The mitochondrial respiratory chain: A metabolic rheostat of innate immune cell-mediated antibacterial responses

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Abstract

Upon microbial infection, cells of the innate immune system undergo profound metabolic reprogramming in order to eradicate pathogens, promote inflammation, and eventually restore tissue homeostasis. Mitochondria are at the core of these adaptations, given their dual role as metabolic hubs and innate immune signaling platforms. The mitochondrial electron transport chain (ETC) is very well characterized at the genetic, molecular, structural, and biochemical level. In contrast, the role for mitochondrial ETC and metabolites beyond fulfilling cellular ATP synthesis in innate immune cell biology was not understood until recently. Here we discuss the latest advances in our understanding of immune functions of mitochondria and particularly the mitochondrial respiratory chain.

Keywords Electron transport chain, metabolism, innate immunity, bacterial infection, pattern-recognition receptors

Introduction: of the importance of metabolism in the innate immune system.

The immune system of jawed vertebrates can be conceptually divided into two branches; the innate and the adaptive immune system (Litman et al., 2010). Although evolutionarily distinct, the innate and adaptive immune system are functionally closely intertwined (Boehm, 2012). Professional phagocytes, such as macrophages or dendritic cells (DCs) continuously probe their environment for microbial invaders. They express a wide array of 'pattern recognition receptors' (PRRs), a large group of germline-encoded immune receptors that detect highly conserved molecules in microorganisms, called 'pathogen associated molecular patterns' (PAMPs) (Janeway, 1989). Ligation of PRRs, such as Toll-like receptors (TLRs), leads to rapid activation of intracellular signaling cascades eliciting pro-inflammatory and antimicrobial responses (**Figure 1**). Upon activation, some members of the NOD-like protein (NLR) family of PRRs can assemble into large cytosolic protein complexes, termed inflammasomes (Martinon et al., 2009). In antigen presenting cells (APCs) such as DCs, PRR activation promotes antigen processing and presentation to cells from the adaptive arm of immunity (i.e T cells and B cells), upregulation of co-stimulatory molecules and cytokine production. Other professional phagocytes including macrophages, monocytes and neutrophils show a high capacity to engulf and destroy microbes such as bacteria. Nevertheless, they also have the capacity to present antigen and produce various cytokines and chemokines upon PRR activation to further tailor immune responses mediated by adaptive immune cells such as T cells or B cells. Thus, PRRs represent a molecular link between innate and adaptive immunity (Iwasaki and Medzhitov, 2010).

Microbes can trigger distinct and specific set of PRRs. The nature of the PRRs engaged during infection shapes the innate immune responses generated by skewing innate immune cells differentiation, which rely on complex molecular events. These events are largely viewed as a succession of phosphorylation events eventually leading to controlled gene transcription. However, early observations noted a profound switch to glucose fermentation upon activation of human and murine professional phagocytes (Chance et al., 1955; Newsholme et al., 1986; Oren et al., n.d.; Sbarra and Karnovsky, 1960, 1959) and recent studies have further

delineated the complex metabolic reprogramming that takes place upon immune cell activation (see other reviews in this issue of *Mitochondrion*) including macrophages and dendritic cells (Gleeson and Sheedy, 2016; O'Neill and Pearce, 2015). As the main metabolic hubs of animal cells, mitochondria are naturally positioned at the center of these 'immunometabolic' regulations. Intriguingly, mitochondria also serve as critical signaling platforms for PRRs including RIG-I like receptors (RLR), or NLR protein 3 (NLRP3). Moreover, release of mitochondria-derived molecules during infection can activate PRRs (Krüger et al., 2015; Shimada et al., 2012; Zhou et al., 2011). Hence, intricate molecular interactions between mitochondria and immune signaling pathways unfold during infection. In contrast, potential interactions between mitochondria or mitochondrial-derived metabolites and invading pathogens are less well studied. Innate immune cell differentiation thus results from specific combinations of signaling events and metabolic adaptations that are dictated by the biochemical composition and infectious features of the microbe encountered. Mitochondria have been found to play important function in both PRR signaling and metabolism of innate immune cells but whether these two faces of mitochondria are interconnected is only started to be understood.

A growing number of studies delineates the metabolic events that occur during innate immune cell activation placing mitochondria at the crossroads of immune signaling and metabolic adaptations. These metabolic pathways generate reductive intermediates that are eventually oxidized in the electron transport chain (ETC). While this process was generally considered as a means to supply sufficient ATP to resting professional phagocytes and APCs, it is becoming increasingly clear that the ETC's contribution in innate immunity extends beyond ATP synthesis when cells become activated. Important progress has been made in our understanding of metabolic adaptation in cells of the adaptive immune system and T cell in particular. We strongly encourage the readers willing to understand the metabolic aspects of adaptive immune cells to refer to comprehensive reviews published in this issue of *Mitochondrion* and elsewhere (Buck et al., 2015; Mills et al., 2017). Here we review recent works highlighting the unexpected antibacterial innate immune functions of the mitochondrial respiratory chain and mitochondrial metabolism.

Metabolic flux variations upon microbial encounter

Microbial encounters initiate complex activation and differentiation programs in phagocytes, which critically shape the nature of the immune response that will be generated. In the middle of last century, several laboratories made the observation that the phagocytes change their metabolic state upon activation raising the possibility that those metabolic adaptations would be part of this differentiation program (Chance et al., 1955; Oren et al., n.d.; Sbarra and Karnovsky, 1960, 1959). Changes in metabolic pathways upon immune cell activation have been reviewed elsewhere (Gleeson and Sheedy, 2016; Mills et al., 2017; O'Neill et al., 2016), and we will only provide a brief overview of the main metabolic adjustments found in macrophages upon microbial stimulation. The majority of studies investigating metabolic changes in macrophages have used selected microbial ligands, most commonly TLR4 agonist lipopolysaccharides (LPS) as a proxy for bacterial infection.

