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## Mitochondria: Diversity in the regulation of NLRP3 inflammasome

Prajwal Gurung<sup>1</sup>, John R. Lukens<sup>1,2</sup>, and Thirumala-Devi Kanneganti<sup>1</sup>

<sup>1</sup>Department of Immunology, St. Jude Children's Research Hospital, Memphis, TN, 38105, USA

<sup>2</sup>Center for Brain Immunology and Glia (BIG), Department of Neuroscience, University of Virginia, Charlottesville, VA, 22908, USA

### Abstract

Recent studies have identified new roles for mitochondria in the regulation of autoinflammatory processes. Emerging data suggests that the release of danger signals from mitochondria in response to stress and infection promotes the formation of the inflammatory signaling platform known as inflammasomes. Activation of inflammasomes by damaged mitochondria results in caspase-1-dependent secretion of the inflammatory cytokines IL-1 $\beta$  and IL-18, and an inflammatory form of cell death referred to as pyroptosis. Here, we review recently described mechanisms that have been proposed to be involved in mitochondria-mediated regulation of inflammasome activation and inflammation. In addition, we highlight how aberrant regulation of mitochondria-induced inflammasome activation centrally contributes to the inflammatory process that are responsible for obesity and associated metabolic diseases.

### Keywords

Mitochondria; NLRP3; inflammasome; metabolic disease

### Historical perspective on the origin and importance of mitochondria

In the early 1960s it was proposed that the presence of mitochondria in cells were the result of evolutionary symbiosis, which is often referred to as endosymbiosis [1]. Several studies have argued that mitochondria were originally separate bacteria that ended up in a symbiotic relationship within mammalian cells during the course of evolution [2]. The mitochondrion is a major organelle required for numerous functions within the cell and is essential for survival. One of the earliest functions attributed to mitochondria is involvement in the glycolysis/TCA cycle/electron transport chain (**see Glossary**) that generates energy in the

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Corresponding author: Thirumala-Devi Kanneganti (Thirumala-Devi.Kanneganti@StJude.org).

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form of ATP (Figure 1A). Defects in the electron transport chain (transport system that generates the major amount of energy through oxidative phosphorylation) are usually detrimental to the host demonstrating the importance of mitochondria. One such example is cyanide poisoning that inhibits the ability of mitochondria to use oxygen to generate ATP [3]. Thus it was not surprising when in the late 1990s scientists discovered a role for mitochondria in promoting cell death (Figure 1B). Contrary to previous held beliefs that the loss of mitochondrial function and ATP produced induced cell death, it is now well established that apoptosis is actually an active process that requires energy. Several recent studies have identified a dynamic role for mitochondria in cell death, especially the role of mitochondria and mitochondrial products in the execution of an inflammatory form of cell death that is mediated by Nod-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome activation referred to as pyroptotic cell death. In this manuscript we will review the crucial role played by mitochondria in the regulation and activation of the NLRP3 inflammasome, discuss recently described modulators of these pathways and lastly detail metabolic disorders that result from mitochondrial dysfunction in context of the NLRP3 inflammasome.

### **Metabolic pathways, mitochondria and the generation of energy**

To fuel the cellular processes that are required to support life, food must be transformed into ATP. Three major metabolic pathways are involved in the stepwise generation of ATP; Glycolysis, the Tricarboxylic acid (TCA) cycle (also known as the Krebs cycle or citric acid cycle) and the Electron transport chain (also known as oxidative phosphorylation) (Figure 1A). Glycolysis, where glucose is converted into two pyruvate molecules, takes place in the cytoplasm. This process yields two ATP and two NADH molecules [4]. The pyruvates released in this step are shuttled into the TCA cycle, which takes place in the mitochondrial matrix. The TCA cycle generates two ATP, eight NADH and two FADH<sub>2</sub> molecules [5]. The NADH and FADH<sub>2</sub> molecules are then used in the electron transport chain, located in the inner membrane of mitochondria, to generate additional ATP [6]. In total, 32 ATP molecules are generated by the electron transport chain system. Therefore one glucose molecule generates a total of 34 ATP molecules within the mitochondria. Even though electron transport chain is an efficient ATP generating process, harmful reactive oxygen species (ROS) are generated as by-products of these reactions. Certain genetic conditions that cause imbalances in the cell metabolic pathways can result in the accumulation of ROS, which can induce cell damage and ultimately death.

