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Opinion

The NLRP3 inflammasome: regulation by metabolic signals

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Macrophages undergo profound metabolic reprogramming upon sensing infectious and sterile stimuli. This metabolic shift supports and regulates essential innate immune functions, including activation of the NLRP3 inflammasome. Within distinct metabolic networks, key enzymes play pivotal roles to control flux restraining detrimental inflammasome signaling. However, depending on the metabolic cues, specific enzymes and metabolites result in inflammasome activation outcomes which contrast other metabolic steps in the pathway. We posit that understanding which metabolic steps commit to discrete inflammasome fates will broaden our understanding of metabolic checkpoints to maintain homeostasis and offer better therapeutic options in human disease.

Highlights

Glycolytic enzymes differentially regulate NLRP3 inflammasome activation.

Itaconate post-translationally modifies kelch-like ECH-associated protein 1 (KEAP1), NLRP3, and gasdermin D (GSDMD) proteins to dampen both the NLRP3 inflammasome and pyroptosis.

Succination of GSDMD and GSDME blocks pyroptosis and apoptosis, respectively.

Fatty acid synthesis and fatty acid oxidation regulate the priming and activation of the NLRP3 inflammasome.

Significance

Several metabolic pathways are instrumental in modulating myeloid cell responses to inflammation and new findings are revealing further molecular details regarding inflammasome regulation. By targeting various proteins, itaconate – a metabolite derived from the TCA cycle – is gaining significant attention because it can dampen the NLRP3 inflammasome and lower pyroptotic cell death.

Activation of the NLRP3 inflammasome, and regulation by metabolic pathways

The **inflammasome** (see Glossary) is a high-molecular-weight multiprotein complex that is assembled primarily by members of the NLR family in response to the cytosolic presence of distinct inciting stimuli [1,2]. First recognized for their role in host defense, inflammasomes play crucial roles in contributing to and often instigating the development of inflammatory, autoimmune, and metabolic diseases [3–5]. Often, the assembly of the inflammasome complex is triggered in response to specific stimuli. For example, mouse NLRP1 is activated upon proteolytic cleavage by the bacterial lethal toxin protease, thus promoting downstream host defense mechanisms. By contrast, the widely studied NLRP3 inflammasome assembles in response to a range of pathogen-associated and more frequently endogenous damage-associated molecular patterns [6]. Consequently, the formation of the NLRP3 inflammasome is tightly regulated by a two-step mechanism consisting of priming and activation signals [7,8]. Although the stepwise mechanistic details remain ambiguous, broadly, NLRP3 complexes together with the adaptor protein ASC (apoptosis-associated speck-like protein) upon activation, which in turn binds to pro-caspase-1 through interaction of the PYD-PYD and CARD-CARD domains, respectively. Consequently, caspase-1 undergoes proximity-induced self-cleavage and activation, leading to the maturation of inflammatory IL-1β and IL-18 cytokines [9,10]. Additionally, active caspase-1 processes the pore-forming protein **gasdermin D** (GSDMD), resulting in an inflammatory cell death termed **pyroptosis** [11–14]. The activating stimuli for NLRP3 include a broad range of mediators that appear during infection [1,15], tissue damage [16], or metabolic stress [17]. In particular, the NLRP3 inflammasome is uniquely regulated by metabolic pathways that upkeep homeostasis. As such, aberrations in the metabolic state have been linked to activation of the NLRP3 inflammasome. This has led to NLRP3 being proposed as a sensor of cellular homeostasis [18].

Myeloid cells, particularly macrophages, are the main cell type involved in inflammasome assembly. Ubiquitously expressed in various mammalian tissues and organs, macrophages can acquire contrasting and heterogeneous functions (for instance in the paradigm for proinflammatory M1 versus anti-inflammatory M2 macrophage phenotypes, although this nomenclature has now

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become obsolete); insights have been gained into metabolic pathway changes that occur during macrophage activation (i.e., immunometabolism) [19–21]. The metabolic pathways are particularly well characterized in M1-like proinflammatory macrophages and comprise: (i) increased aerobic **glycolysis** and pentose phosphate pathway activation; (ii) truncated mitochondrial **tricarboxylic acid cycle (TCA)** with concomitant accumulation of citrate and succinate; (iii) increased lipid metabolism; and (iv) selective amino acid uptake and usage [22]. While metabolic pathways and metabolites predominantly provide energy and substrates for cellular processes, how metabolic reprogramming instructs immune functions, including the NLRP3 inflammasome has been significantly studied and appreciated in recent years [23]. This was partly fueled by the study of metabolic diseases, which provided some of the first clues into the elaborate relationship between metabolism and the NLRP3 inflammasome [24,25]. On a cellular level, this relationship may involve changes to pathway flux by the activity of individual enzymes or an adjustment to the availability of different metabolites. In this Opinion, we propose that distinct metabolic steps commit to discrete inflammasome fates. We discuss recent findings, particularly focusing on a set of glycolytic enzymes, TCA cycle metabolites, and lipid pathways that reveal contrasting NLRP3 inflammasome outcomes. An increased understanding of this area can broaden the avenues for treating NLRP3-related disorders.

Glycolysis and the NLRP3 inflammasome

Upon acute infection or tissue damage, metabolic reprogramming similar to the Warburg effect, occurs to support phagocytic and inflammatory functions [26]. The preference by activated cells to undergo aerobic glycolysis also supplies metabolic intermediates that define the activation status of inflammatory pathways. The earliest studies established glycolytic enzymes that supported inflammasome activation, which have been recently contested [27]. Thus, understanding how the glycolytic burst regulates inflammasomes can help pave the way for establishing novel therapeutic avenues to treat certain inflammatory diseases.