Activation of PRRs by bacterial components strongly increases phagocyte's glycolytic flux, and levels of different glycolytic intermediates are highly elevated in activated macrophages (Everts et al., 2014; Everts et al., 2012; Jha et al., 2015). Increased in glycolytic flux and lactate production is found in macrophages upon infection with different bacterial species including *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) (Garaude et al., 2016; Palsson-McDermott et al., 2015; Tannahill et al., 2013), *Mycobacterium tuberculosis* (Mehrotra et al., 2014; Tannahill et al., 2013), or *Escherichia coli* (*E. coli*) (Garaude et al., 2016). Interestingly, this increase in glycolysis and lactate production is observed even under unlimited access to oxygen (O'Neill and Pearce, 2015). For this reason, this induction of aerobic glycolysis has been repeatedly compared to the 'Warburg effect' observed in cancer cells. Surprisingly, concomitant to this increase in glycolysis, a decrease in mitochondrial oxygen consumption was observed suggesting that glucose catabolism was diverted from its use by the mitochondria (Everts et al., 2012; Jha et al., 2015; Krawczyk et al., 2010; Tannahill et al., 2013). However, recent studies revealed that activation of TLRs others than TLR4 could increase mitochondrial oxygen consumption and engage distinct

metabolic programs in human monocytes (Lachmandas et al., 2016) and plasmacytoid dendritic cells (D. Wu et al., 2016). Therefore, while the increased glycolytic flux is clearly a hallmark of many activated innate immune cells (macrophage, DCs, monocytes or neutrophils) other more subtle metabolic reprogramming events are likely to unfold depending on the nature of the microbial stimulus and the PRRs engaged, as well as involved cell type.

The pentose phosphate pathway (PPP), which diverts glycolytic intermediates towards nucleotide and amino acid production, is also augmented by LPS/TLR4 stimulation (Jha et al., 2015; Tannahill et al., 2013). It generates reducing equivalents of NAPDH, which are required for fatty acid synthesis and contribute to cellular redox homeostasis in macrophage. A recent study by Haschemi *et al*. highlighted some molecular details about PPP regulation and functional consequences during murine macrophage activation. This study showed that a substantial portion of glycolysis-derived carbons is routed towards the PPP upon LPS stimulation (Haschemi et al., 2012). The authors notably identified the carbohydrate kinase-like protein (CARKL) as a sedoheptulose kinase (converting the PPP metabolite sedoheptulose to sedoheptulose-7-phosphate) that can repress LPS-induced inflammatory cytokines production by macrophage. CARKL is downregulated in LPS-treated macrophages (also called M1 macrophages or inflammatory macrophages) but upregulated in IL4-activated macrophage (or M2 macrophage), which are essential to fight parasite infection and can modulate immune responses through the production of regulatory cytokines such as IL-10. Consistently, the authors found that loss of CARKL induced a mild activation in resting macrophage while its over-expression sensitized macrophage to M2 polarization. This suggests that CARKL may exert a pivotal function in macrophage polarization.

The role of fatty acid metabolism during professional phagocyte activation is less well understood. Fatty acid oxidation (FAO) is augmented in mouse and human monocytes primed with activators of the NLRP3 inflammasome, involving mitochondrial NADPH oxidase 4 (NOX4)-dependent ROS production (Moon et al., 2016). However, IL4-activated macrophage have been shown to use FAO contrary to macrophages induced by stimulation with LPS and Interferon–γ (Huang et al., 2014) . Pearce an colleagues used etomoxir, an inhibitor of carnitine-palmitoyl

transferase 1 (CPT1), a key enzyme in the long-chain fatty acid oxidation to show that lipolysis of triacylglycerol substrates by phagocytes was important for oxidative phosphorylation elevation and expression of genes that define IL-4-activated macrophages. In contrast, the genetic deletion of *Cpt2*, which also strongly diminished long-chain fatty acid oxidation in the mitochondria, did not alter IL-4-stimulated macrophage polarization as shown by Finkel and co-workers (Nomura et al., 2016). Fatty acid synthesis seems in contrast to be closely related to inflammatory phenotypes in macrophages. It is augmented in LPS-stimulated macrophages (Feingold et al., 2012) and fatty acid synthase is induced during sepsis through a mechanism that requires mitochondrial uncoupling protein 2 (UCP2) (Moon et al., 2015). More work is clearly needed to further define the exact contributions of FAO and fatty acid synthesis to the functions of macrophages and other innate immune cells during infection (Namgaladze and Brüne, 2014).

While metabolism of various amino acids is likely to contribute to immune function (O'Neill et al., 2016), glutamine has emerged as the most prominent amino acid in macrophage and DC metabolic reprogramming. Its catabolism (glutaminolysis) has been found to extensively replenish the TCA cycle and the hexosamine pathway while glycolytic product pyruvate is diverted from entry into the mitochondrion in murine macrophages (Jha et al., 2015; Tannahill et al., 2013). In LPS-primed macrophages, glutamine is thought to contribute to succinate accumulation through anaplerosis of α-ketoglutarate (Jha et al., 2015; Tannahill et al., 2013) and is required for production of the inflammatory cytokine IL-1β (Wallace and Keast, 1992) and the generation of nitric oxide (NO) through arginine synthesis in murine macrophages and human monocytes (Murphy and Newsholmes, 1998), thereby contributing to antimicrobial functions of macrophages. In addition NO can inhibit mitochondrial respiration and ATP production through nitrosylation of the iron-sulfur containing ETC complex I, II and IV (Beltran et al., 2000) thus accounting for OXPHOS shut down observed in LPS-stimulated macrophages and DCs. Consistently, glutamine deficiency reduced inflammasome activation (He et al., 2016). Glutamine-derived succinate can also be used to generate nitric oxides (Bellows and Jaffe, 1999), which in turn can inhibit the mitochondrial respiratory chain in LPS stimulated DCs (Everts et al., 2012). However, whether glutamine catabolism can contribute to other metabolic

