC) converts α-ketoglutarate to succinyl-CoA, and this yields the second molecule of NADH while another carbon unit is lost as CO_2 (15). In the fifth reaction, catalyzed by succinyl-CoA synthetase, cleavage of the thioester bond of succinyl-CoA is coupled to the phosphorylation of a purine nucleoside diphosphate (usually GDP but also ADP) and the formation of succinate (16).

Reactions of four-carbon compounds constitute the final stage (composed of three reactions) of the Krebs cycle: the regeneration of oxaloacetate. The first step in the regeneration of oxaloacetate (and the sixth of the cycle) is the oxidation of succinate to fumarate by succinate dehydrogenase (SDH), also referred to as complex II of the ETC (17). This also generates $FADH_2$, and electrons are then transferred from $FADH_2$ to coenzyme Q (CoQ) (17). The seventh step of the cycle is the stereospecific hydration of fumarate to L-malate by fumarate hydratase (18). Finally, the eighth (and last) step of the Krebs cycle is the oxidation of malate to oxaloacetate by malate dehydrogenase

and the reduction of NAD^+ to form the third and final molecule of NADH (19). And so, one full turn of the cycle is complete. The key outputs of the Krebs cycle from a biochemical point of view are the 3 NADH molecules and the $FADH_2$ that will feed the ETC (**Figure 1**). As we shall see, however, these intermediates in the Krebs cycle are also used as signals during macrophage activation.

The main purpose of the ETC is to generate ATP, which then serves as the principal immediate donor of free energy in biological systems. In a typical cell, a molecule of ATP is consumed within a minute of its formation, while a typical human consumes the equivalent of 65 kg of ATP at rest over a period of 24 h (20). However, the total quantity of ATP in the body is limited to approximately 100 g; thus, the turnover of this small quantity of ATP is very high. Strikingly, each molecule of ATP is recycled between 1,000 to 1,500 times per day. To achieve this high degree of turnover, a sophisticated combination of enzyme and coenzyme complexes (ETC) work in tandem to synthesize ATP from ADP and inorganic phosphate, in a process termed oxidative phosphorylation (OXPHOS) (**Figure 2*b***) (9, 10). The transfer of electrons from NADH and $FADH_2$ to O_2 occurs at the inner mitochondrial membrane (IMM), the location of the ETC, and leads to the pumping of protons (H^+) out of the mitochondrial matrix into the intermembrane space (9, 10). This unequal distribution of protons generates a pH gradient and transmembrane electrical potential (electrochemical gradient) that creates a proton-motive force. ATP is synthesized when protons flow back to the mitochondrial matrix (9, 10). As such, the electron motive force generated by the Krebs cycle is converted to a proton motive force and then into phosphoryl transfer potential (9, 10). The conversion of the electron motive force to the proton motive force is achieved by the respiratory chain, which consists of three electron-driven proton pumps—NADH-Q oxidoreductase (complex I), Q-cytochrome *c* oxidoreductase (complex III), and cytochrome *c* oxidase (complex IV) (9, 10). Special electron carriers are also involved in ferrying the electrons from one complex to the next. The first of these is CoQ, also referred to as ubiquinone because it is a hydrophobic ubiquitous quinone that diffuses rapidly within the IMM and shuttles electrons between complex I and complex III (9, 10). SDH (complex II) also interacts and reduces CoQ to generate the proton motive force, although it is not a proton pump itself, and unlike other Krebs cycle enzymes, it directly associates with the IMM and the ETC (17). SDH therefore provides an integral physical link between the Krebs cycle and ATP formation. The second of the special electron carriers is cytochrome *c*, a small soluble protein that shuttles electrons from complex III to complex IV, the final proton pump of the chain and the site of O_2 reduction to H_2O (9, 10). The final phase of OXPHOS is carried out by the F_0F_1-ATP synthase, an ATP-synthesizing complex driven by the flow of protons back into the mitochondrial matrix, thus converting the proton motive force to phosphoryl transfer potential (converting ADP to ATP) (9, 10). OXPHOS is a vivid demonstration that proton gradients are an incontrovertible currency of free energy in biological systems. As we will see below, both the electrochemical proton gradient and the ETC can be also harnessed for signaling purposes during macrophage activation, largely via the generation of reactive oxygen species (ROS).

3. KREBS CYCLE REWIRING

As the Krebs cycle is an amphibolic pathway, meaning it participates in anabolic and catabolic processes, the diversion of nutrients to replenish Krebs cycle intermediates, termed anaplerosis, and the removal of intermediates to feed biosynthetic pathways, termed cataplerosis, is necessary to ensure functionality of the cycle and to support important cellular processes (6) (**Figure 1**). Anaplerosis and cataplerosis describe reciprocal and correlative reactions that can be dictated by changes in the microenvironment of the cell, such as hypoxia, or in response to extracellular

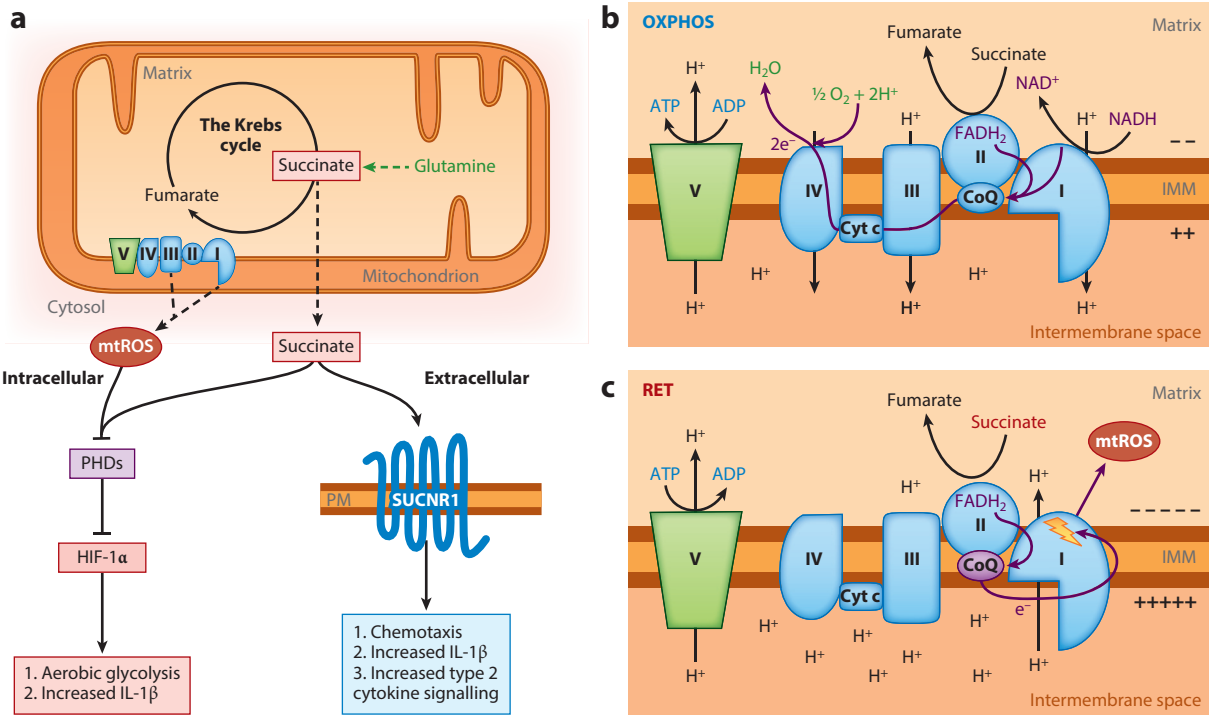

Figure 2

Succinate and mitochondrial reactive oxygen species (mtROS) as signals during macrophage activation. Succinate accumulation in pro-inflammatory macrophages acts via three primary mechanisms to regulate IL-1β production. (*a*) Firstly, succinate, derived in part from glutamine anaplerosis, can be exported from mitochondria into the cytosol where it directly inhibits prolyl hydroxylase (PHD) activity to stabilize hypoxia-inducible factor-1α (HIF-1α). (*b*) Secondly, lipopolysaccharide reprograms oxidative phosphorylation (OXPHOS), which enables succinate oxidation by complex II to drive reverse electron transport (RET). (*c*) RET promotes mtROS production from complex I, which inhibits PHD activity and stabilizes HIF-1α. The repurposing of OXPHOS to RET is aided by glycolytic adenosine triphosphate (ATP), complex V, and inner mitochondrial membrane (IMM) hyperpolarization. Thirdly, succinate can also be secreted from the cell where it can bind succinate receptor 1 (SUCNR1), which acts as an autocrine and paracrine sensor for succinate, as part of a positive feed-forward loop to sustain IL-1β and promote chemotaxis (*a*). Extracellular succinate and SUCNR1 can also be anti-inflammatory in certain contexts by synergizing with type II cytokine signaling to promote anti-inflammatory gene expression. Other abbreviations: ADP, adenosine diphosphate; CoQ, coenzyme Q; Cyt c, cytochrome *c*; $FADH_2$, flavin adenine dinucleotide; NADH, nicotinamide adenine dinucleotide; PM, plasma membrane.