Glycolytic enzymes differentially regulate the NLRP3 inflammasome

The role of glycolysis in inflammasome activation ranges from merely promoting the NLRP3 inflammasome during homeostasis, to more complex functions that have been attributed to various glycolytic enzymes. First, glycolytic enzymes independently contribute to several physiological processes. Second, the state of cells and the point at which glycolysis is disrupted are more important than previously appreciated [28]. Broadly, equilibrium within the glycolytic pathway can limit NLRP3 inflammasome activation; yet, perturbations at marked points can inhibit or promote NLRP3 activation. For instance, blockade of hexokinase 1 (HK1) and pyruvate kinase M2 (PKM2), which respectively catalyze the first and terminal steps in the glycolytic pathway, restricts canonical NLRP3 activation in mouse bone marrow-derived macrophages (BMDMs) [29,30] (Figure 1). By contrast, HK2 inhibition via N-acetylglucosamine (NAG; a sugar subunit of Gram-positive bacterial cell wall peptidoglycan) results in HK2 dissociation from mitochondrial outer membranes and boosts inflammasome activation in mouse BMDMs [31]. The latter discovery was made exposing BMDMs to NAG sensing by HK2, resulting in elevated cytosolic HK2 expression and enzymatic activity (Figure 1) [31]. Likewise, direct microinjection of sugars into the cytosol of primary macrophages triggered the release of GFP-tagged HK2 into the cytosol [31]. Furthermore, transfection of HK2 enzymatic product, glucose-6-phosphate (G6P; which regulates glycolysis by feedback inhibition) in LPS-primed mouse BMDMs, elevated NLRP3-dependent IL-1β secretion [31]. It is plausible that G6P accumulation drives excess NAD^+ and activation of the inflammasome; however, this remains to be fully assessed [32,33]. In contrast to the effects seen with **ATP** and nigericin, the mitochondrial membrane potential and integrity remains intact upon HK2 inhibition [31]. While HK2 can contribute to Toll-like receptor 2 (TLR2)-driven glycolysis in dendritic cells (DCs) [34], an earlier study failed to detect HK2 expression in mouse BMDMs [30]. Despite these

Glossary

ATP: molecule that acts as a source of energy for most cellular functions; produced during glycolysis and the TCA cycle.
Alkylation: post-translational modification in which an alkyl group is enzymatically added to help regulate various cellular processes.
Anaplerosis: process of replenishing TCA cycle intermediates.
β-Oxidation: fatty acid oxidation; catabolic mechanism by which acetyl-CoA is generated in the mitochondrial matrix through the breakdown of fatty acids.
Fatty acids: key components of many lipids, comprising either saturated or unsaturated forms. They consist of carbon chains from 4 to 28 in length, harboring varied configurations.
Gasdermin D: pore-forming protein that triggers pyroptosis upon assembly of the inflammasome.
Glycolytic flux: variable change between metabolites fructose 6-phosphate and fructose-1,6-bisphosphate.
Glycolysis: key cytosolic process in which glucose is converted into pyruvate, water, and hydrogen. This is the first step in the process to produce ATP and leads to the TCA cycle.
Hypoxia-inducible factors (HIFs): family of transcription factors that mediate metabolic and cellular responses to environmental changes.
Inflammasome: multiprotein intracellular complex consisting of pro-caspase 1, adapter protein ASC, and a cytosolic pattern recognition receptor.
Lipid droplets: lipid storage organelles consisting of a hydrophobic core of neutral lipids enclosed by a phospholipid monolayer.
Metabolic flux: rate or turnover of metabolites through a metabolic pathway.
Mitophagy: cell death that occurs in response to abnormal or damaged mitochondria.
Oxidative phosphorylation (OXPHOS): enzymatic process in which carbon sources are oxidized to generate ATP in mitochondria via the electron transport chain.
Pyroptosis: programmed cell death triggered by a broad array of signals, downstream of inflammasome assembly. During pyroptosis, proinflammatory cytokines IL-1β and IL-18 are released to help other proinflammatory signaling processes.

discrepancies, tight regulation of the inflammasome seems to occur at the level of HK because the two isoforms can regulate NLRP3 inflammasome. Mammalian tissues express four HK isoforms, with HK4 (glucokinase) having the least affinity for glucose; HK2 is the predominant form in insulin-sensitive tissues such as skeletal muscle and adipose tissue [35]. These observations suggest that the expression of the HK1 and HK2 in mammalian inflammasome-competent tissues might modulate net inflammasome activity.

NLRP3 inflammasome and glycolytic flux

Productive glycolysis results in the conversion of NAD^+ to NADH, lactate secretion, and ATP production. Inhibition of GAPDH and α-enolase, resulting in disruption of glycolytic flux, triggers the NLRP3 inflammasome in a K^+-independent manner in mouse BMDMs [33] (Figure 1). In agreement, a study using modeling and GAPDH *in vitro* flux assays in cell lines suggested that under nutrient-rich conditions, flux through GAPDH, and not enzymes in upper glycolysis, may be rate-limiting [36]. Similarly, mouse infection with *Salmonella enterica* Typhimurium serovar, which disrupts **glycolytic flux** (measured by increased NAD^+/NADH concentrations), elevated NLRP3 activation in BMDMs [33,37]. Notably, this result was restricted to *Salmonella* grown under SPI-2-inducing conditions that limited activation to the NLRP3 inflammasome. Conversely, supplementation with glycolytic end-product pyruvate reduced inflammasome foci in primary mouse and human macrophages [33,38,39]. However, inhibition of the NLRP3 inflammasome with pyruvate was not complete, suggesting that a compensatory role for other inflammasomes might exist, or that *Salmonella* might use pyruvate, which remains to be further investigated [33,38,39]. Nevertheless, these studies propose that NLRP3 can sense changes in glycolytic flux. However, targeting entry into glycolysis by 2-deoxyglucose (2-DG), which targets HK, has exhibited contrasting results on NLRP3 activation [31,33,40]. This may be partly because 2-DG is a competitive inhibitor of HK; therefore, it might be sensitive to the presence of the preferred HK substrate, glucose. Aldolase A (ALDOA) (fructose bis-phosphate), which monitors glycolytic flux, regulates NLRP3 inflammasome by participating in the formation of AXIN-based AMPK-activation complex on the lysosomal surface, as shown for example, in mouse bone marrow-derived DCs [41–43]. ALDOA inhibition removes damaged mitochondria through parkin-dependent **mitophagy** in response to NLRP3 agonists in such myeloid cells [41–43] (Figure 1). These findings suggest that depending on the enzyme and the glycolytic step in the pathway, glycolytic flux can regulate the NLRP3 inflammasome in discrete ways.