pathways in phagocytes still needs to be further documents. A recent study nonetheless revealed some interesting features of the use of glutamine in macrophages. Liu et al. showed that α-ketoglutarate produced by glutaminolysis accumulates in IL-4-stimulated macrophages in which it induces Jumonji domain-containing protein 3 (Jmjd3)-dependent epigenetic reprogramming and instructs cells to augment FAO. In contrast, α-ketoglutarate represses the NF-kB pathway in LPS-primed macrophages through prolyl-hydroxylase (PHD)-dependent post-translational modification of IKKb. The ratio between glutamine-derived α-ketoglutarate and succinate controls macrophage differentiation. A high α-ketoglutarate/succinate ratio promotes IL-4-induced macrophage differentiation, whereas a low ratio supports inflammatory macrophage generation. Hence, glutamine metabolism is a critical a key metabolic checkpoint of macrophage fate and endotoxin tolerance (Liu et al., 2017).

Adaptations of the mitochondrial Electron Transport Chain (ETC) during microbial infection

ETC adaptations as a response to fuel source fluctuations

Most catabolic processes converge on the mitochondrial ETC by supplying electrons through the reductive equivalents of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH). The ETC comprises two electron carriers (coenzyme Q [CoQ]/ubiquinone and cytochrome c) and four respiratory complexes (complex I-IV [CI-CIV]). Respiratory complexes I, III, and IV can assemble as large molecular supercomplexes (SCs) in the mitochondrial inner membrane (for a comprehensive review see (Enriquez, 2016)). The transfer of electrons through the respiratory complexes generates a H^+ gradient that is used by the H^+-ATP synthase (CV) to generate ATP. The supramolecular organization of the electron transport chain and its functional implications are a subject of passionate debate (Cogliati et al., 2016; Lapuente-Brun et al., n.d.; Mourier et al., 2014; Pérez-Pérez et al., 2016). Nevertheless, recent studies have provided detailed insights into the sequential assembly of the respiratory chain (Cogliati et al., 2016; Guerrero-Castillo et al., 2016; Mick et al., 2012; Stroud et al., 2016) and resolved the structure of the so-called respirasome (a supercomplex composed of

CI, a dimer CIII and CIV) (Gu et al., 2016; Letts et al., 2016; M. Wu et al., 2016) and CI (Fiedorczuk et al., 2016). The dynamic assembly of respiratory complexes into supercomplexes may provide a structural environment favorable to assembly of CI (Enriquez, 2016; Genova and Lenaz, 2014; Lapuente-Brun et al., n.d.), stabilize the individual respiratory complexes (Acín-Pérez et al., 2004), prevent ROS production by sequestrating reactive radicals (Maranzana et al., 2013), or organize electron flux to optimize the use of available substrate required for ATP synthesis and oxygen consumption (Guarás et al., 2016; Lapuente-Brun et al., n.d.). Switch in the nature of the fuel that feed cellular metabolism alters the intramitochondrial ratios of $FADH_2$ and NADH (herein F/N ratios) (Speijer, 2016), which may require adjustment of respiratory chain super-assembly. The predicted F/N ratio is lowest for glucose oxidative breakdown with a ratio of 0.2 (one molecule of $FADH_2$ formed per five molecules of NADH), but is high (0.5) for saturated long-chain fatty acids with one molecule of $FADH_2$ per two NADH molecules (Speijer, 2011). Interestingly, fasting modifies SCs distribution and ETC activity (Lapuente-Brun et al., n.d.), and shifting nutrient substrate from glucose to fatty acids can downregulate CI content (Guarás et al., 2016). Mechanistically, it was proposed that accumulation of the reduced form of CoQ can generate a reverse electron transfer leading to oxidation of CI subunits (Chouchani et al., 2016). The resulting degradation of CI is likely to modify the stoichiometry of SCs to adjust the electron flow within the ETC (Guarás et al., 2016). Nutrient availability also regulates mitochondrial dynamics. However, the natural occurrence and functional roles supercomplexes still remain to be fully established.

Architectural changes of the ETC and their role in innate immune responses

Given the profound metabolic changes during activation of innate immune cells, it could be assumed that the architecture and the activity of the ETC would also vary in such conditions. A first indication came from observations in macrophages infected with *Listeria monocytogenes,* an intracellular Gram-positive pathogen that causes listeriosis. It was found that *Listeria* infection causes transient mitochondrial network fragmentation (Stavru et al., 2011). These alterations require pore-forming toxin listeriolysin O (LLO), but are independent of dynamin-like protein 1 (Drp1) or Opa1, two well-known proteins involved in mitochondrial

fusion/fission dynamics (Stavru et al., 2013). In contrast, the obligate intracellular bacterium *Chlamydia trachomatis* maintains mitochondrial network integrity by inhibiting Drp1-induced mitochondrial fragmentation (Chowdhury et al., 2017). Mitochondrial dynamics play a particularly important role in antiviral immunity. It was observed that RIG-I (a member of the RIG-I-like receptors or RLR), its downstream adaptors mitochondrial antiviral signaling (MAVS) and mitochondria localize around centers of viral replication upon infection by single-stranded RNA Sendai virus or 5'ppp-RNA detection (Onoguchi et al., 2010). MAVS is a RLR-signaling adaptor that translocates at the mitochondria upon viral infection to form a mitochondrial antiviral signaling platform and induce type-I interferons (IFN) essential for fighting viral infection (West et al., 2011b). Consistent with the link between mitochondrial dynamics and MAVS signaling, both mitofusin proteins (MFN) 1 and 2, two well-characterized fusion regulators, can interact with MAVS (Castanier et al., 2010; Yasukawa et al., 2009). Although the respective role of MFN1 and MFN2 in RLR signaling and the formation of MAVS platform is still not clear, other proteins involved in mitochondrial dynamics have been found to interact with proteins of the RLR/MAVS pathway during viral infection (West et al., 2011b) supporting the notion that mitochondrial dynamics is closely linked to innate immune signaling. However, whether this also occurs upon bacterial infection is still poorly appreciated. Additional research should clarify the role of mitochondrial dynamics related protein in innate immune signaling and their contribution to phagocyte-mediated antibacterial responses. Nevertheless, taken together those studies suggested that the alterations of the mitochondrial network induced by bacterial infection and inflammation are likely to affect the bioenergetics and immune signaling of innate immune cells. Accordingly, significant changes in the expression of mitochondrial carriers including the dicarboxylate carrier DIC, the citrate carriers CIC or the adenine-nucleotide carriers ACC have been associated with inflammation (Iacobazzi et al., 2017). Adaptations of the ETC are therefore likely to occur to meet the strong energetic requirements and metabolic adjustments of activated immune cells.