signals, such as hormones or inflammatory stimuli (6, 21). However, a concept first proposed in the 1970s (22, 23) has now reemerged whereby the Krebs cycle can undergo remodeling to support the nonmetabolic functions of its intermediates and their derivatives. This rewiring is often achieved through modulation of the expression and/or activity of specific Krebs cycle enzymes and both anaplerotic and cataplerotic sequences.

An early example of this concept comes from the study of prostate secretory epithelial cells, which have the specialized function and capability of accumulating extraordinarily high levels of citrate (24–26). The accumulated citrate is then secreted to form an important component of human prostatic fluid (semen) often reaching concentrations of up to 150 mM, an intriguing phenomenon first described in 1929 (25). This striking characteristic of prostate acinar epithelial cells is achieved by their unique ability to acquire high levels of zinc intracellularly via elevated expression of ZIP1 (SLC39A1), the major endogenous zinc uptake transporter. Increased mitochondrial

zinc then acts as a competitive inhibitor of mitochondrial aconitase, which is not a typical rate-limiting enzyme in mammalian metabolism, by specifically targeting the citrate to *cis*-aconitate reaction and truncating the Krebs cycle (24, 25). Three key anaplerotic sequences are also employed and required to support citrate accumulation. These include an increase in the expression of pyruvate dehydrogenase (PDH), to increase acetyl-CoA synthesis from pyruvate, the aspartate transporter (EAAC1), to enhance aspartate uptake, and the aspartate aminotransferase (GOT2), to increase the synthesis of oxaloacetate from aspartate (24). These anaplerotic sequences ensure the replenishment of the six-carbon units lost upon citrate secretion. Importantly, these enzymes and ZIP1 are positively regulated at the transcriptional level by the hormones testosterone and prolactin to optimize the operation of this metabolic pathway in prostate acinar epithelial cells (24, 26). Therefore, a major reproductive function of these hormones is to rewire the Krebs cycle in order to promote citrate accumulation and secretion by the prostate. Given this example, it should not come as a huge surprise that a cell type as important and phenotypically plastic as the macrophage might require a rewiring of the Krebs cycle.

3.1. Krebs Cycle Rewiring in Macrophages

Hormonal regulation of zinc accumulation, citrate production, and Krebs cycle remodeling is quite a rare example of how the Krebs cycle and its derivatives can be repurposed by environmental cues for nonmetabolic roles. However, this concept has gained real traction in recent years with the discovery that classically activated macrophages suppress OXPHOS and dramatically rewire the Krebs cycle, as identified primarily by the accumulation of succinate and itaconate (27–29) (**Figures 2** and **3**). It has now been proposed that two metabolic breakpoints occur in the Krebs cycle upon proinflammatory activation of macrophages (28, 29).

The first breakpoint occurs at IDH, due to transcriptional repression of *Idh* mRNA levels, and was first suggested by Tannahil et al. (27). In this landmark study, the authors generated a comprehensive metabolic map of LPS-activated macrophages by combining unbiased transcriptional and metabolic datasets, demonstrating that downregulation of many mitochondrial genes directly correlates with the expression profile of altered metabolites (27). This breakpoint was confirmed by Jha et al. (28, 29) using an integrated high-throughput transcriptional and metabolic profiling analysis pipeline (CoMBI-T). While the authors also observed transcriptional downregulation of *Idh* mRNA and an increase in the citrate:α-ketoglutarate ratio, they went one step further to validate this breakpoint by performing stable isotopic experiments using uniformly labeled glucose (U-^{13}C-glucose) and glutamine (U-^{13}C-glutamine) (28, 29). Consistent with the CoMBI-T results, ~20% of the total citrate pool contained glucose-derived carbon, whereas α-ketoglutarate had none (28, 29). Likewise, a loss of partially labeled α-ketoglutarate from U-^{13}C-glutamine was also observed, indicative of interrupted Krebs cycle activity (28, 29). Reverse flow through IDH was also undetectably low based on the absence of 5 carbon labeling in citrate from U-^{13}C-glutamine (28, 29). Importantly, the authors of these studies also noted that in the context of *Idh* downregulation, *cis*-aconitate is redirected toward itaconate (28, 29) and citrate toward lipid synthesis (27). Two recent studies have found that downregulation of *Idh* mRNA does not result in the downregulation of IDH protein levels (30, 31). However, IDH enzymatic activity is suppressed by NO-mediated cysteine nitrosation (30), and the inhibition of IDH activity is driven by autocrine type I interferon signaling (31), which is known to positively regulate nitric oxide synthase 2 (*Nos2*) expression in macrophages (32). As such, both type I interferon signaling and the induction of *Nos2* and NO synthesis are crucial mediators of Krebs cycle rewiring and the first metabolic breakpoint in macrophages.