### **The role of mitochondria in cell death**

Cell death regulated by mitochondria-associated proteins is termed 'mitochondrial apoptosis' (Figure 1B). The B cell lymphoma (BCL) protein family includes members that are both pro- and anti-apoptotic molecules, and regulate cell death by controlling mitochondrial outer membrane permeabilization (MOMP). The BCL-2 family members BAX, BAK and BOK are known to be pro-apoptotic and promote MOMP [7–9]. The BH3-only protein BID assists BAX activation and oligomerization on the mitochondrial outer membrane to promote MOMP [10]. BCL-2 was one of the first mitochondria-associated proteins shown to regulate apoptosis by inhibiting BAX-mediated initiation of MOMP [11]. Alongside BCL-2, family members known for their anti-apoptotic functions include BCL-

XL, BCL-W, MCL-1, BCL-RAMBO BCL-L10 and BCL-G [12]. Furthermore, BH3-only proteins such as BIM, BID, BAD, NOXA and PUMA promote mitochondrial apoptosis by inhibiting BCL-2, or directly inducing MOMP [12]. Thus, members of BCL-2 family tightly regulate mitochondrial apoptosis. Apart from BCL-2 family members, accumulation of ROS and increased calcium mobilization are also known to damage mitochondria through MOMP [13]. The final outcome of MOMP is the release of effector molecules from mitochondria that induce apoptosis through the activation of executioner caspases. The major effectors that mediate mitochondrial apoptosis as a result of MOMP are cytochrome c, apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (SMAC) and OMI (serine protease also known as HtrA2) [14]. All of these proteins are normally located within the intermembrane space of mitochondria, and when released into the cytoplasm they induce caspase-dependent apoptosis. While cytochrome c and AIF directly induce apoptotic protease activating factor 1 (APAF-1) oligomerization to promote formation of the apoptosome, SMAC and OMI act indirectly by sequestering inhibitor of apoptosis (IAP) proteins to release APAF-1 [14, 15]. Although caspase-dependent apoptosis is the main pathway for mitochondrial apoptosis, cell death can occur independent of caspases once mitochondrial function is perturbed [16].

Recent studies have now shown that cell death pathways are interconnected with inflammation, especially with the flurry of reports on caspase-8 promoting inflammasome activation [18].

### **Nod-like receptors and inflammasomes**

Nod-like receptors (NLRs) are an evolutionary conserved family of receptors that reside in the cytoplasm and recognize pathogen and danger associated molecular patterns (PAMPS and DAMPs). Of the 22 NLRs in humans and 34 NLRs in mice, only a handful have been formally characterized to date [19]. While NLRs can engage several signaling pathways upon ligand recognition, certain NLRs including NLRP1b, NLRP2, NLRP3, NLRC4, NLRP6, NLRP7 and NLRP12 form multimeric protein complexes comprising of adaptor apoptosis-associated speck-like protein containing a CARD (ASC) and caspase-1, termed inflammasomes [18, 20, 21]. However, it should be noted that the precise roles for NLRP2, NLRP6, NLRP7 and NLRP12 in inflammasome complex formation still remain incompletely characterized and thus future studies are needed to define their specific roles in inflammasome activation. Assembly of the inflammasome triggers autocleavage and activation of the caspase-1. Biologically active caspase-1 then processes the proinflammatory cytokines pro-IL-1 $\beta$  and pro-IL-18 into their bioactive mature forms. In addition, caspase-1 activation also provokes an inflammatory pyroptotic cell death.

NLRP3 is the best-characterized inflammasome, mainly because of its ability to be activated by a wide variety of ligands. Activation of the NLRP3 inflammasome requires two signals; a priming signal that is necessary for the upregulation of NLRP3 and pro-IL-1 $\beta$  followed by an activation signal that prompts NLRP3 to assemble the inflammasome complex [22]. Several DAMPs such as ATP, alum hydroxide, silica crystals, urea crystals, nigericin and active infections with bacteria, viruses and fungi activate the NLRP3 inflammasome [23]. How NLRP3 recognizes these different ligands and whether a common signal converges

downstream of PAMPs and DAMPs to activate NLRP3 has been a longstanding question in the field. Some of the proposed common events downstream from PAMPs and DAMPs are mitochondrial dysfunction and ROS generation [24, 25]. Several reports propose distinct mechanisms that explain the central role of mitochondria in the activation of the NLRP3 inflammasome. These studies provide mechanistic insight to explain how both mitochondrial dysfunction and gain-of-function mutations in *NLRP3* can result in similar metabolic disorders in patients.