TCA cycle metabolites regulate NLRP3 inflammasome

Acetyl-CoA is generated by the oxidation of pyruvate (glycolysis), long-chain **fatty acids** (**β-oxidation**), or certain amino acids before it is catabolized through the TCA cycle in the mitochondria producing NADH and, to a lesser extent, ATP [44]. Derived NADH serves as an electron donor for the electron transport chain (ETC) to generate a mitochondrial membrane potential which, in the presence of oxygen, phosphorylates ADP to ATP in a process known as oxidative phosphorylation (OXPHOS). TCA-coupled mitochondrial OXPHOS is the most effective energy-producing catabolic process [45]. In addition, TCA intermediates play important roles in cell signaling, including modulating activation of the NLRP3 inflammasome [46]. However, specific functions for individual metabolites in this pathway remain ambiguous.

Succinate

Macrophage activation increases intracellular succinate, stabilizing the transcription factor **hypoxia-inducible factor (HIF)**-1α which specifically results in elevated *Il1b* expression; also, succinate is partially generated by glutamine-dependent **anaplerosis** by α-ketoglutarate in the absence of glycolysis [40,47]. Accordingly, knockdown by siRNA of glutamine transporter *Slc3a2 or* exposure to 2-DG reduces IL-1β expression in mouse BMDMs and human peripheral

Reactive oxygen species (ROS): oxygen-derived molecules harboring an oxidizing effect and produced in response to oxygen metabolism, cytokine signaling, and microbial invasion. Examples of ROS include peroxide, superoxide, and the hydroxyl radical.
Stable isotope tracing: method for quantifying metabolic rates of specific metabolites by using stable (nonradioactive) isotopes with a heavier mass than the naturally occurring isotope.
Tricarboxylic acid (TCA) cycle: cycle of aerobic reactions which generate ATP via the oxidation of acetyl-CoA.

blood mononuclear cells (PBMCs), respectively [40]. Conversely, prolyl hydroxylase, which hydrolyses HIF-1α and generates succinate only as a byproduct, is impaired when cytosolic succinate rises, arguing that intracellular succinate concentrations crucially regulate IL-1β production [40]. However, IL-1β production may also correlate with the efficiency of succinate oxidation by succinate dehydrogenase (SDH) [48] (Figure 2). SDH is also a part of the mitochondrial ETC (as complex II). Thus, since BMDMs from *Irg1*$^{-/-}$ mice lacking the ability to synthesize itaconate, an endogenous SDH inhibitor, also display elevated HIF-1α (and IL-1β expression) compared to wild-type BMDMs, this suggests that the direction and efficiency of the ETC in activated macrophages, rather than succinate accumulation, is equally important in IL-1β production [48]. As such, the mechanistic underpinnings of HIF-1α–IL-1β signaling remain ambiguous and warrant further investigation.

Itaconate

As mentioned, SDH is endogenously targeted by itaconate synthesized from the decarboxylation of *cis*-aconitate by the enzyme immune-responsive gene 1 (*Irg1*). While experiments in *Irg1*$^{-/-}$ mouse BMDMs revealed elevated NLRP3 activation (measured from increased IL-1β and IL-18 secretion) [48], interpreting the specific functions of itaconate from studies using itaconate derivatives has posed challenges. Depending on the duration and the step of exposure (3 vs 12 h; before or after LPS exposure), itaconate derivatives dimethyl itaconate and 4-octyl itaconate (4-OI) can affect the priming and activation steps of the NLRP3 inflammasome in primary mouse and human macrophages [48–50]. However, whether different derivatives enter cells, remain stable, are converted into itaconate, and finally induce increased succinate amounts in cells has been debated, with not much consensus [49,51,52]. Therefore, the reliability of itaconate derivatives to recapitulate the physiological effects of intracellular itaconate on NLRP3 regulation is somewhat doubtful. This underscores the need to determine optimal exogenous tools for itaconate research.