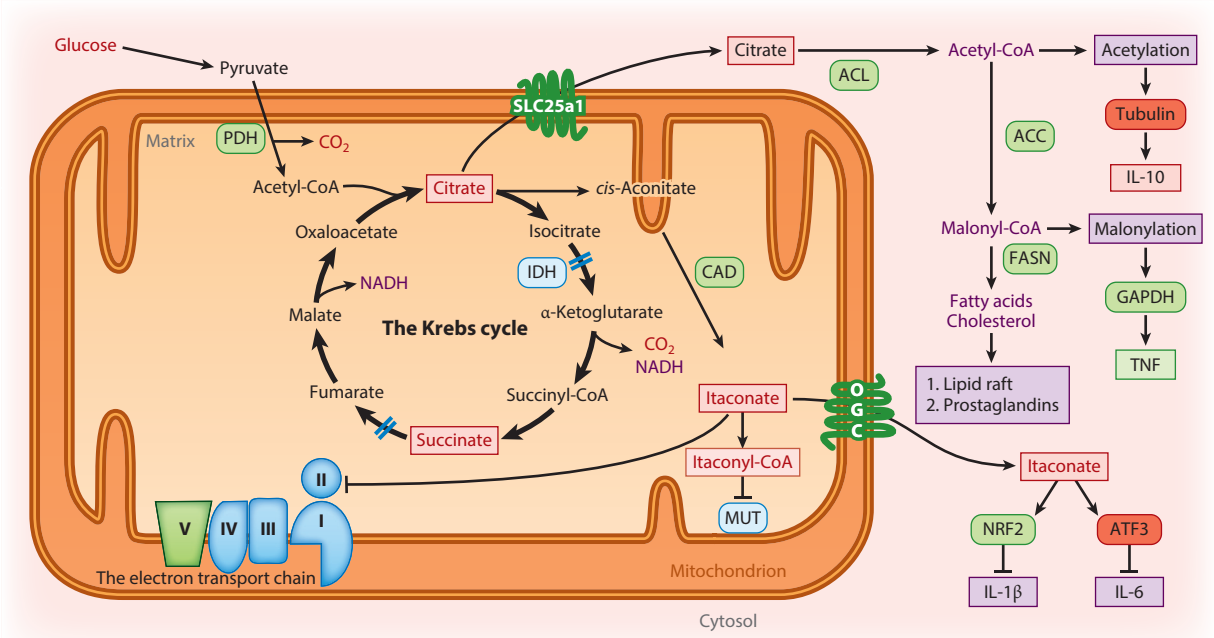

Figure 3

Citrate and itaconate as inflammatory signals during macrophage activation. In macrophages, citrate synthesized from glucose-derived pyruvate and pyruvate dehydrogenase (PDH) can be exported from mitochondria via the mitochondrial citrate carrier SLC25a1. In the cytosol, citrate can be converted to acetyl-CoA and oxaloacetate by the ATP-citrate lyase (ACL). Increased cytosolic acetyl-CoA can then be used as a cofactor by acetyltransferases, such as MEC17, which acetylates tubulin during pro-inflammatory macrophage activation to increase the anti-inflammatory cytokine IL-10. Acetyl-CoA can also be converted to malonyl-CoA via acetyl-CoA carboxylase (ACC). Similar to what occurs with acetylation, malonyl-CoA can be used to malonylate protein lysine residues, such as observed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH), or it can feed into fatty acid and cholesterol synthesis through fatty acid synthase (FASN). Malonylation is an important pro-inflammatory signal that promotes tumor necrosis factor (TNF) translation and secretion in response to lipopolysaccharide. Fatty acids and cholesterol from FASN are also essential for lipid raft formation, TLR4 signaling, and the production/secretion of cytokines and prostaglandins. In the mitochondrial matrix, citrate can be converted to itaconate, via *cis*-aconitate, by the enzyme *cis*-aconitate decarboxylase (CAD). Increased itaconate levels result in the competitive inhibition of complex II, impaired succinate oxidation, and a decrease in oxidative phosphorylation. Increased itaconate also results in elevated itaconyl-CoA levels, which inhibits the vitamin B_{12}-dependent enzyme, methylmalonyl-CoA mutase (MUT). Itaconate can also be exported from mitochondria by the oxoglutarate carrier (OGC), whereby it can stabilize the antioxidant and anti-inflammatory transcription factors NRF2 and ATF3 to decrease the pro-inflammatory cytokines IL-1β and IL-6, respectively. Other abbreviations: IDH, isocitrate dehydrogenase; NADH, nicotinamide adenine dinucleotide.

The second metabolic breakpoint in the Krebs cycle is proposed to occur at SDH (28, 29) (**Figures 2** and **3**). While no change in the expression of SDH subunits was detected, U-^{13}C-glutamine tracing experiments suggested inefficient succinate-to-fumarate transition, as ~35% of the total pool of succinate, but only 22% of the total pool of malate, contained glutamine-derived carbon (28, 29). Importantly, succinate was previously shown to accumulate to high levels in response to LPS stimulation, a process that was largely dependent on glutamine anaplerosis (27). Mechanistically, it was suggested that NO-based inhibition of SDH activity might account for impaired succinate oxidation (28), although more recent studies have identified itaconate-mediated SDH inhibition as the cause of this metabolic breakpoint (33, 34), a discovery that is discussed in detail below. Importantly, these studies nicely demonstrated how the coordination of anaplerotic/cataplerotic sequences and Krebs cycle enzyme functionality act to remodel the Krebs

cycle. Anaplerosis occurs via glutamine feeding the Krebs cycle at α-ketoglutarate, while cataplerosis includes withdrawal of citrate for lipid synthesis and *cis*-aconitate for itaconate production.

More recently, the Krebs cycle has been shown to undergo two stages of remodeling, which are the dynamic and temporal nature of this process. The first stage is characterized by the above metabolic breakpoints with succinate and itaconate transiently accumulating (35). The second, later, stage is marked by the subsistence of these metabolites, which is largely driven by the inhibition of the pyruvate dehydrogenase complex (PDHC) and the OGDC (35). PDHC is a large, highly integrated complex of three distinct enzymes that belongs to a family of homologous complexes that includes OGDC (36–38). These complexes are giant, with molecular masses ranging from 4 million to 10 million Da (36, 37). The three distinct enzymes are the pyruvate dehydrogenase component (E_1), dihydrolipoyl transacetylase (E_2), and dihydrolipoyl dehydrogenase (E_3). E_1 catalyzes the oxidative decarboxylation of pyruvate (or α-ketoglutarate in the case of OGDC), releasing CO_2 in the process (36, 37). E_2 uses lipoic acid as a catalytic cofactor (lipoamide as a prosthetic group) and catalyzes the transfer of an acetyl group to CoA (stoichiometric cofactor) (36, 37). Finally, E_3 catalyzes the regeneration of the oxidized form of lipoamide (36, 37). The core of the complex is formed by E_2 and consists of eight catalytic trimers (α_3) with a small domain at the amino terminus that contains the flexible lipoamide cofactor (36, 37). The lipoamide domain is followed by a domain that interacts with E_3 and a large transacetylase domain (36, 37). E_1 is an $\alpha_2\beta_2$ tetramer, of which there are 24 copies, while E_3 is an αβ dimer, of which there are 12 copies (36, 37). E_1 and E_3 surround the E_2 core. The key to the efficient catalysis for both PDHC and OGDC is the flexible lipoamide arm of E_2 that carries substrate from active site to active site; thus, the structural integration of three distinct enzymes and the lipoamide arm makes the coordinated catalysis of a complex reaction possible. In macrophages, inhibition of PDHC and OGDC in the second stage of remodeling is achieved by altering the lipoylation state of their E_2 subunits (35). PDHC activity is further inhibited by the phosphorylation of its E_1 subunit. As such, it can be proposed that a third metabolic breakpoint occurs in the Krebs cycle at OGDC. This study therefore elucidates a dynamic picture of Krebs cycle remodeling in proinflammatory macrophages, while uncovering key mechanistic insights into how this remodeling is achieved.

In a resting state, macrophages utilize an intact Krebs cycle and respire normally (4, 28, 39). Similarly, in an alternatively activated state, macrophages maintain robust oxidative Krebs cycle activity while increasing OXPHOS and ATP levels (28, 40) (**Figure 4**). The enhancement of Krebs cycle activity after IL-4 stimulation is largely believed to be driven by anaplerotic rewiring of glutamine metabolism (28, 40, 41). In fact, one-third of all carbons in Krebs cycle metabolites in M[IL-4] macrophages originate from glutamine, as determined by U-^{13}C-glutamine tracing (28). Glutamine deprivation is also associated with downregulated Krebs cycle activity and M[IL-4] transcriptional signature in alternatively activated macrophages (28). Furthermore, IL-4-induced PPARγ transcriptional activity is essential for alternative activation via modulation of Krebs cycle enzyme and respiratory chain gene expression and glutamine metabolism (41). Together these studies provide compelling support for a causal link between this anaplerotic sequence, Krebs cycle and OXPHOS functionality, and alternative macrophage activation. How specific Krebs cycle metabolites are generated by rewiring coordinate pro- and anti-inflammatory macrophage gene expression programs is now discussed in turn and is summarized in **Table 1**.

4. THE ROLE OF SUCCINATE AND α-KETOGLUTARATE AS INFLAMMATORY SIGNALS

Succinate has recently emerged as a key player in macrophage activation (27, 42, 43). Mechanistically, succinate has been shown to act on several pathways to exert its immunomodulatory