It was originally postulated that the overall mitochondrial oxygen consumption by the respiratory chain of macrophages, DCs and monocytes is reduced following TLR- (Krawczyk et al., 2010; Tannahill et al., 2013) and Dectin-1

(Cheng et al., 2014) activation. Thus it has been general wisdom that during infections, the ETC might serve other roles than sustaining metabolic fluxes and generating ATP, which would then mainly arise from glycolysis (Mills et al., 2017). However, an increase in glucose-dependent oxygen consumption was observed within few hours after LPS stimulation of DCs (Bart Everts et al., 2014; Everts et al., 2012) suggesting that mitochondrial respiration is required for innate immune cell activation. In fact, phagocytosis of living *Escherichia coli* by macrophages can the maximal respiration capacity and induces changes in the architecture of the ETC within two hours post-infection leading to a decrease in the abundance of CI and CI-containing SCs (Garaude et al., 2016). Consequently, CI activity is decreased upon *E. coli* infection. However, the overall mitochondrial respiratory capacity is maintained at least for several hours after bacterial stimulation. This is explained by an increase in mitochondrial glycerol-3-phosphate dehydrogenase activity, which allows mitochondrial recycling of cytosolic NADH and supply of electrons to the ETC (Garaude et al., 2016). CII activity is also strongly increased and contributes to mitochondrial respiration and ATP synthesis during bacterial infection (Garaude et al., 2016). Interestingly, the observed ETC adaptations were absent in macrophages stimulated with TLR4 agonist LPS or with heat killed bacteria. We have previously shown that murine phagocytes possess the intrinsic capacity to discriminate viable from dead bacteria through the recognition of specialized PAMPs, which we refer to as *vita*-PAMPs (Sander et al., 2012). Bacterial RNA was identified as the first *vita*-PAMP, recognition of which signals microbial viability to the immune system and elicits pronounced immune responses. A signature response of macrophages and DC to live bacteria or bacterial RNA is the activation of the NLRP3 inflammasome. When we investigated the ETC adaptations following bacterial stimulation we found that bacterial RNA or live bacteria, but not their killed counterparts, initiated ETC adaptations through activation of the phagosomal NADPH oxidase and the ROS-dependent tyrosine kinase Fgr, which was shown to phosphorylate the tyrosine 604 of the CII subunit succinate dehydrogenase subunit A (SDHA) (Acin-Perez et al., 2014; Garaude et al., 2016). The partner phosphatase of Fgr was recently identified (Nath et al., 2015), highlighting the potential of pharmaceutical manipulation of post-translational modifications of CII activity to control inflammation and other metabolic-related

diseases (Bezawork-Geleta et al., 2017). Hence, we demonstrate that specific innate immune signals that convey an increased level of infectious threat to the immune system induce profound metabolic alterations beyond the well-described glycolytic switch (Garaude et al., 2016).

This study suggested that innate immune receptor-mediated endosomal ROS production during bacterial infection directly increases mitochondrial ETC capacity but also enhance mitochondrial CII-dependent ATP synthesis, challenging the idea that ATP demand is mostly covered by anaerobic glycolysis. Nevertheless, it remains to be fully established whether metabolic flux modulation and the subsequent changes in the proportion of reductive equivalents NADH and $FADH_2$ contribute to those adaptations. First evidence came from a recent study by O'Neill and colleagues. They found that LPS/TLR4-activated murine macrophages accumulate succinate (Mills et al., 2016). As a consequence, TLR4-induced succinate oxidation by succinate dehydrogenase (SDH - which is also CII) and elevation of the mitochondrial membrane potential drives a reverse electron transfer (RET) that promotes mitochondrial ROS production by CI. This was dampened by the over-expression of an alternative oxidase (AOX) from *Ciona intestinalis*, which restores the normal flow of electrons through CoQ thereby avoiding RET (El-Khoury et al., 2014). It is tentative to speculate here that upon LPS stimulation, the increase ATP synthesis through glycolysis allows RET to occur assuming that mitochondrial ETC is not uncoupled. This would place ATP demand as an important driver of mitochondrial changes in LPS-activated phagocytes. This study also confirmed the importance of CII for macrophage activation upon innate immune receptor stimulation, and placed CI at the core of immune signaling. Complex I deficiency is a well-known cause of rare neurological and metabolic disorders. However, a recent study by Jin *et al* based on the deletion of the CI subunit Ndufs4 highlighted a key function of CI in macrophage differentiation and contribution to inflammatory responses (Jin et al., 2014). The global Ndusf4 loss results in lethal encephalomyopathy (Kruse et al., 2008) and decreases bone resorption and increases bone mass (Jin et al., 2014), while Ndfus4-deficient macrophages exhibit a pro-inflammatory phenotype, which can be attenuated by deletion of TLR2 and TLR4 again highlighting the relationship between innate immune receptors and the ETC (Jin et al., 2014). Together with the observation

that Gram-negative bacterial infections leads to a decrease in CI (Garaude et al., 2016) and that LPS stimulation induce RET and ROS production at CI (Mills et al., 2016), the study of Jin et al. further suggests that CI functions as a regulator of inflammatory responses by macrophages and that its downregulation or dysregulation act as an inflammatory signal.