By contrast, in independent studies, mouse macrophages exposed to high concentrations of unmodified itaconate (itaconic acid), show intracellular itaconate accumulation, specifically resulting in NLRP3 inhibition [52,53]. Several mechanisms have been proposed for the action of itaconate (sometimes using itaconate derivatives) on NLRP3 activation (Figure 2). Itaconate alkylates cysteine residues (i.e., 2,3-dicarboxypropylation or itaconation) on kelch-like ECH-associated protein 1 (KEAP1), allowing NRF2 to accumulate and increase the expression of downstream anti-inflammatory genes (such as heme oxygenase 1 and glutathione) and decrease IL-1β and IL-6 transcription in mouse and human macrophages [54–56]. Itaconation of the NLRP3 Cys548 residue disrupted NLRP3 and NEK7 interaction in mouse BMDMs [57]. The itaconated residue was revealed by tandem mass spectrometry of murine NLRP3 immunoprecipitated from 4-OI-treated NLRP3-overexpressing HEK293 cells [57]. Itaconation of GSDMD Cys77 residue in synergism with the expression of inducible nitric oxide synthase provided late tolerance to pyroptosis in mouse BMDMs [58]. In the latter study, while caspase-1 activity was observed in a fluorogenic FLICA assay, the processed caspase-1 band was not observed by conventional immunoblotting. Whether endogenous itaconate can modify residues that are identified using derivatives remains to be determined to fully establish the varied mechanisms by which itaconate can regulate NLRP3 activation. It is also plausible that intracellular itaconate modifies additional residues in inflammasome components which may offer novel insights into inflammasome assembly. Indeed, a study using an itaconate-alkyne probe, which recapitulated endogenous itaconate properties, identified the above Cys548 residue and several new itaconated sites on NLRP3, AIM2, and GSDMD in the RAW264.7 mouse macrophage cell line in culture [59]. These studies indicate that itaconate can regulate the NLRP3 inflammasome by several mechanisms that might be present in other inflammasomes or be involved in the specific regulation of downstream pyroptosis.