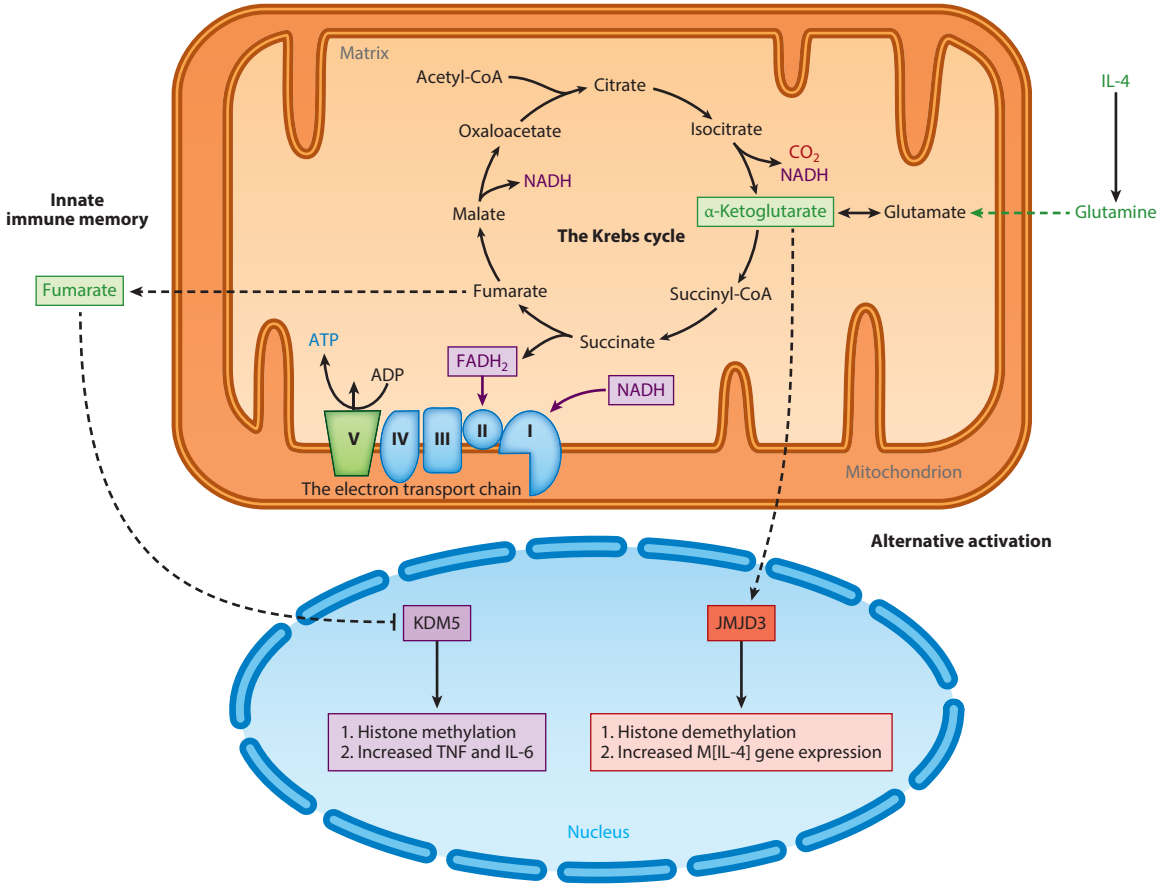

Figure 4

α-Ketoglutarate and fumarate remodel the epigenetic landscape of macrophages. Glutamine-derived α-ketoglutarate and fumarate are required for epigenome remodeling in alternatively activated macrophages and during innate immune memory, respectively. IL-4 induces glutamine anaplerosis during alternative activation to enhance oxidative phosphorylation and ATP production. This also increases α-ketoglutarate levels, which are then used as a cofactor by the histone demethylase JMJD3 to promote an anti-inflammatory M[IL-4] gene expression program. Monocytes primed with β-glucan (used to induce innate immune memory) undergo Krebs cycle rewiring, which results in fumarate accumulation derived from glutamine anaplerosis. Fumarate then acts to inhibit the KDM5 family of histone demethylases, which alters histone methylation and results in increased TNF and IL-6 production upon rechallenge with other inflammatory stimuli, such as lipopolysaccharide. Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; $FADH_2$, flavin adenine dinucleotide; JMJD3, Jumonji-C-domain-containing histone demethylase 3; NADH, nicotinamide adenine dinucleotide.

Succinylation: a posttranslational modification of protein lysine residues by the Krebs cycle intermediate succinyl-CoA

properties. Intracellularly, succinate can accumulate, undergo mitochondrial export and act to stabilize hypoxia-inducible factor-1α (HIF-1α) and/or undergo oxidation by SDH to drive mitochondrial ROS (mtROS) generation from complex I (27, 42). Dysregulated succinate metabolism can also result in the accumulation of succinyl-CoA and lysine succinylation, a recently identified posttranslational modification (PTM) (27, 44). Importantly, the balance between succinate and α-ketoglutarate levels intracellularly can also regulate members of the α-ketoglutarate-dependent dioxygenases (α-KGDDs) involved in epigenome remodeling and innate immune memory (45). A role for fumarate in this process is also discussed here (46). Extracellularly, succinate can ligate the G protein–coupled receptor (GPCR) succinate receptor 1 (SUCNR1) to elicit downstream signaling cascades and regulate macrophage effector functions (43). This section discusses in

Table 1 Overview of Krebs cycle and Krebs cycle–derived metabolite signaling

Metabolite(s)	Synthesis (enzyme)	Cellular target(s) and/or PTM in macrophage	Signaling pathway(s) and/or processes	Inflammatory outcome(s)
Succinate	Succinyl-CoA synthetase	SUCNR1 **α-KGDDs:** PHDs JMJDs	G_i- and G_q-coupled HIF-1α Stabilization Histone methylation	Increased IL-1β (69) Chemotaxis (69, 71, 72) Increased type 2 cytokine signaling (43) Increased IL-1β (27, 42) Increased proinflammatory gene expression (45)
α-Ketoglutarate	Isocitrate dehydrogenase	**α-KGDDs:** PHDs JMJDs TETs	 HIF-1α Inhibition Histone demethylation DNA demethylation	 Decreased IL-1β (27) Increased anti-inflammatory and M[IL-4] gene expression (45, 65)
Succinyl-CoA	α-Ketoglutarate dehydrogenase complex	**Succinylation:** PKM2	 HIF-1α activity	 Increased IL-1β (62)
Fumarate	Succinate dehydrogenase	**α-KGDDs:** JMJDs	 Histone methylation	 Increased TNF and IL-6 (46)
Citrate	Citrate synthase	SLC25a1	Downstream citrate metabolism	Increased ROS, NO and prostaglandins (74, 75)
Acetyl-CoA	ATP-citrate lyase	**Acetylation:** Histone H3 α-Tubulin	 Transcription p38 kinase	 Increased IL-6 (84, 85) Increased IL-10 (88)
Malonyl-CoA	Acetyl-CoA carboxylase	**Malonylation:** GAPDH	 Glycolysis, RNA binding	 Increased TNF (91)
Itaconate (and derivatives[a])	*cis*-Aconitate decarboxylase	SDH **2,3-dicarboxypropylation:** KEAP1 GSH LDHA Several others	Succinate oxidation NRF2 ATF3	Inhibits proinflammatory cytokine production (34) Inhibits IL-1β (99) Inhibits IL-6 (100) Unknown
Itaconyl-CoA	Succinyl-CoA synthetase?	MUT	Mitochondrial B_{12} metabolism	Unknown (103)

Abbreviations: α-KGGD, α-ketoglutarate-dependent dioxygenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; HIF-1α, hypoxia-inducible factor-1α; JMJD, Jumonji-C-domain-containing histone demethylase; KEAP1, kelch-like ECH-associated protein 1; LDHA, lactate dehydrogenase A; MUT, methylmalonyl-CoA mutase; PHD, prolyl hydroxylase; PKM2, pyruvate kinase M2; PTM, posttranslational modification; SDH, succinate dehydrogenase; SUCNR1, succinate receptor 1; TET, ten eleven translocation.

[a]Mainly refers to the itaconate derivatives, dimethyl itaconate and 4-octyl itaconate.

detail the current work carried out on both the intracellular and extracellular functions of succinate and α-ketoglutarate.

α-Ketoglutarate-dependent dioxygenases (α-KGDDs): nonheme, iron-containing enzymes that consume oxygen and α-ketoglutarate as cosubstrates and catalyze a wide range of oxygenation reactions

Succinate receptor (SUCNR1): a GPCR with the Krebs cycle intermediate succinate as its cognate ligand

Hypoxia-inducible factor 1 (HIF-1): a dimeric protein complex that plays an integral role in the body's response to low oxygen concentrations, or hypoxia

Prolyl hydroxylases (PHDs): a family of α-KGDDs that hydroxylate HIF-1α and promote its degradation by the 26S proteasome

4.1. Intracellular Succinate Signaling

A key discovery in the field of immunometabolism was the identification of succinate accumulation in macrophages treated with LPS and its signaling via the transcription factor HIF-1α (27) (**Figure 2*a***). HIF-1 is a highly conserved member of the basic helix-loop-helix (bHLH) family of transcriptional regulators, central in directing the response to hypoxia (47). HIF-1 was first discovered by Semenza & Wang (48) in 1992, when it was found to bind the human erythropoietin gene under hypoxic conditions. Since then, a crucial role for HIF-1 in the regulation of numerous cellular processes has emerged. Specifically, HIF-1 coordinates the expression of genes involved in cellular metabolism, angiogenesis, inflammation, erythropoiesis, and proliferation by binding hypoxia response elements (HREs) in the promoters of its target genes (47, 49). A key outcome of its activation is the orchestrating of a transcriptional switch in the metabolic phenotype of the cell from the use of OXPHOS to glycolysis (glycolytic reprogramming) (47).