4- The mitochondrial Electron Transport Chain (ETC): an immune rheostat during infection (~600words)

ETC byproducts as innate immune signals

Several byproducts of mitochondrial activity have been implicated in inflammatory processes (**Figure 2**). Shadel and co-workers recently found that perturbation of oxidative phosphorylation by moderate mitochondrial DNA (mtDNA) stress engages cytosolic signaling to initiate antiviral immune responses (West et al., 2015). Heterozygous 'transcription factor A, mitochondrial' (TFAM) deficiency leads to aberrant packaging of mtDNA and induces its escape to the cytosol where it is sensed by the innate immune receptor cGAS, which promotes STING-IRF3-dependent induction of interferon-stimulated genes. Mitochondrial ROS (mtROS) are generated as a byproduct of ETC activity or dysfunction, and mtROS have also been identified as a critical stimulator of cytosolic immune receptors [8]. Phagocytosis of microorganisms like bacteria elicits a respiratory burst leading to the generation of high amounts of ROS, which critically contribute to bacterial clearance (Lambeth, 2004). Although ROS generated by the phagosomal NADPH oxidase are considered the main component of the antimicrobial response in phagocytes, mtROS have recently emerged as important contributors to the bactericidal capacity and immune signaling in innate immune cells (West et al., 2011a). As many as 10 possible sites of ROS production have been proposed in mitochondria (Quinlan et al., 2013) with CI being one of the most relevant for macrophages (Kelly et al., 2015; Mills et al., 2016). In phagocytes, expression of mitochondrial uncoupling protein 2 (UCP2) dampens mtROS production (Arsenijevic et al., 2000) and TLR4 engagement decreased UCP2 expression in LPS-primed murine macrophages (Kizaki et al., 2002). Consequently, *Ucp2*$^{-/-}$ mice are more resistant to infection by intracellular pathogens *L. monocytogenes* (Rousset et

al., 2006) and *Toxoplasma gondii* (Arsenijevic et al., 2000), and UCP2-deficient macrophages exhibit increased killing of *S.* Typhimurium (Arsenijevic et al., 2000). Finally, a recent study by Kawataba and colleagues shows that treatment of mouse embryonic fibroblasts (MEFs) with chemical uncoupling molecules that decrease mitochondrial membrane potential lowers type IFN production (Koshiba et al., 2011). Precisely, they observed that the increased abundance of UCP-2 leads to proton leakage from mitochondria that impairs MAVS-dependent signaling. This suggests that the regulation of the mitochondrial ETC could directly influence the outcomes of innate immune signaling. Importantly, these studies highlights the notion that induction of RET, CI-mediated ROS production and subsequent increase in antimicrobial capacity and inflammatory responses by activated macrophages all require dampening of mitochondrial uncoupling mechanisms.

In further support of the important role of mitochondrial byproducts, West *et al.* observed that mitochondria co-localize and potentially interact with bacteria-containing phagosomes (West et al., 2011a). They proposed that mitochondrial ROS production is induced by cell surface TLRs (e.g. TLR2 or TLR4), in a process that requires the TLR-adaptor TRAF6 (TNF receptor associated factor 6) and the mitochondrial protein ECSIT (evolutionary conserved signaling intermediate), which was originally described as a CI assembly factor (Vogel et al., 2007). Hence, accumulating evidence suggests a direct interaction between mitochondria, or their metabolic byproducts and invading pathogens. Aside from their proposed bactericidal role, mtROS have been reported to be involved in the activation of key innate immune modules. NLRP3 is a member of the cytosolic Nod-like receptors (NLRs) family. Upon activation, NLRP3 assembles into a large cytosolic protein complex termed the inflammasome. Inflammasomes serve as a platform for autocatalytic activation of pro-inflammatory caspases, most prominently Caspase-1, which processes inflammatory cytokines IL-1β and IL-18 (Martinon et al., 2009). The NLRP3-inflammasome is activated by a variety of endogenous and exogenous stimuli during bacterial infection including bacterial pore-forming toxins or bacterial nucleic acids (Martinon et al., 2009; Sander et al., 2012). Similar to the observed changes in ETC SC composition, NLRP3 is also activated by bacterial RNA (Kanneganti et al., 2006; Sander et al., 2012). ROS production is common to many activators of NLRP3 inflammasome and mtROS seem to be critically involved in

NLPR3 inflammasome activation. ETC inhibition promotes mtROS generation, which increases NLRP3 activation and IL-1β secretion (Zhou et al., 2011). Accordingly, removal of dysfunctional ROS-producing mitochondria by directed autophagy (mitophagy) inhibits NLRP3 activation and IL-1β release (Nakahira et al., 2011). However, the precise molecular mechanisms that links mtROS to NLRP3 inflammasome activation remain unresolved. Macrophages deficient for the cytosolic superoxide dismutase 1 (SOD1) produce less IL-1β due to the inhibition of caspase-1 by oxidation and glutathionylation of specific cysteines (Meissner et al., 2008). In addition, mtROS generation may be dispensable for some NLRP3 activators. The oxazolidinone antibiotics linezolid, used in the treatment of methicillin-resistant *S. aureus* infections, can activate the NLRP3 inflammasome independently of ROS (Ament et al., 2002). In this context, NLRP3 activation occurs via its binding to the mitochondrial-specific phospholipid cardiolipin (Iyer et al., 2013). In addition to their role in NLRP3 inflammasome activation, mtROS also regulate LPS-mediated production of different cytokines including proIL-1b (Kelly et al., 2015; Mills et al., 2016; Tannahill et al., 2013) by stabilizing hypoxia-inducible factor 1a (HIF1a) (Tannahill et al., 2013). HIF1a has thus emerged as a key regulator of metabolic reprogramming in innate immune cells as exemplified by its important role in human monocyte functional adaptations occurring in sepsis (Shalova et al., 2015).