## Mitochondria, inflammation and regulation of the NLRP3 inflammasome

The role of mitochondria in inflammation was initially suggested from studies that discovered a role for the mitochondrial antiviral signaling protein (MAVS) in eliciting antiviral interferon responses during viral infections [26–28]. With the discovery of the NLRP3 inflammasome and pyroptotic cell death in the last decade, the involvement of mitochondria in these pathways has similarly evolved. Several distinct pathways emanating from mitochondria, and in particular mitochondrial dysfunction, have been proposed to modulate NLRP3 inflammation activation (Figure 2). Even though these pathways are shown as individual separate pathways in Figure 2 and discussed below likewise, it is important to note that these pathways are interconnected and might not be mutually exclusive.

### Mitochondrial ROS

Mitochondria are the major source of cellular ROS. The electron transport chain in the mitochondrial inner membrane is critically involved in the generation of energy, where oxygen acts as an electron acceptor. When the electron transport chain breaks down, ROS can accumulate to toxic levels within cells. Several studies have shown that ATP- and monosodium urate (MSU) crystal-induced ROS production activates inflammasomes [29, 30]. ROS generated during phagocytosis of silica and asbestos particles activated NLRP3 inflammasome formation in macrophages [31]. Conversely, treatment of macrophages with the ROS inhibitors N-acetyl-L-cystine [32] or (2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate (APDC) [31] can inhibit silica and asbestos-induced NLRP3 inflammasome activation. Furthermore, inhibition of mitochondrial complex-I by rotenone or complex-III by antimycin A induces robust ROS production by mitochondria [33, 34]. This enhanced ROS production is sufficient to drive NLRP3 inflammasome activation, suggesting mitochondrial ROS as a direct activator of the NLRP3 inflammasome [25].

ROS activation is not an absolute requirement for activation of all NLRP3 inflammasomes. In particular, stimulation of macrophages with linezolid (from the oxazolidinone class of antibiotics) or infection of macrophages with influenza and encephalomyocarditis viruses does not require ROS for activation of the NLRP3 inflammasome [35, 36]. Additionally, whether ROS production is a prerequisite or just a consequence of inflammasome activation remains obscure. Future studies looking at a time lapse release/production of ROS following stimulation of macrophages during NLRP3 inflammasome activation at a single cell level will be instrumental in defining the dynamics and requirement of ROS production.

## Calcium mobilization and potential role for mitochondria

Calcium ( $\text{Ca}^{2+}$ ) is a secondary messenger that plays pivotal roles in the regulation of multiple signaling pathways within cells. Aberrant fluxes in  $\text{Ca}^{2+}$  can result in catastrophic cellular events and thus cells deploy multiple strategies to control intracellular  $\text{Ca}^{2+}$  levels. In particular, mitochondria play a pivotal role in regulating  $\text{Ca}^{2+}$  levels by accepting  $\text{Ca}^{2+}$  that is released from the endoplasmic reticulum [37]. While a controlled level of  $\text{Ca}^{2+}$  storage within the mitochondria modulates  $\text{Ca}^{2+}$  signaling,  $\text{Ca}^{2+}$  overload can cause mitochondrial dysfunction. A role for  $\text{Ca}^{2+}$  influx to the cytoplasm in NLRP3 inflammasome activation is substantiated by studies using the  $\text{Ca}^{2+}$ -chelating agent BAPTA-AM [38, 39]. Incubation of macrophages with BAPTA-AM before co-stimulation with lipopolysaccharide (LPS) and ATP or infection with *Mycobacterium abscessus* inhibited NLRP3 inflammasome activation in a dose dependent manner [38, 39]. Additional studies confirmed that  $\text{Ca}^{2+}$  influx into the cytoplasm following ATP, ultraviolet B radiation (UBV), and cholesterol-dependent cytolysins treatment activates the NLRP3 inflammasome in LPS primed macrophages [40–42]. Furthermore,  $\text{Ca}^{2+}$  detection by the  $\text{Ca}^{2+}$ -sensing receptor (CaSR) directly promotes NLRP3 inflammasome activation [43, 44]. Mounting evidence now suggests that an influx of  $\text{Ca}^{2+}$  into the cytoplasm is a common proximal event involved in activation of the NLRP3 inflammasome [44, 45]. A recent study suggested that  $\text{K}^+$  efflux could be upstream of  $\text{Ca}^{2+}$  influx during NLRP3 inflammasome activation [46], although how these two pathways may be linked is still ambiguous and will need further investigation.