During hypoxia, HIF-1 forms a heterodimeric complex consisting of an oxygen-sensitive α subunit and a constitutively active β subunit (47). Under normoxic conditions (when O_2 levels are high), HIF-1α is tightly regulated by prolyl hydroxylases (PHDs), a family of α-KGDDs (50). Enzymatically, PHD1, 2, and 3 utilize α-ketoglutarate and molecular oxygen to hydroxylate conserved proline residues in HIF-1α, generating succinate in the process (50). Hydroxylation of HIF-1α provides a recognition site for the binding of an E3 ubiquitin ligase, the tumor suppressor protein Von Hippel–Lindau (VHL) (50, 51). The binding of VHL results in the ubiquitination of HIF-1α and its subsequent degradation via the 26S proteasome (51). Under hypoxic conditions, HIF-1α degradation is prevented, enabling its translocation to the nucleus and dimerization with HIF-1β and the binding of target genes (47).

In 2005, Selak and colleagues (52) identified a novel mitochondrion-to-cytosol signaling pathway linking the mitochondrial dysfunction observed in tumors with SDH mutations and the activation of HIF-1α. Importantly, inefficient SDH activity enabled succinate to accumulate to high levels and competitively inhibit PHDs, even in the absence of VHL mutations, an important oncogenic event in tumor formation (52). As such, this represented the first identification of succinate as an oncometabolite (52). Analogous to this, Tannahill et al. (27) demonstrated that under normoxic conditions LPS-induced succinate accumulation stabilized HIF-1α in macrophages. This study also suggested that succinate behaves as a danger signal or alarmin to sustain the production of IL-1β via direct binding of succinate-induced HIF-1α to an HRE element in its promoter (27). As such, enhanced *Il1b* transcription was identified as a key inflammatory output from succinate accumulation. Furthermore, LPS-induced HIF-1α activation is essential for the glycolytic reprogramming event observed in macrophages following stimulation (27). This study was the first to identify the importance of Krebs cycle rewiring in governing macrophage metabolic remodeling and emphasized the importance of Krebs cycle intermediates as inflammatory signaling molecules.

More recently, succinate oxidation by LPS-activated macrophages was shown to inhibit PHD activity indirectly via mtROS resulting in HIF-1α stabilization and IL-1β production (42) (**Figure 2*a***). Like succinate, ROS are critical regulators of PHD enzymatic activity under normoxic conditions (53, 54). Although the physiological relevance of mtROS in HIF-1α stabilization is not without its controversy (55), normoxic mtROS production that arises from loss of SDHB from complex II has previously been suggested to inhibit PHDs by inducing nonenzymatic decarboxylation of α-ketoglutarate (54). However, PHD activity is also highly dependent on iron (Fe^{2+}) as a cofactor, and so ROS-mediated oxidation of Fe^{2+} to Fe^{3+} has also been proposed as a potential

inhibitory mechanism (53). Interestingly, Mills et al. (42) found that limiting succinate oxidation in activated macrophages with the prodrug dimethyl malonate (DMM), which releases the potent complex II inhibitor malonate, potently blocks mtROS production from complex I while repressing LPS-induced HIF-1α and IL-1β. In a reciprocal manner, succinate oxidation limits the production of the anti-inflammatory cytokines IL-10 and IL-1RA, an impairment overcome by SDH inhibition with DMM (42). However, the precise role of HIF-1α in regulating IL-10 and IL-1RA remains to be explored. In line with this study, complex I–mediated mtROS generation in macrophages has previously been shown to promote IL-1β production while limiting IL-10, a process inhibited by the antidiabetes drug metformin (56). In addition to succinate oxidation by SDH, mtROS production by complex I was dependent on increased mitochondrial membrane potential (electrochemical gradient) (42). Hyperpolarization of the IMM is thought to be driven by the hydrolysis of glycolytic ATP by the reverse activity of ATP synthase, as inhibition of hexokinase with 2-deoxyglucose (2-DG), or the F_0F_1-ATP synthase with oligomycin, is sufficient to lower membrane potential and impair LPS-induced IL-1β production (27, 42). Treatment of macrophages with the proton ionophore uncoupling agent carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), which dissipates mitochondrial membrane potential, was also sufficient to block mtROS, HIF-1α, and IL-1β levels (42).

Reverse electron transport (RET): when electrons from succinate flow backward to complex I and generate reactive oxygen species

Mills et al. (42) argue that succinate-induced mtROS production from complex I in this setting is a result of reverse electron transport (RET) (**Figure 2*c***). This process was first observed in a model of ischemia-reperfusion (IR) injury, whereby succinate accumulates during ischemia due to a reversal in SDH directionality (57). Upon reperfusion, however, SDH rapidly oxidizes succinate, resulting in excessive reduction of the CoQ pool. This forces electrons to flow backward through complex I to generate superoxide (O_2^-), an event inhibited by DMM or the complex I inhibitor rotenone (42, 57). To further support a role for RET, macrophages expressing an alternative oxidase (AOX) from *Ciona intestinalis* were employed (42). Importantly, AOX enables the oxidation of excess electrons built up in the Q pool. In agreement with RET, AOX expression in macrophages impairs LPS-induced mtROS production, HIF-1α stabilization, and IL-1β production (42). Complex I–derived mtROS, caused by deletion of the essential subunit Ndufs4, has previously been shown to promote inflammation and potentiate macrophage activation even in the absence of stimulation, highlighting the relevance of this site in ROS production (58). The importance of complex II and succinate oxidation in innate immune signaling was also further exemplified by Garaude et al. (59), who nicely demonstrated the transient remodeling of complex I–containing supercomplexes in activated macrophages, switching the relative contributions of complex I and complex II to respiration. Importantly, the authors demonstrated an impairment in the antibactericidal activity of macrophages and an inhibition of IL-1β in *Escherichia coli*–infected mice treated with DMM (59). While no direct evidence was presented, the authors suggested an impairment in RET might account for their observed phenotype (59). It is also important to note that a role for complex III–derived mtROS in regulating proinflammatory macrophage activation has recently been identified (60). As such, the dynamics of mtROS production and the relative contributions of RET, complex I, and complex III to this process will require further investigation. Taken together, these observations highlight an essential role for the ETC and mtROS signaling in the regulation of macrophage activation and inflammation.

4.2. Succinylation and Inflammatory Signaling

As mentioned, dysregulated succinate metabolism and succinyl-CoA accumulation can result in lysine succinylation, such as that observed in LPS-activated macrophages (27). This modification induces a 100-Da change in mass and masks the positive charge of the lysine side chain,

likely resulting in a significant conformational change in the target protein (44). Succinylation is largely thought to occur nonenzymatically; however, mitochondrion-localized SIRT5 is a known desuccinylase that can enzymatically remove succinyl groups and regulate signaling processes (61). Interestingly, succinylation is known to regulate macrophage function in response to LPS. Specifically, hypersuccinylation of lysine 311 on pyruvate kinase M2 (PKM2), a key glycolytic enzyme previously known to interact with HIF-1α and regulate aerobic glycolysis in macrophages, inhibits its pyruvate kinase activity by promoting its tetramer-to-dimer transition (62, 63). Importantly, Wang et al. (62) showed that SIRT5 desuccinylates PKM2 to prevent its entry into the nucleus, the formation of a HIF-1α/PKM2 complex, and IL-1β induction. Furthermore, the authors showed that SIRT5-deficient mice are more susceptible to dextran sodium sulfate (DSS)-induced colitis and display elevated levels of IL-1β in vivo. This study, coupled with the identification of a plethora of substrates that constitute the succinylome, suggests this PTM could have far-reaching consequences in altering protein function and as a signal in innate immunity.