TCA cycle-derived metabolites are essential components of immune responses

Mitochondrial-derived metabolites generated in the Krebs cycle have important inflammatory signaling functions (Mills et al., 2017), and many of these metabolites accumulate in macrophages upon PRR ligation (Jha et al., 2015). Accumulation of succinate is associated with an enhanced production of pro-IL-1β and diminution of IL-10 in LPS-stimulated macrophages(Mills et al., 2016; Tannahill et al., 2013). Several mechanisms have been proposed to explain how succinate regulates cytokine production. First, succinate is structurally similar to a-ketoglutarate and has been shown to inhibit dioxygenases including the HIF1a regulators prolyl 4-hydroxylasess (PHD) (Hewitson et al., 2007; Selak et al., 2005). Second, succinate oxidation by CII and subsequent mtROS generation from CI leads to HIF-1a activation, which in turn induces *IL1B* transcription. Consequently, the

pharmacological inhibition of CII inhibits LPS- or bacteria-induced production of IL-1β and boosts IL-10 expression(Garaude et al., 2016; Mills et al., 2016). Fumarate, another TCA cycle metabolite and enzymatic product of CII also accumulates in activated innate immune cells. Work from Netea and co-workers has previously demonstrated that fumarate accumulates in monocytes activated by Beta-glucans due to replenishment of the Krebs cycle through glutaminolysis (Arts et al., 2016). In this context, fumarate was shown to inhibit KDM5 histone demethylases leading to epigenetic imprinting and altered cytokine expression profiles, a hall-mark of the recently proposed form of innate immune memory, also referred to as trained immunity (Arts et al., 2016; Netea et al., 2016). The link between metabolic adaptations and epigenetic imprinting has long since been noted in metabolic disorders, and it will be exciting to further investigate its role in immunity. Interestingly, fumarate also exerts direct antibacterial capacities. We could show that fumarate impairs the growth of *E. coli* and *S.* Typhimurium (Garaude et al., 2016). Dimethyl-fumarate (DMF) is an approved drug for the treatment of multiple sclerosis and psoriasis (Al-Jaderi and Maghazachi, 2016) but its potential as an antimicrobial drug has not been explored so far. Taken together, these studies place CII activation and subsequent fumarate production at the crossroads of many inflammatory signaling pathways, and moreover deliver further evidence for a direct interaction between mitochondrial molecules and invading pathogens. Another important TCA cycle metabolite driving inflammatory responses is citrate. The accumulation of citrate in TLR-activated innate immune cells is thought to support fatty acid biosynthesis, which is essential for the production of proinflammatory cytokine in DCs (B Everts et al., 2014). Citrate accumulation is likely caused by a decreased isocitrate dehydrogenase expression in LPS-activated cells. This also leads to accumulation of itaconate by decarboxylation of cis-aconitate, a citrate derivative and substrate of isocitrate dehydrogenase, by 'immune responsive gene-1' (IRG1). TLR4 activation strongly enhances IRG-1 expression in macrophages leading to increased production of itaconate (Michelucci et al., 2013; Naujoks et al., 2016). Recent studies have shown that itaconate limits the deleterious effects of excessive ROS production caused by sustained SDH (CII) activity (Lampropoulou et al., 2016). Pre-treatment of macrophages with itaconate prior to LPS stimulation inhibits SDH, increases

succinate levels, limits HIF-1a expression, and reduces inflammatory cytokine production. Similar to fumarate, itaconate posseses direct antimicrobial effects. IRG-1 dependent production of itaconate was shown to inhibit *in vitro* growth of several bacterial pathogens including *Legionella pneumophila*, *S. enterica* or *M. tuberculosis* (Michelucci et al., 2013; Naujoks et al., 2016). The expression of IRG-1 is in turn regulated by interferons, which are produced upon PRR activation. The antibacterial effects of itaconate may be at least partially explained by the inhibition of the glyoxylate shunt, an essential pathway in fatty acid- and acetate-dependent bacteria, which is absent in mammals. It was thus proposed that itaconate impairs microbial metabolism while sparing host cells from the potentially detrimental induction of metabolism-dependent inflammatory responses.

Concluding remarks (~150words)

Recent work revisiting old observations on metabolic reprogramming of phagocytes during infection and inflammation has placed mitochondria, and particularly the ETC, at the center of the newly discovered immuno-metabolic adaptations. The mitochondrial respiratory chain has the capacity to adjust its architecture and activity in response to innate immune signals, and thereby regulate immune responses. These findings may provide novel options for controlling immune responses. Manipulation of specific features of mitochondrial metabolism could represent a valid strategy to fine-tune immune responses. It is becoming clear that mitochondrial-derived metabolic intermediates, redox molecules, and ROS play essential roles in antimicrobial immune responses. More research is needed to deepen our understanding of the intricate cross-regulations between the mitochondrial respiratory chain and innate immune responses during infection.