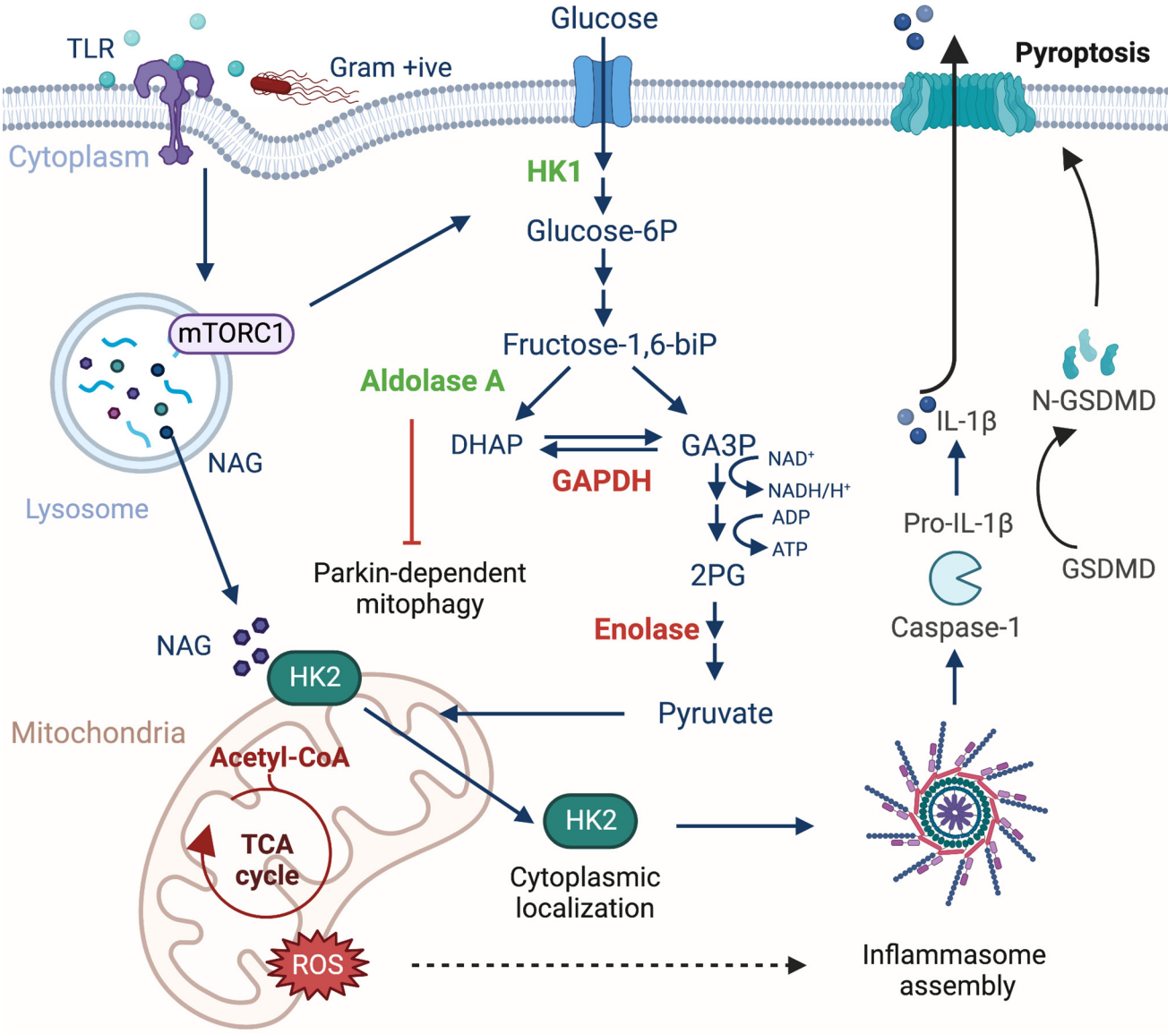

Figure 1. Glycolytic enzymes differentially regulate the mammalian NLRP3 inflammasome. Glycolysis begins with the regulated uptake of glucose into the cytoplasm. Pathway signaling results in the breakdown of glucose in a series of chemical reactions within the cytoplasm to form pyruvate. The pyruvate thus formed diffuses into the mitochondria where it enters the TCA cycle. TLR ligation triggers mTORC1- and HK1-dependent glycolysis which is essential for NLRP3 inflammasome activation [30]. By contrast, NAG, a sugar subunit of Gram-positive bacterial cell wall peptidoglycan, is sensed by HK2, resulting in HK2 inhibition and dissociation from the mitochondria, triggering inflammasome activation [31]. Other enzymes in glycolysis have also been shown to regulate the NLRP3 inflammasome. The activity of GAPDH and α-enolase (in red, bold) limits the NLRP3 inflammasome and the inhibition of the two enzymes disrupts glycolytic flux and elevates activation of the NLRP3 inflammasome [33]. By contrast, aldolase A (in green, bold) activity promotes activation of the NLRP3 inflammasome as aldolase A inhibition removed damaged mitochondria through parkin-dependent mitophagy [41]. Activation of the NLRP3 inflammasome results in caspase-1 cleavage leading to the induction of GSDMD-dependent pyroptosis and IL-1β secretion [6]. Abbreviations: 2PG, 2-phosphoglycerate; DHAP, dihydroxyacetone phosphate; GA3P, glyceraldehyde 3-phosphate; HK, hexokinase; mTORC1, mammalian target of rapamycin complex 1; NAG, N-acetylglucosamine; TCA cycle, tricarboxylic acid cycle. Created with BioRender.com.

Fumarate

Fumarate can block GSDMD processing, oligomerization, and pyroptosis [60]. Chemoproteomic approaches on BMDMs exposed to cell-permeable fumaric acid with an alkyne handle (for target

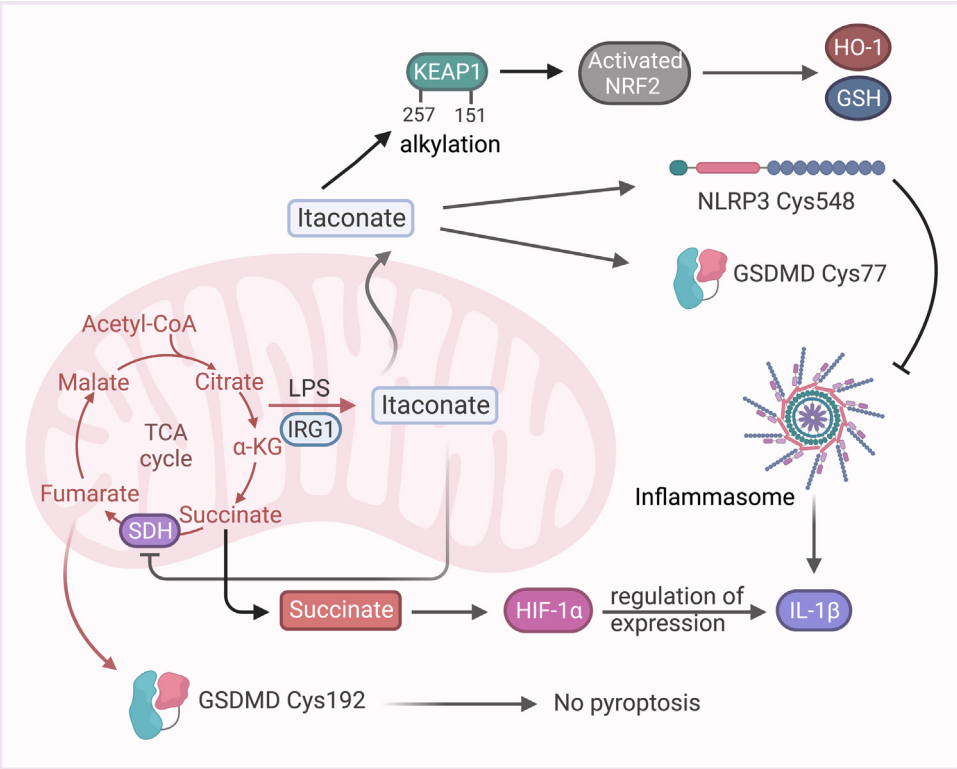

Figure 2. TCA cycle metabolites regulate the mammalian NLRP3 inflammasome. Activated macrophages show increased amounts of succinate resulting in IL-1β production either through HIF-1α stabilization, or through increased production of itaconate, which inhibits SDH [40]. Itaconate is synthesized from the decarboxylation of *cis*-aconitate by the enzyme IRG1. Itaconate inhibits the NLRP3 inflammasome in different ways by **alkylation** of cysteine residues of KEAP1, NLRP3, and GSDMD. Itaconation of KEAP1 at several cysteine residues results in NRF2 accumulation resulting in the expression of anti-inflammatory genes such as heme oxygenase 1 and glutathione [49]. Itaconation of NLRP3 Cys548 residue disrupts its interaction with NEK7 resulting in reduced inflammasome activation [57]. Itaconation of Cys77 residue in GSDMD provides late tolerance to pyroptosis [58]. Fumarate also modifies (succination) GSDMD Cys192 thereby inhibiting GSDMD oligomerization and pyroptosis [60]. Abbreviations: GSH, glutathione; HIF-1α, hypoxia-inducible factor 1α; HO-1, heme oxygenase 1; IRG, immune-responsive gene 1; KEAP1, Kelch ECH associating protein 1; NRF2, nuclear factor erythroid 2-related factor 2; NEK7, NIMA-related kinase 7; SDH, succinate dehydrogenase; TCA cycle, tricarboxylic acid cycle. Created with BioRender.com.

identification) revealed that the probe reacted with GSDMD [60]. Mass spectrometry of recombinant GSDMD treated with fumaric acid led to the identification of succinated cysteine residues (mouse Cys192 position, Cys191 residue in humans). The modification prevented GSDMD and caspase-1 interactions while the processing of the cysteine protease remained unhindered in BMDMs [60] (Figure 2). Consequently, the fumarate analog dimethylfumarate blocked cell death and lactate dehrdrogenase (LDH) release in response NLRP3 activation, but also in response to NLRC4 and AIM2 stimulation [60]. Fumarate similarly blocked the processing of GSDME (succination at Cys45 position), which drove apoptosis in mouse cells lacking GSDMD [60,61]. Notably, some of the succination sites were also itaconated in these studies [59,60], which suggests a potential preference for these sites, or it may reflect the structural similarity between the two TCA metabolites [62]