Most of the studies looking at the role of  $\text{Ca}^{2+}$  overload suggest a direct role for  $\text{Ca}^{2+}$  in NLRP3 inflammasome activation, although these studies are not without caveats.  $\text{Ca}^{2+}$  is a secondary messenger required for various cellular functions and is abundantly present within the cellular cytoplasm. Why does this  $\text{Ca}^{2+}$  present within the cytoplasm not activate NLRP3 inflammasomes? Is there a  $\text{Ca}^{2+}$  threshold that determines activation of the NLRP3 inflammasome? One hypothesis that could possibly explain these discrepancies is that  $\text{Ca}^{2+}$  overload causes mitochondrial dysfunction and mitochondrial dysfunction eventually induces NLRP3 inflammasome activation. Whether  $\text{Ca}^{2+}$  induced NLRP3 inflammasome activation requires mitochondria remains unknown and experiments with cells lacking mitochondria could answer these questions.

## Reductions in $\text{NAD}^+$ trigger NLRP3 inflammasome assembly

Inducers of the NLRP3 inflammasome such as ATP and nigericin have been shown to cause mitochondrial damage. Moreover, ATP- and ROS-induced damage to mitochondria causes reductions in cytoplasmic levels of  $\text{NAD}^+$  [47]. Misawa *et al.* further showed that the reduced  $\text{NAD}^+$  induces  $\alpha$ -tubulin dependent assembly of mitochondria that ultimately promotes recruitment of ASC and NLRP3 [47]. This study clearly demonstrated that mitochondrial dysfunction-associated reduction in  $\text{NAD}^+$  promotes NLRP3 inflammasome activation. Several other models have been described where specific molecules on mitochondria such as cardiolipin and mitofusins promote NLRP3 inflammasome assembly. Further investigations are required to delineate whether  $\text{NAD}^+$  is a specific mechanism or a precursor to these pathways.

### Cardiolipin in NLRP3 inflammasome activation

Cardiolipins are lipids that facilitate optimal oxidative phosphorylation in mitochondria [48]. Interestingly, cardiolipin deficient cells are resistant to cell death [49]. Recent studies by Iyer *et al.* show that cardiolipins are critical modulators of NLRP3 inflammasome activation [35]. Mitochondrial destabilization induces externalization of cardiolipin to the outer membrane of the mitochondria [50]. Iyer *et al.* showed that cardiolipin on the mitochondrial outer membrane directly binds to the leucine rich repeats (LRR) of NLRP3 and activates the NLRP3 inflammasome [35]. Knockdown of cardiolipin synthase (CLS; the enzyme that synthesizes cardiolipin) using siRNA, although only ~50% efficient, resulted in a significant reduction in caspase-1 activation and subsequent IL-1 $\beta$  release [35]. In a cell free system, addition of cardiolipin is sufficient to activate the NLRP3 inflammasome and induce caspase-1 cleavage. This study was the first to show that mitochondria-associated cardiolipin is required for recruitment and activation of the NLRP3 inflammasome. Molecular insights into how cardiolipin binding and its ligand activity induce NLRP3 inflammasome activation are needed. Studying CLS-deficient cells and mice will ultimately be required to establish the direct role of cardiolipin in cell death and NLRP3 inflammasome activation.

### Mitofusins in NLRP3 inflammasome activation

Mitofusin 1 and 2 are mitochondrial outer membrane associated proteins that are crucial for mitochondrial fusion. Mice lacking either mitofusin 1 or mitofusin 2 are embryonic lethal, demonstrating the critical roles played by these proteins *in vivo* [51, 52]. *In vitro* studies with cells lacking either mitofusin 1 or 2 have corroborated their roles in mitochondrial fusion and loss of mitochondrial membrane potential [51, 52]. A recent study has now implicated mitofusins in the activation of the NLRP3 inflammasome during influenza and encephalomyocarditis virus (EMCV) infections [36]. Influenza and EMCV infections induce a loss of mitochondrial membrane potential, which is essential for NLRP3-mitofusin 1 and NLRP3-mitofusin 2 interactions. Biochemical analysis identified that the 4,3 hydrophobic heptad repeat (HR) region 1 of mitofusin 2 interacts with NLRP3 [36]. Knockdown of mitofusin 2 significantly reduced caspase-1 activation and IL-1 $\beta$  production in response to viral infections. While this study clearly suggests mitofusins as potential docking sites for NLRP3 on the mitochondria, whether mitofusins directly activate NLRP3 or merely act as a docking site for inflammasome assembly needs to be further investigated. Even though mitofusin 1 was able to equally recruit NLRP3, its role in NLRP3 inflammasome activation remains obscure, and whether NLRP3-mitofusin 1- mitofusin 2 are present in the same complex is not known. Lastly, the roles for both mitofusin 1 and mitofusin 2 need to be confirmed using cells deficient in these molecules and by generating mitofusin 1 and mitofusin 2 conditional knockout mice.