Jumonji-C-domain-containing histone demethylases (JMJDs): a family of α-KGDDs involved in histone demethylation

Ten eleven translocases (TETs): a family of α-KGDDs involved in DNA demethylation

4.3. Succinate, α-Ketoglutarate, Fumarate, and Epigenetic Reprogramming

In addition to the PHDs, α-ketoglutarate is an important cofactor for the α-KGDD family of Jumonji-C-domain-containing histone demethylases (JMJDs), involved in histone demethylation, and the ten eleven translocation (TET) family of 5mC hydroxylases, which play a role in DNA demethylation (64). Like the PHDs, these enzymes can undergo competitive inhibition by succinate, and so the ratio of α-ketoglutarate to succinate can often be a key determinant of their enzymatic activity, thus enabling them to remodel the epigenome (45). As previously mentioned, glutamine anaplerosis of the Krebs cycle represents an important metabolic module governing the alternative activation of macrophages in response to IL-4 (28). Liu et al. (45) took this observation further when they uncovered an important signaling role for glutamine-derived α-ketoglutarate, which facilitates an IL-4-induced gene expression program via JMJD3-driven epigenetic changes (**Figure 4**). Furthermore, the authors found that α-ketoglutarate suppresses the classical activation of macrophages (as a low α-ketoglutarate/succinate ratio strengthens the proinflammatory phenotype) and supports endotoxin tolerance after classical activation (45). Recently, melatonin was shown to alleviate inflammation associated with obesity in part by increasing the ratio of anti- to proinflammatory adipose tissue macrophages (ATMs) (65). Mechanistically, this was achieved by the targeting of ATMs with adipocyte-derived exosomal α-ketoglutarate and TET-mediated DNA demethylation (65).

Epigenetic remodeling has also been implicated as an important mechanism governing innate immune memory (also known as trained immunity) (66, 67). Innate immune memory is an emerging concept in immunology describing a change in the reactivity of immune cells previously exposed to various inflammatory stimuli. This change in reactivity allows macrophages to respond more robustly to subsequent, but potentially unrelated, insults. At the molecular level, stimuli, such as the fungal wall component β-glucan from *Candida albicans*, alter histone methylation patterns that persist after resolution of the infection and enhance cytokine production upon reinfection (66). Like LPS, β-glucan induces Krebs cycle rewiring and glutamine anaplerosis in human monocytes, which acts to increase intracellular fumarate levels (46). Fumarate, like succinate, is a competitive inhibitor of the JMJD and TET families of α-KGDDs that can modulate the epigenome (46). Importantly, Arts et al. (46) showed that glutamine-derived fumarate acts to inhibit the KDM5 family of histone demethylases after β-glucan training (**Figure 4**). Inhibition of these demethylases subsequently increases the levels of H3K4me3, a marker of active gene transcription, at the promoters of the proinflammatory cytokines TNF and IL-6, boosting their production upon restimulation with LPS (46). As such, the balancing of succinate, α-ketoglutarate,

and fumarate levels in macrophages can have profound functional consequences for macrophage polarization states and innate immune memory by altering the epigenetic landscape of the cell.

4.4. Extracellular Succinate Signaling

In 2004, He and colleagues (68) discovered unexpectedly that the Krebs cycle intermediates succinate and α-ketoglutarate are the cognate ligands for the orphan GPCRs GPR91 (also SUCNR1) and GPR99 (also OXGR1), respectively. Furthermore, SUCNR1 was found to be a G_i- and G_q-coupled GPCR highly expressed in the kidney, liver, spleen, and small intestine (68). Importantly, the authors demonstrated that succinate acts as a hypertensive agent to increase blood pressure in animals, in part through SUCNR1 and the renin-angiotensin system (68). Thus, by acting as a ligand for SUCNR1, succinate was found to have an unexpected signaling function independent of its role in bioenergetics (68). Since this seminal discovery, subsequent studies have found SUCNR1 to be expressed on the plasma membrane of macrophages, with important consequences for their function during a variety of inflammatory contexts (43, 69) (**Figure 2*a***).

In a recent study, Littlewood-Evans et al. (69) demonstrated that macrophages release succinate into the extracellular milieu when activated, while simultaneously upregulating SUCNR1. Intriguingly, SUCNR1 then acts as an autocrine and paracrine sensor for extracellular succinate to augment IL-β production, which in turn increases SUCNR1 levels, fueling this cycle of cytokine production (69). SUCNR1-deficient mice also show reduced macrophage activation and production of IL-1β in response to LPS and in a model of antigen-induced arthritis (69). Treatment of macrophages with synovial fluid from rheumatoid arthritis patients, which is abundant in succinate (70), elicits IL-1β release, suggesting that chronically elevated succinate is pathological in this setting (69). Succinate levels are also elevated in other chronic inflammatory settings, such as in mouse adipose tissue during models of diet-induced obesity or in the plasma of patients with type 2 diabetes (43, 71). Interestingly, SUCNR1-deficient mice were reported to have significantly reduced numbers of ATMs compared to adipocytes and improved glucose tolerance compared with wild-type mice fed a high-fat diet, owing in part to a reduction in macrophage chemotaxis (71). Succinate-induced chemotaxis has also been implicated in the pathogenesis of chronic neuroinflammation during experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis (MS) (72). Specifically, infiltration of damaging mononuclear phagocytes (MP) is decreased via the transplantation of neural stem cells (NSCs), which scavenge succinate from the cerebrospinal fluid (72). Mechanistically, inflammatory phagocytes release succinate that ligates SUCNR1 on the surface of NSCs to upregulate the expression of the dicarboxylate cotransporters SLC13a3 and SLC13a5, and induce the secretion of the anti-inflammatory lipid mediator prostaglandin E2 (PGE2) (72). Upregulation of solute carrier family 13 member 3 (SLC13a3) and SLC13a5 scavenges succinate away from inflammatory macrophages and microglial cells, preventing further infiltration and secondary CNS damage (72). These findings suggest that SUCNR1-induced chemotaxis promotes the infiltration of macrophages into various tissues during chronic inflammatory settings and presents itself as a promising target to combat inflammatory disease.

In contrast to these studies, Keiran et al. (43) have found that activation of SUCNR1 has a critical role in the anti-inflammatory response in macrophages during alternative activation and obesity. Specifically, SUCNR1 expression was increased in M[IL-4]-polarized macrophages and decreased in M[LPS]-polarized macrophages, in contrast to reports by Littlewood-Evans and colleagues (69), while succinate-SUCNR1 signaling promoted an anti-inflammatory phenotype by augmenting their response to type 2 cytokines (43). Transgenic mice that had SUCNR1 deleted specifically from the myeloid lineage also displayed signs of tissue inflammation and glucose intolerance when fed a normal chow diet, while the metabolic consequences of obesity,

such as insulin insensitivity, were also exacerbated (43). Furthermore, myeloid-specific deletion of SUCNR1 impaired adipose-tissue browning in response to cold exposure. As such, an unexpected role for succinate in limiting inflammation during obesity was identified. Whether extracellular succinate is proinflammatory or anti-inflammatory during inflammation could therefore be context dependent.

cis-aconitate decarboxylase (CAD): metabolic enzyme that catalyzes the synthesis of itaconate from *cis*-aconitate

Immunoresponsive gene 1 (IRG1): metabolic enzyme that catalyzes the synthesis of itaconate from *cis*-aconitate

5. THE ROLE OF CITRATE AND ITACONATE AS INFLAMMATORY SIGNALS

The last set of metabolites we consider within the context of Krebs cycle rewiring in macrophages are citrate and itaconate, which are synthesized in the mitochondrial matrix by citrate synthase (12) and *cis*-aconitate decarboxylase (CAD) (73), also known as immunoresponsive gene 1 (IRG1), respectively. Intriguing roles for these metabolites have been reported in macrophage function and are discussed below.