Salmonella-derived TCA metabolites can also modulate host inflammasome activation [63]. For instance, infection of wildtype (or *Nlrc4*$^{-/-}$) mouse BMDMs with a *Salmonella* sp. strain that was

deficient in the enzyme aconitase (encoded by *acnB*, and responsible for catalyzing the conversion of citrate to the itaconate precursor aconitate), enhanced LDH release and NLRP3-dependent IL-1β secretion [63]. Consistent with *in vitro* inflammasome responses, mice infected orally with *acnB* mutant *Salmonella,* at day 7, exhibited elevated serum IL-18 while splenic burden was significantly reduced compared to controls [63]. To persist in the host, other pathogens such as *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* also required TCA metabolites, as documented in various murine models of lung infection [64,65]. Indeed, both host and microbe-derived TCA metabolites serve as ammunition in macrophage inflammatory responses [66,67]. However, whether increased citrate production by *Salmonella acnB* mutant could modulate NLRP3 inflammasome was not fully explored in the previous study [63]. It has been hypothesized that inflammasome regulation by TCA metabolites might also contribute to promoting long-term infections [66,67]. Thus, the immunomodulatory role of TCA metabolites might be beneficial for the treatment of NLRP3-related sterile inflammatory diseases (possibly in rheumatoid arthritis) but could also jeopardize host immune response and pathogen clearance, both of which remain conjectural.

Overall, even as succinate and the efficiency of succinate oxidation promotes IL-1β, TCA metabolites itaconate and fumarate inhibit NLRP3 inflammasome and **pyroptotic** cell death, thus highlighting the immunomodulatory properties of distinct TCA metabolites.

Inflammasome regulation by lipid pathways

The macrophage lipid metabolic program is linked to pathways such as those discussed in the preceding text, which together, frame a wider metabolic network to supports macrophage function and regulate the NLRP3 inflammasome. For instance, glycolysis provides glycerol-3-phosphate for triglyceride synthesis, supporting the formation of **lipid droplets** which are a source of inflammatory mediators and antimicrobial proteins [68,69].

While saturated fatty acids (SFAs) activate the NLRP3 inflammasome, monounsaturated fatty acids such as oleic acid (OA), either counteract SFA-mediated NLRP3 activation or directly downregulate NLRP3 activation *in vitro* and *in vivo* [54,70–72]. The role of OA-mediated inhibition of the NLRP3 inflammasome remains unclear, although OA might decrease the intracellular amounts of SFA through triglyceride synthesis and lipid droplet formation as suggested in murine macrophages [73] (Figure 3). Dietary polyunsaturated fatty acids (PUFAs), including docosahexaenoic acid (DHA), can be sensed by and dampen NLRP3 activation in LPS-primed human macrophages [55,56]. DHA also downregulates *Il1b* and *Nlrp3* gene expression during silica-induced inflammasome activation *in vitro* and in mouse Kupffer cells *in vivo* [74,75].

Fatty acid oxidation and synthesis can regulate the inflammasome

Fatty acids (FAs) by a process called β-oxidation (or fatty acid oxidation; FAO) are oxidized in the mitochondria to acetyl-Co-A, which is further catabolized through the TCA cycle to generate ATP through OXPHOS. NOX4 upregulates carnitine palmitoyltransferase 1A (CPT1A), the enzyme that regulates FA entry into the mitochondrial matrix, enhancing FAO and NLRP3 inflammasome activation [76]. Conversely, downregulation of CPT1A and the consequent inhibition of FAO blunts NLRP3 inflammasome activation in *Hif2a*$^{-/-}$ macrophages and alleviates obesity-induced insulin resistance in myeloid-specific *Hif2a* knockout mice relative to controls [77]. Accordingly, pharmacological blockade of FAO in macrophages has restrained the progression of atherosclerosis and gouty inflammation in mice [78,79]. FAs can be anabolized *de novo* in the cell cytoplasm and endoplasmic reticulum. *De novo* FAS is governed by the enzymatic activity of fatty acid synthase (FASN), which is upregulated in pro-inflammatory macrophages and is essential for NLRP3 inflammasome priming [80]. Using quantitative lipidomics, TLR stimulation or *M. tuberculosis*