### Mitochondrial DNA

Bacterial and viral RNA can also activate the NLRP3 inflammasome [53]. Similarly, self and foreign DNA in the cytoplasm have also been shown to activate the AIM2 inflammasome [54, 55]. Mitochondrial DNA is released into the cytoplasm following intrinsic cell death, resulting in inflammation [56, 57]. Following on from these reports, two

separate studies have now shown that mitochondrial DNA in the cytoplasm activates the NLRP3 inflammasome [17, 58]. Nakahira *et al.* suggested that mitochondrial ROS is required for the release of mitochondrial DNA and activation of the NLRP3 inflammasome [59]. Shamida *et al.* further showed that mitochondrial death is a prerequisite for the release of oxidized mitochondrial DNA into the cytoplasm [58]. The oxidized mitochondrial DNA directly bound NLRP3 to activate the inflammasome. Further supporting a direct role for mitochondrial DNA, cells lacking mitochondrial DNA (generated by ethidium bromide treatment) were unable to provoke NLRP3 inflammasome activation [17]. Interestingly, NLRP3 is required for the release of mitochondrial DNA during inflammasome activation [17]. This suggests that mitochondrial DNA might act in a positive feedback loop to potentiate NLRP3 inflammasome activation. In contrast, a recent report demonstrated that mitochondrial apoptosis is totally dispensable for NLRP3 inflammasome activation using a series of genetically deficient mouse models [60]. Additional studies are needed to clarify different ways mitochondrial apoptosis may be involved in the activation of the NLRP3 inflammasome.

### **Mitochondrial antiviral signaling protein (MAVS) in NLRP3 inflammasome activation**

MAVS are central adapter molecules required for transmitting interferon responses and signaling events downstream of nucleic acid sensors such as retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated protein 5 (MDA-5) [26–28]. The discovery that MAVS are localized in the mitochondrial membrane implicated mitochondria in innate immune signaling [27]. MAVS form prion like aggregates on the outer membrane of mitochondria to perpetuate antiviral signaling [61], and it is the caspase activation and recruitment domains (CARDs) in MAVS that are necessary and sufficient to form these aggregates *in vitro* [61]. Interestingly, MAVS has also been reported to be required for NLRP3 inflammasome activation in response to non-crystalline NLRP3 activators such as ATP, nigericin and poly I:C [62]. Indeed, MAVS were required for recruitment of NLRP3 to the mitochondria, although surprisingly this recruitment did not require ASC and was solely dependent on the pyrin domain of NLRP3 [62]. Although the requirement of MAVS for NLRP3 inflammasome activation in response to poly I:C has been corroborated [63, 64], several groups have been unable to recapitulate a role for MAVS in NLRP3 inflammasome activation in response to ATP or nigericin [60, 63, 64]. Biochemical studies evaluating the interactions of MAVS with NLRP3 during stimulation with NLRP3 agonists will shed light onto how MAVS are specifically required for poly I:C induced NLRP3 inflammasome activation. Moreover, studies elucidating the contribution of the mitochondrial membrane in these interactions will be important to fully understand the specific nature of MAVS in activating NLRP3 inflammasome.

### **Negative regulators of mitochondrial damage and NLRP3 inflammasome activation**

Mitochondria have emerged as central regulators of NLRP3 inflammasome activation and a plethora of studies have elucidated the various mitochondrial components required to trigger inflammasome signaling. Studies examining the mitochondrial pathways and components that negatively regulate NLRP3 inflammasome activation are scarce. Autophagy is a

fundamental cellular process that is required for clearance of unwanted or damaged organelles within the cell. Autophagy is also a mechanism for the cell to recycle critical nutrients. Inhibition of autophagy machinery by 3-methyladenine (3-MA) instigated activation of the NLRP3 inflammasome [25]. Nakahira *et al.* showed that autophagy regulates the clearance of damaged mitochondria from the cytoplasm, a process now known as mitophagy [59]. Genetic ablation of LC3B or Beclin 1, both critical modulators of autophagy, results in the accumulation of damaged mitochondria and enhanced NLRP3 inflammasome activation during ATP stimulation of macrophages [59]. As such, mice deficient in LC3B or Beclin 1 are highly susceptible to sepsis and LPS-induced endotoxemia. Similarly, mice deficient in Atg16L1, a critical component of autophagy machinery, show enhanced NLRP3 inflammasome activation [65].