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Disclosure statement

The authors declare no competing financial interests

Figure legends

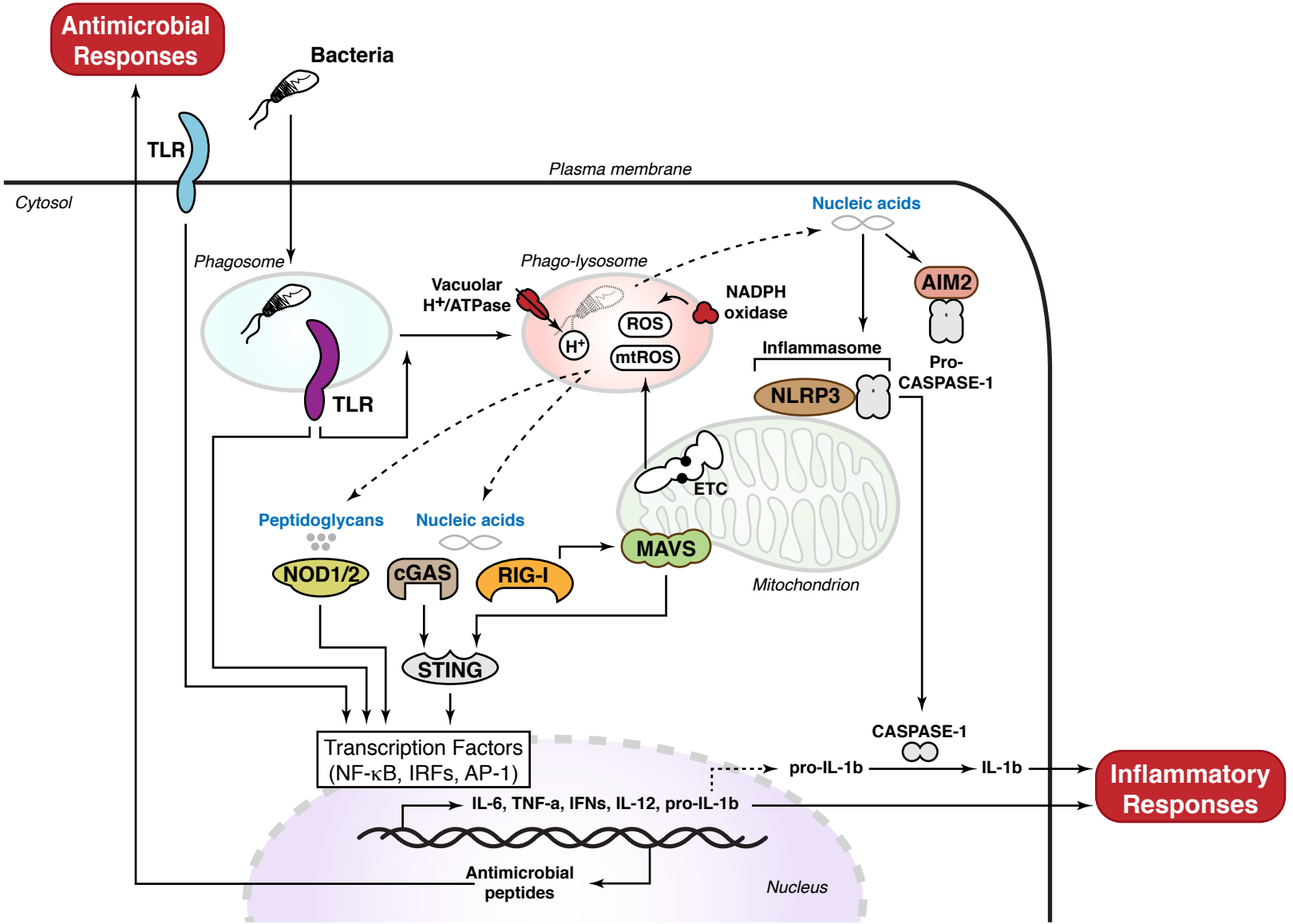
Figure 1: *Schematic view of pattern recognition receptor signaling and mitochondria in antibacterial immunity.*

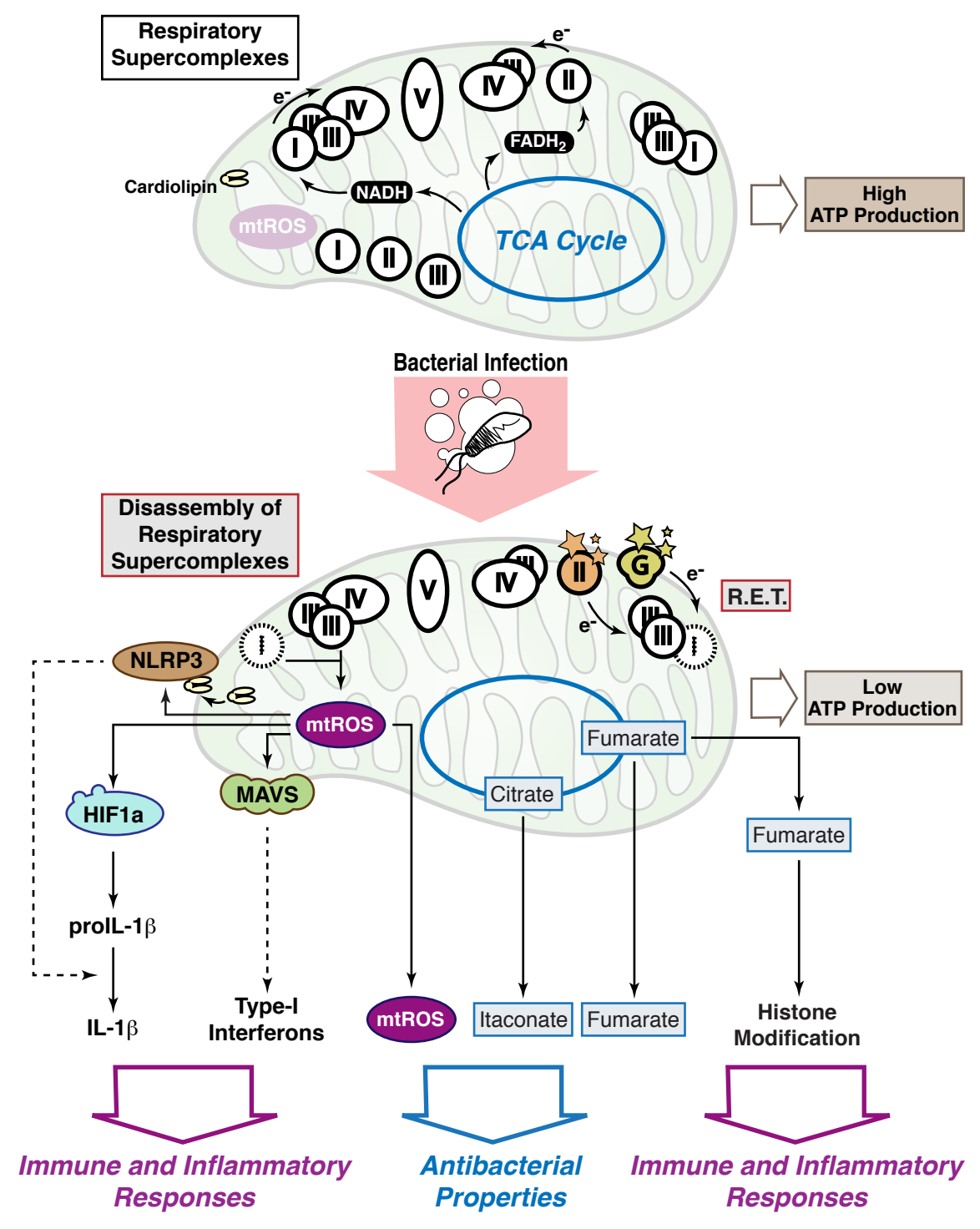
Professional phagocytes express a vast array of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) located on the cell surfaces and in the endosomal compartment, whereas as NOD-like receptors (NLRs) and other PRRs survey the cytosol. Upon ligation, TLRs and other PRRs including NOD1 and -2 activate various signaling cascades generally converging on NF-κB, MAPK, and IRFs, leading to massive transcriptional reprogramming and the production of cytokines like interleukin (IL)-6, tumor necrosis factor (TNF), pro-IL-1β and/or type-I interferons (IFNs). Upon activation, several NLRs assemble into large multimeric protein complexes termed inflammasomes, which catalyze the activation of Caspase-1. Active Caspase-1 in turn cleaves the proforms of IL-1β and IL-18 leading to the release of active IL-1β. Phagocytosis of bacteria leads to the activation of surface-, endosomal, and cytosolic PRRs. Induction of phagosomal ROS by TLRs contributes to bacterial killing. Mitochondria colocalize with bacteria-containing phagosomes, and mitochondrial (mt) ROS may further promote endosomal PRR signaling. Degradation of internalized bacteria and subsequent