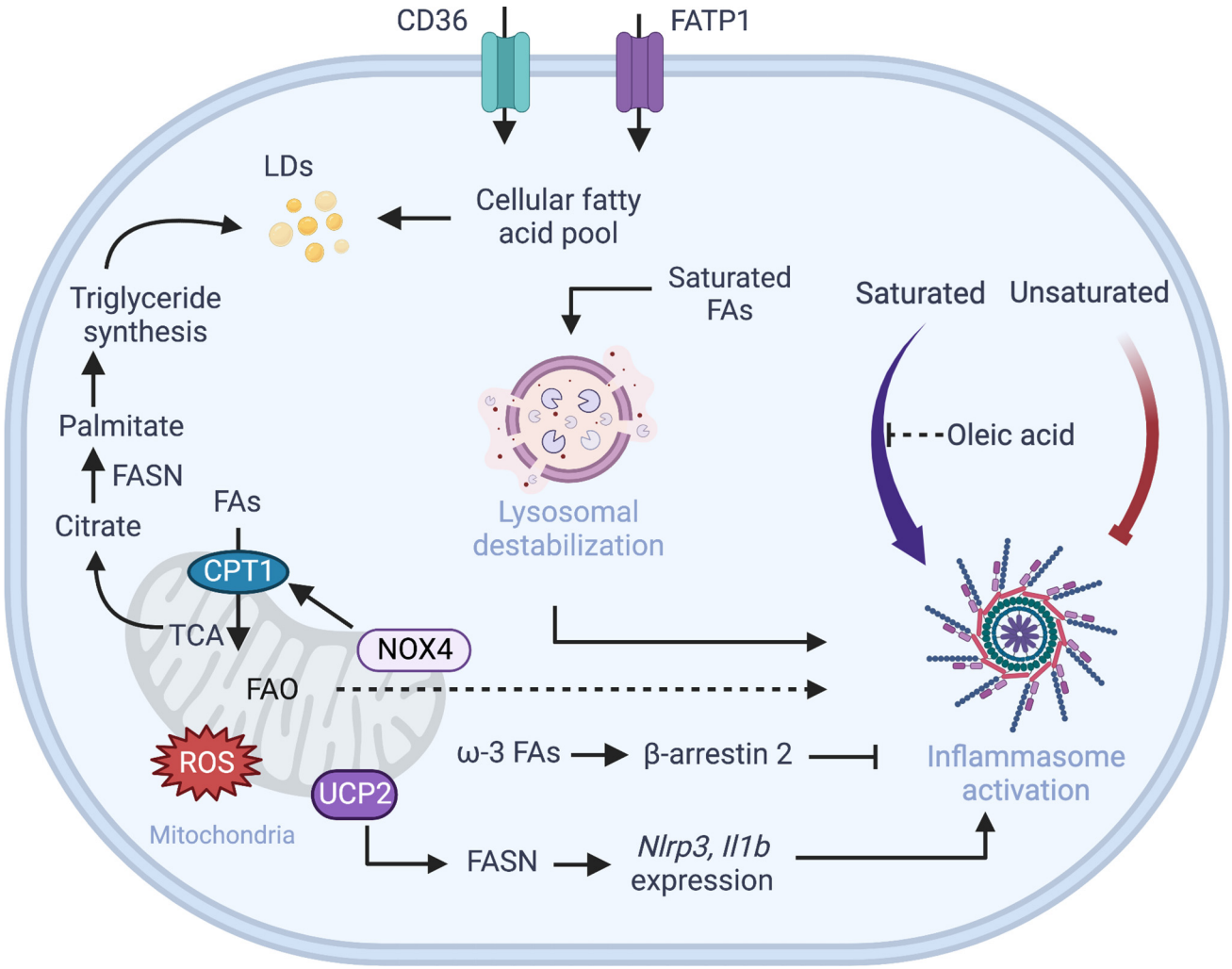

Figure 3. FAO and synthesis regulate the mammalian NLRP3 inflammasome. Macrophages acquire essential FAs through various pathways, including uptake of non-essential FAs by FATP1, as well as oxidized low-density lipoproteins and very-low-density lipoproteins via CD36 or scavenger receptors. The mitochondrial UCP2 regulates the NLRP3 inflammasome through a key driver of fatty acid synthesis, FASN which regulates NLRP3 and IL-1β expression [80]. Typically, saturated FAs activate the NLRP3 inflammasome, while unsaturated FAs inhibit NLRP3 inflammasome activation [70–72]. Oleic acid, a monounsaturated fatty acid, can counteract NLRP3 activation by saturated FAs. PUFAs abolish NLRP3 inflammasome activation by interacting with β-arrestin-2 [73]. Similar to FA synthesis, FAO promotes NLRP3 inflammasome activation [76]. NOX4 can regulate NLRP3 inflammasome activation by controlling the expression of CPT1A; a pivotal enzyme in the FAO pathway [76]. Saturated FAs may also lead to crystal formation resulting in NLRP3 inflammasome activation through lysosomal destabilization. However, triglyceride synthesis and lipid droplet formation may decrease the intracellular amounts of saturated FAs [68,73]. Abbreviations; CPT1A, carnitine palmitoyltransferase 1A; FAO, fatty acid oxidation; FASN, fatty acid synthase FATP1, fatty acid transport protein 1; NOX4, NADPH oxidase 4; PUFA, polyunsaturated ω-3 fatty acid; UCP2, uncoupling protein-2. Created with BioRender.com.

infection in BMDMs has been shown to elevate cellular FAs [81]. *M. tuberculosis* infection induced *de novo* synthesis of PUFA precursors in macrophages, while ablation of PUFA biosynthesis downregulated *Il1b* in mouse BMDMs [81]. This study was relevant as it showed that *M. tuberculosis* competes for the usage of host PUFA, hampering macrophage function, including inflammasome activation [81]. Collectively, these findings show that FA biosynthesis and FA oxidation can regulate NLRP3 activation. However, the underlying mechanisms for these processes remain unclear.

Concluding remarks

Cellular metabolism serves to supply building blocks and generate metabolic energy for cell growth and survival. In activated macrophages, metabolic pathways additionally determine immune fates by shaping cells to either achieve activating or resolving inflammatory functions. While the well-studied NLRP3 inflammasome is typically activated in response to metabolic perturbations, the full repertoire of metabolic changes that temper the NLRP3 inflammasome remains unknown. In this Opinion, we discussed how major metabolic pathways may regulate NLRP3 inflammasome activation (Table 1,

Outstanding questions

How can metabolic flux during inflammasome activation be precisely quantified? Most studies either interrupt discrete steps or utilize exogenous supplementation assays to study the effect of individual enzymes and metabolites. Studies using stable isotope-based metabolic flux analysis (for example, ^{13}C) are needed to help unveil physiological metabolic rates during inflammasome activation.

What is the physiological significance of cysteine modification carried out by itaconate and fumarate? Do they modify other inflammasome components?