5.1. Extramitochondrial Citrate Metabolism and Inflammation

In a manner like that observed in prostate secretory epithelial cells, a key functional outcome of Krebs cycle rewiring in macrophages is to facilitate citrate synthesis and export from mitochondria (74, 75). To achieve this, proinflammatory macrophages sustain pyruvate oxidation through PDH and increase glutamine uptake and anaplerosis, as demonstrated using stable-isotope-assisted metabolomics (76). This reprogramming event is quite surprising given that HIF-1α stabilization can also result in the induction of pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates and inhibits PDH enzymatic activity in other contexts, such as during tumor hypoxia (76, 77). In macrophages, however, PDK1 expression is repressed by LPS stimulation via an as yet unidentified mechanism, thus enabling glucose-derived citrate synthesis (76). In addition to sustaining PDH activity, LPS increases the expression of the mitochondrial citrate carrier, SLC25a1, in an NF-κB-dependent manner (74). SLC25a1 can then catalyze the exchange of mitochondrial citrate for cytosolic malate, leading to an efflux of citrate from the mitochondria and its accumulation in the cytosol (74) (**Figure 3**). Functionally, inhibition of SLC25a1 in activated macrophages with the inhibitor 1,2,3-benzentricarboxylic acid (BTA) or through genetic silencing, leads to a marked reduction in ROS, NO, and prostaglandin production (74). This suggests that the efflux of citrate from mitochondria is an essential proinflammatory signal during macrophage activation.

In the cytosol, citrate can be converted to acetyl-CoA and oxaloacetate by ATP-citrate lyase (ACL) (75, 78) (**Figure 3**). Like SLC25a1, ACL is transcriptionally upregulated in response to LPS and functionally required for ROS, NO, and prostaglandin synthesis in activated macrophages (75). Although the precise mechanism by which citrate regulates these processes is unknown, acetyl-CoA produced in this manner is essential for de novo lipogenesis and as a cofactor for acetyltransferases, a family of enzymes that catalyze the transfer of acetyl groups to protein lysine residues (76, 78). Acetyl-CoA can also be converted to malonyl-CoA via acetyl-CoA carboxylase (ACC), which is then used by fatty acid synthase (FASN), along with acetyl-CoA, as the primary substrates for palmitate synthesis (79). Importantly, FASN has recently been identified as a key regulator of classical macrophage activation (79). Firstly, during diet-induced obesity, deletion of FASN prevents macrophage adhesion, migration, and activation in vivo by altering plasma membrane order and composition, primarily by reducing cholesterol retention, and impairing RHO GTPase trafficking (80). Secondly, FASN-derived acetoacetyl-CoA, synthesized from the condensation of acetyl-CoA and malonyl-CoA, was unexpectedly required for endogenous cholesterol synthesis and enabled TLR4 recruitment to lipid rafts in response to LPS stimulation (79). TLR4 recruitment to lipid rafts is an important cellular event that facilitates signal transduction and

proinflammatory macrophage activation during infection (79). Furthermore, C13*orf*31 (FAMIN), a recently identified protein that forms a complex with FASN on peroxisomes and promotes de novo lipogenesis, was also found to regulate ROS and cytokine production in LPS-activated macrophages (81). As such, inhibition of SLC25a1, ACL, and downstream citrate metabolism may modulate ROS, cytokine, and prostaglandin levels by impairing de novo lipogenesis, TLR4 receptor signaling, and NF-κB activation.

Malonylation: a posttranslational modification of protein lysine residues by malonyl-CoA, which is derived from the Krebs cycle intermediate citrate

5.2. Acetylation and Inflammatory Signaling

Acetylation can also modulate macrophage function. ACL-derived acetyl-CoA has been shown to drive histone acetylation, a process that can have profound effects on cellular function (78, 82, 83). Importantly, histone acetyltransferases (HATs) can play a major role in the epigenetic regulation of gene expression by modifying chromatin structure (82). Specifically, acetylation of lysine residues on H3 is associated with the expression of IL-6 in virus-infected (84) and paraquat (PQ)-treated (85) macrophages, MMP-1 and MMP-3 secretion in *Mycobacterium tuberculosis*–infected macrophages (86), and elevated IL-12p40 promoter activity (87). Despite this, there are only limited reports on the involvement of HATs in specific gene activation during macrophage activation, and this remains an open area of investigation. Acetylation can also regulate the function of proteins outside of histone modifications (88). Specifically, acetylation of the cytoskeletal protein α-tubulin, by the tubulin acetyltransferase MEC17, was shown to regulate IL-10 induction in LPS-activated macrophages (88) (**Figure 3**). As such, acetylation can serve as an important signal to govern both pro- and anti-inflammatory cytokine production in activated macrophages.

5.3. Malonylation and Inflammatory Signaling

Like succinyl-CoA and acetyl-CoA, malonyl-CoA can also modify protein lysine residues, in a process termed malonylation. Protein lysine malonylation is a highly conserved protein modification; however, it remains largely understudied (89). In two models of type 2 diabetes, proteomic analysis of liver tissue was used to identify 573 malonylated lysine sites from 268 proteins, many of which were metabolic enzymes involved in glycolysis, e.g., glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and lipid metabolism, e.g., ATP-citrate lyase (ACLY) (90). Recently, Galván-Peña et al. (91) showed an increase in the levels of malonyl-CoA and protein malonylation in activated macrophages. One of the targets identified also included GAPDH. In addition to its role in glycolysis, GAPDH also acts as an RNA-binding protein to posttranscriptionally regulate mRNAs containing ARE elements in their 3′ untranslated region (91, 92). In macrophages, GAPDH is known to posttranscriptionally repress TNF translation; however, the signals that govern this process remain unclear (91, 92). Interestingly, Galván-Peña and colleagues (91) showed that upon LPS stimulation GAPDH undergoes malonylation on lysine 213, leading to its dissociation from *TNF* mRNA and promoting its translation (**Figure 3**). Furthermore, malonylation of GAPDH was also shown to increase its enzymatic activity and engagement in the glycolytic pathway (91). As such, malonylation was shown for the first time to be an important inflammatory signal in macrophages regulating both metabolic reprogramming and cytokine production.

5.4. Itaconate as an Immunomodulatory Metabolite

In addition to citrate formation, a key functional outcome of IDH repression and PDH activity is the synthesis of the antibacterial metabolite itaconate (76) (**Figure 3**). Itaconate is synthesized from the Krebs cycle intermediate *cis*-aconitate by CAD, an enzyme whose expression is induced in response to inflammatory macrophage activation and largely restricted to cells of the

myeloid lineage (73). For decades, itaconic acid, also called methylenesuccinic acid, produced by the fungus *Aspergillus terreus* has been used in the industrial arena for the synthesis of various polymers and was not generally classified as a mammalian metabolite (93). However, in 2011 it was detected in primary murine macrophages that had been activated with LPS (93) and in the lungs of *M. tuberculosis*–infected mice (94). Following this discovery, Michelucci et al. (73) were able to demonstrate that *Irg1*, now aconitate decarboxylase 1 (*Acod1*), which is highly expressed in mammalian macrophages during inflammation, was the sought-after CAD (73). Furthermore, the authors observed a reduction in antimicrobial activity during bacterial infection upon loss of itaconate synthesis (73). While itaconic acid is a known antimicrobial metabolite that can inhibit isocitrate lyase activity and the glyoxylate shunt (see 95–97), it has recently emerged as a novel regulator of macrophage metabolism and function.

2,3-Dicarboxypropylation: a novel posttranslation modification whereby itaconate modifies protein cysteine residues and glutathione

Nuclear factor erythroid 2–related factor 2 (NRF2): the master antioxidant transcription factor that protects against oxidative damage triggered by injury or inflammation

As mentioned, itaconate has recently been identified as a novel anti-inflammatory metabolite that tempers the inflammatory response in macrophages, independent of its antibactericidal activity (34, 98–100). The immunomodulatory properties of itaconate were first observed by Lampropoulou and colleagues (34) in 2016, when they noted an increase in inflammatory cytokine production in *Acod1*-deficient macrophages. In addition, the authors were able to demonstrate an inhibition of LPS-induced IL-6, IL-1β, and IL-12p40, as well as other proinflammatory mediators, in macrophages pretreated with the cell-permeable itaconate derivative dimethyl itaconate (DMI) (34). As such, this study was the first to highlight the anti-inflammatory action of itaconate during macrophage activation.