release of bacterial nucleic acids leads to the activation of cytosolic and endosomal PRRs including TLR7/8, TLR9, cGAS, and RIG-I, all of which induce activate IRFs and induce the production of type-I IFNs. Bacterial nucleic acids also activate the NLRP3- and AIM2- inflammasomes. NLRP3 and other PRRS, as well as PRR adaptor proteins like MAVS localize to mitochondria.

Figure 2: *The mitochondrial electron transport chain (ETC) is at the core of innate immune responses against bacteria.* In macrophage, TCA cycle produces reductive equivalents of NADH and $FADH_2$ that provide electrons to the ETC, which in turn produces sufficient ATP to fulfill cellular energetic demands of resting cells. A significant proportion of the ETC is assembled in supercomplexes (SCs) including the respirasome ($CI/CII_2/CIV$). Mitochondrial ROS (mtROS) production is low in resting macrophage. The engagement of innate immune receptors including Toll-like receptors (TLRs) and NOD-like receptors (NLRs) during infection by live Gram-negative bacteria is thought to divert mitochondrial metabolism from energy production towards anabolic and signaling functions. Levels of SCs are decreased, probably due to destabilization of the complex I (CI) and is associated with a decrease in CI activity. In turn, CII and glycerol-3-phosphate dehydrogenase (G3PDH, here signalized as G) activities are augmented and provide electrons to the ETC and generate reverse electron transfer, accounting for CI destabilization and mitochondrial ROS (mtROS) production. MtROS can then activate hypoxia-induced factor 1a (HIF1α) to increase expression of the pro-inflammatory cytokine pro-IL-1b, and contribute to NLRP3 activation, which assembles the inflammasome to convert pro-IL-1β into the mature biologically active cytokine IL-1β. MtROS can also promote the recruitment of mitochondrial anti-viral signaling protein (MAVS) to the mitochondrial outer membrane where it interacts with stimulator of inferno genes (STING) to stimulate the production of antimicrobial Type-I interferons. MtROS also exert direct antibacterial properties when released in the microbe-containing phagosomes. Cardiolipin translocation from the inner membrane to the outer membrane in damaged mitochondria – as it can be induced by different bacterial infection – provides a docking site for NLRP3. microbes that trigger mitochondrial damages. Reprogramming of the tricarboxylic acid (TCA) cycle lead the accumulation of citrate, which can be metabolized into itaconate thereby

contributing to antibacterial capacities of macrophages. Fumarate also accumulates due to increased CII activity and has been show to possess antibacterial properties.


Antimicrobial Responses
Bacteria
TLR
Plasma membrane
Cytosol
Nucleic acids
Phagosome
Phago-lysosome
Vacuolar H+/ATPase
NADPH oxidase
ROS
mtROS
H+
AIM2
Inflammasome
Pro-CASPASE-1
NLRP3
TLR
ETC
Peptidoglycans
Nucleic acids
NOD1/2
cGAS
RIG-I
MAVS
Mitochondrion
STING
Transcription Factors (NF-κB, IRFs, AP-1)
CASPASE-1
pro-IL-1b
IL-1b
Inflammatory Responses
IL-6, TNF-a, IFNs, IL-12, pro-IL-1b
Antimicrobial peptides
Nucleus


Respiratory Supercomplexes
e-
e-
I
III
IV
V
II
FADH2
Cardiolipin
NADH
mtROS
TCA Cycle
High ATP Production
Bacterial Infection
Disassembly of Respiratory Supercomplexes
G
e-
e-
R.E.T.
NLRP3
mtROS
Low ATP Production
Fumarate
HIF1a
MAVS
Citrate
Fumarate
proIL-1β
IL-1β
Type-I Interferons
mtROS
Itaconate
Fumarate
Histone Modification
Immune and Inflammatory Responses
Antibacterial Properties
Immune and Inflammatory Responses