How do cells coordinate metabolic fluxes in different organelles for optimal inflammasome activation? NLRP3 activation is dependent on the integration of signals from multiple organelles. As metabolic precursors are spatially compartmentalized, evaluating metabolic rates in relevant organelles can help identify mechanistic underpinnings of the metabolic control of inflammasome activation.

Does metabolism calibrate the activation of non-NLRP3 inflammasomes, for example NLRC4 and AIM2? What are the mechanistic details for this, and what outcomes are achieved?

Do pathogens target host cellular metabolism to specifically modulate NLRP3 activation? What host and pathogen factors are involved? Pathogens use astute methods for their replication and nutritional needs within the cells they infect. Understanding the precise adaptations of specific pathogens can further help efforts to prevent infection and transmissibility.

Key table

Table 1. Summary of the input of distinct metabolites and enzymes to NLRP3 inflammasome activation

Glycolytic enzymes/ metabolites	Cell type and species	Mechanism	Refs
HK1	Mouse BMDMs	shRNA knockdown of hexokinase 1 inhibits the NLRP3 inflammasome.	[30]
HK2	Mouse BMDMs	Bacterial N-acetylglucosamine induces dissociation of hexokinase 2 from mitochondria activating the NLRP3 inflammasome.	[31]
Glucose-6-phosphate	Human THP-1 and PBMCs	Glucose-6-phosphate dehydrogenase (G6PD) deficiency, either through pharmacological inhibition or knockout, leads to inhibition of NLRP3 inflammasome via decreased ROS production.	[32]
Aldolase A	Mouse J774A.1 and BMDMs, Human THP-1	ALDOA is a monitor of glycolytic flux and works to support NLRP3 activation.	[41]
GAPDH and α-enolase	Mouse BMDMs	Chemical proteomics identified GAPDH and α-enolase as targets of the NLRP3 activator GB111-NH2.	[33]
PKM2	Mouse BMDMs, Human THP-1	shRNA knockdown of PKM2 or pharmacological inhibition of the PKM2-EIF2AK2 pathway attenuates NLRP3 and AIM2 inflammasomes.	[29]
Pyruvate	Mouse peritoneal macrophages, Human THP-1 and PBMCs	Ethyl pyruvate inhibits NLRP3 inflammasome activation via preservation of mitochondrial integrity.	[38]
LDH	Mouse BMDMs, Human THP-1	LDH inhibition and pyruvate oxidation showed inhibition and activation of NLRP3 inflammasome, respectively.	[39]
TCA metabolites			
Succinate	Mouse BMDMs	Increased amounts of succinate in LPS activated cells showed increased IL-1β via HIF-1α stabilization.	[40]
Itaconate	Mouse BMDMs, Human PBMCs from CAPS patients	Treatment with itaconate derivative 4-OI reduced inflammasome activation while *Irg1*-knockout cells (which cannot produce itaconate) exhibited increased inflammasome activation. Itaconate inhibited NLRP3 and NEK7 interaction.	[57]
	Mouse BMDMs	Itaconate supressed IL-1β secretion and strongly enhanced interferon-β secretion.	[52]
	Mouse RAW264.7 cells	By using a biorthogonal probe, itaconate-alkyne, itaconate induced anti-inflammatory outcomes.	[59]
	Mouse BMDMs, Human THP-1	Itaconate alkylates several cysteine residues on KEAP1, allowing NRF2 to induce antioxidant and anti-inflammatory functions.	[49]
Fumarate	Mouse BMDMs	Supplementation with fumarate analog dimethyl fumarate succinated GSDMD, resulting in inhibition of GSDMD processing, oligomerization, and pyroptosis.	[60]

Key table). We homed in on how distinct enzymes and metabolites within these networks can operate and contrast inflammasome functions, suggesting that the integration of several discrete steps must occur at the physiological and metabolic level to modulate macrophage functions and inflammation outcomes.

The metabolic control of inflammasomes has mostly been deciphered by using conventional cell culture approaches in which either a metabolic enzyme is inhibited, or its substrate/product is exogenously supplemented. However, accurate analysis of **metabolic flux** during inflammasome activation remains largely unexplored. Stable isotope-based metabolic flux analysis (**stable isotope tracing**) can more accurately reveal metabolic rates during inflammasome activation, providing greater insights into the contributions of metabolic alterations to NLRP3-dependent pathology. Currently, how a precise shift in metabolism is sensed and further integrated to initiate a protective or pathogenic inflammasome response, remains unknown. However, variation in the amounts or potency of cellular metabolites might impinge on the roles of the inflammasome during pathological conditions [24,72]. In this context, it is tempting to speculate that metabolite flux in organelles that leads to their compartmentalization might contribute to calibrating NLRP3 inflammasome activation [82]. Indeed, succinate transport from mitochondria that results in increased succinate cytosolic concentrations has been demonstrated to increase IL-1β expression in activated mouse BMDMs [40].

Recent studies on itaconate and fumarate have significantly clarified the roles of TCA metabolites in inflammatory cytokine production, **reactive oxygen species (ROS)** production, and inflammatory disease models. It is therefore evident that metabolic signals tightly regulate the activation of the NLRP3 inflammasome. Some timely key questions include: can we calibrate inflammasome activation by tweaking metabolic pathways *in vivo*? Does the metabolic regulation of the inflammasome extend beyond the NLRP3 sensor? Can inflammasome activation in turn regulate metabolic flux? Though some progress has been made, these and other questions (see Outstanding questions) remain unresolved. However, we argue that by understanding the metabolic control of the NLRP3 inflammasome, we can further advance our identification of putative targets for intervention in inflammatory and autoimmune disorders in which NLRP3 might contribute to an underlying pathology.

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Declaration of interests

No interests are declared.

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