Mechanistically, itaconate is now known to mediate these effects via modulation of macrophage metabolism (**Figure 3**). Firstly, itaconate can competitively inhibit complex II–mediated oxidation of succinate, which enables succinate accumulation in response to LPS and is thought to limit RET-induced inflammation (34). Itaconate was first identified as a competitive SDH inhibitor in 1949 (101), which prompted the initial speculation that itaconate may modulate cellular metabolism. In two independent studies, genetic deletion of *Acod1* and the exogenous addition of itaconate were sufficient to decrease and increase succinate accumulation in macrophages, respectively (33, 34). Itaconate was also shown to competitively inhibit purified SDH (34). Furthermore, the suppression of OXPHOS is lost in *Acod1*-deficient macrophages treated with LPS, and in fact an increase in oxygen consumption rates (OCRs) is observed (34). This increase in OCR is likely due to increased fueling of the Krebs cycle by glutamine in the absence of complex II inhibition. Together these papers provide compelling evidence that CAD-mediated itaconate synthesis promotes succinate accumulation and offer a molecular explanation for the second breakpoint in the TCA cycle.

Secondly, itaconate can bind and inactivate reactive thiol groups of proteins and glutathione, an irreversible nonenzymatic process termed 2,3-dicarboxypropylation (99, 100). This novel function of itaconate, which was only identified in 2018, derives from its unsaturated dicarboxylic acid structure, which makes it a mildly electrophilic alkylating agent when it accumulates to sufficient levels (99, 100). Importantly, the alkylating potential of itaconate and its derivatives has important functional consequences during the inflammatory response. Specifically, it has been proposed that itaconate is exported from mitochondria to the cytosol, whereby it alkylates E3 ubiquitin ligase substrate adaptor, kelch-like ECH-associated protein 1 (KEAP1), and depletes intracellular glutathione (GSH) levels to drive the activation of the anti-inflammatory transcription factor NRF2 (99, 100). Evidence for KEAP1 targeting, the major NRF2 repressor protein, comes primarily from the use of itaconate derivatives, namely, 4-octyl itaconate (4-OI), which has the same electrophilic properties of endogenous itaconate and has been shown to directly alkylate the key redox sensing cysteine, C151 (99). Importantly, mutation of C151 to serine (C151S) in KEAP1 impairs the ability of 4-OI to stabilize NRF2 (99). In addition to NRF2 regulation, GSH

depletion by itaconate, and its more electrophilic derivatives, activates a novel NF-kB inhibitor zeta (Iκbζ)-ATF3 inflammatory axis that regulates IL-6 expression in LPS-tolerized macrophages and LPS-activated macrophages, respectively (100). Furthermore, several other targets of 2,3-dicarboxypropylation were identified in a quantitative proteomic screen in LPS-and 4-OI-treated macrophages, including lactate dehydrogenase A (LDHA) (99). LDHA is an important regulator of aerobic glycolysis, which suggests that this novel PTM may have far-reaching consequences as an inflammatory signal during macrophage activation. In line with this, a recent study generated a more comprehensive list of itaconate and 2,3-dicarboxypropylation targets using S-glycosylation-based cysteine profiling (102). Excitingly, several targets are enzymes in the glycolytic pathway and include aldolase, GAPDH, and LDHA (102). Furthermore, the authors nicely show that 2,3-dicarboxypropylation of aldolase on C73 and C339 inhibits its enzymatic activity in macrophages (102). As such, itaconate can also mediate its anti-inflammatory effect by inhibiting aerobic glycolysis in proinflammatory macrophages.

Activating transcription factor 3 (ATF3): transcription factor induced in response to physiological stress

Itaconate accumulation has also been reported to increase itaconyl-CoA levels in macrophages (103). Intriguingly, two consequences of itaconyl-CoA accumulation are the inhibition of the mitochondrial B_{12}-dependent enzyme methylmalonyl-CoA mutase (MUT) and the abolition of substrate-level phosphorylation (SLP) (104). Inhibition of MUT by itaconyl-CoA results in B_{12} inactivation in LPS-activated macrophages and a reduction in circulating B_{12} levels in patients who present with the homozygous loss of the citramalyl-CoA ligase (CLYBL) (103). However, the consequences of dysregulated B_{12} metabolism and indeed SLP inhibition with regard to macrophage activation and inflammation remain an open area of investigation. It is also tempting to speculate that increased itaconyl-CoA levels could result in the itaconylation of protein lysine residues and thus act as an inflammatory signal akin to acetylation, malonylation, and succinylation. Itaconate has also been shown to protect mice from lethality during infection with *M. tuberculosis* (98) and to create an antiviral metabolic niche in neurons infected with Zika virus (105). While the exploration of itaconate signaling during macrophage activation (and indeed other inflammatory contexts) is still in its infancy, the profound levels to which itaconate accumulates and the findings to date suggest it is a central regulator of macrophage metabolism and function.

6. CONCLUSIONS AND PERSPECTIVES

A striking change has been happening in the field of immunology where metabolic processes are now being considered as important a determinant of immune cell function as the activation of immune cells by cytokines and PRRs. The field of immunometabolism has benefitted greatly from technological advancements and improvements in instrumentation, such as the use of mass spectrometric techniques and computational analysis, over the last 10 years (106, 107). One critical point to consider is our limited understanding of how global metabolism interacts with and influences cellular metabolism in vivo, and the availability of human data is also quite limiting. However, what has become clear is the critical role of the Krebs cycle in immune cell function outside of its role in bioenergetics or general metabolism. Specifically, prominent roles for succinate, α-ketoglutarate, citrate, and itaconate in macrophage activation have been uncovered.

Despite this, two areas that require improvement to better understand the immunomodulatory properties of the Krebs cycle and metabolic reprogramming in immune cells are organelle-specific metabolite analysis in vitro and metabolite profiling in vivo. Importantly, steps have been made in both directions. Although outside of an immune context, these studies may have utility in the study of immunometabolism. Firstly, Chen et al. (108) recently described a method for the rapid and specific isolation of mitochondria and its use in tandem with a database of predicted mitochondrial metabolites (MITObolome). Intriguingly, they were able to utilize this method and compare it to

the whole-cell metabolome across various states of respiratory chain function, revealing extensive compartmentalization of mitochondrial metabolism and signatures unique to the inhibition of each respiratory chain complex (108). Secondly, it is known that mammalian organs exchange metabolites via the circulation, but a comprehensive investigation into this has only recently been carried. Jang et al. (106) compared metabolite concentrations in arterial blood versus draining venous blood from 11 organs in the pig, thus providing a quantitative atlas of interorgan metabolite exchange that could prove fruitful in understanding how organ-specific metabolism interacts with the immune system. Thirdly, stable-isotope tracing in humans has also been used to great effect in assessing metabolic heterogeneity and Krebs cycle functionality within tumors (107) and perhaps could be adapted for use in inflammatory diseases, whereby diseased tissue could be surgically excised. As such, these strategies may be adopted to encompass other tissue and cell types, cellular organelles, and compartments in future studies. Specifically, these strategies may help us better understand the mechanics of Krebs cycle remodeling in macrophages and how this interacts with and influences cellular architecture and signaling, as well as revealing how this process unfolds in tissue-resident macrophage populations in vivo during inflammatory diseases.

Important insights into immunometabolic networks and metabolite signaling may also uncover promising therapeutic targets for autoimmune and inflammatory diseases. For example, dimethyl fumarate, a cell-permeable fumarate derivative used to treat relapsing-remitting MS and psoriasis, targets GAPDH and aerobic glycolysis to mediate its anti-inflammatory effects (109). As such, the use of a modified version of this Krebs cycle intermediate revealed a metabolic module with clinical tractability. The use of the cell-permeable itaconate derivatives, 4-OI and DMI, was also shown to be protective in murine models of sepsis (99) and psoriasis (100), respectively. Therefore, the development of small-molecule drugs based on Krebs cycle intermediates and their derivatives are promising therapeutics themselves. It is therefore inevitable that future therapeutic approaches targeting or based on the Krebs cycle will emerge. Likewise, the use of Krebs cycle metabolites as biomarkers of immune-related disease progression or status may also become integral in a clinical setting, with abnormal levels often observed in diseased tissue such as in the rheumatoid joint (69, 70) or ulcerative colitis (110). It is now clear that the Krebs cycle goes beyond its role in bioenergetics to influence cellular signaling and may serve as the ultimate determinant of macrophage function. The Krebs cycle has therefore been reborn in the macrophage as an immunometabolic hub, the continuing study of which has great potential to deepen our understanding of the role of the macrophage in health and disease.

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