In a separate study involving influenza virus infections, Lupfer *et al.* demonstrated a role for the nucleotide-binding oligomerization domain containing 2 (NOD2) protein and its adaptor receptor interacting protein kinase 2 (RIPK2) in promoting mitophagy to clear damaged mitochondria from the cells [66]. NOD2 and RIPK2-deficient mice are highly susceptible to influenza virus infection as a result of uncontrolled inflammation and hyperactive NLRP3 inflammasomes [66]. Consistent with their role in autophagy [67], NOD2 and RIPK2-deficient cells accumulate damaged mitochondria. Phosphorylation of unc-51 like autophagy activating kinase 1 (ULK1) is an important event that promotes mitophagy [68]. Mechanistic studies showed that RIPK2 promotes ULK1 phosphorylation to initiate the formation of the autophagosome and clearance of damaged mitochondria [66]. These studies clearly highlight the need for future work to examine regulatory pathways that purge damaged mitochondria during inflammation, infection and cellular stress. Any defects in these pathways can promote uncontrolled inflammation and immunopathology that can be detrimental to the host. Thus, understanding the molecular mechanisms of how these pathways can be intersected will be helpful in designing potential therapeutics.

## **Mitochondria dysfunction-induced inflammasome activation drives obesity-associated disease**

Mitochondria play essential roles in the maintenance of metabolic health and disruption of mitochondrial activity is believed to be a leading cause of obesity and associated metabolic disorders. Obesity-driven inflammation provokes a perpetual cycle of metabolic dysfunction and contributes to the development of obesity-related disorders including type 2 diabetes mellitus (T2DM), hepatosteatosis, dyslipidemia and atherosclerosis [69]. Under healthy conditions, mitochondria contribute to the regulation of metabolic homeostasis by supplying cells with ATP, controlling energy expenditure, coordinating the disposal of ROS and regulating intrinsic cell death. However, nutrient overload that results from excessive consumption of energy rich foods and lack of physical activity can cause the accumulation of partially oxidized substrates (such as fatty acids) in mitochondria and aberrant mitochondrial ROS production. Unchecked ROS production and the buildup of improperly oxidized substrates ultimately lead to impaired mitochondrial function [70]. Indeed, mitochondria in obese individuals exhibit altered morphology, decreased ATP output, impaired bioenergetics and diminished fatty acid oxidation [71–77].



Caloric restriction and exercise can promote improved mitochondrial bioenergetics efficiency and biogenesis [78]. Reductions in ROS production and proliferation of mitochondria are believed to be responsible for the enhanced mitochondrial activity that results from restrictions in caloric intake [79, 80]. Mitochondrial dysfunction has also been reported to contribute to other metabolic syndrome disorders that are associated with obesity. For instance, defective mitochondrial activity is believed to be involved in T2DM pathogenesis [74]. T2DM is associated with impaired mitochondrial respiration and bioenergetics [73, 75], and inherited defects in mitochondrial function are reported to contribute to the increased risk of severe insulin-resistance in offspring from parents with T2DM [76].

Although defective mitochondrial activity has been extensively linked to obesity-associated disease, how aberrant regulation of mitochondria mechanistically contributes to the induction of the inflammatory responses that are responsible for driving obesity and metabolic dysfunction had remained poorly characterized until recently. The NLRP3 inflammasome can sense danger-associated signals that are induced by defective mitochondria. An NLRP3 inflammasome-mediated inflammatory cascade then promotes adipose tissue inflammation and metabolic dysfunction. Specifically, it was discovered that the NLRP3 inflammasome can detect various obesity-associated DAMPs including ROS, damaged mitochondria, saturated fatty acids and ceramides; and that the activation of caspase-1 in the inflammasome complex spurs inflammatory signaling [24, 81–84]. Additionally, the development of adipose tissue inflammation and metabolic impairment in both diet-induced and genetic models of obesity is associated with deregulated caspase-1 activation and hyperinflammatory secretion of the inflammasome-derived cytokines IL-1 $\beta$  and IL-18 in various metabolic organs [81, 83, 85].

Defective regulation of mitochondrial activity has recently been identified as a central instigator of NLRP3 inflammasome-mediated inflammation in obesity-associated disease. Caloric overload causes adipocytes to undergo considerable differentiation and expansion in order to accommodate the storage of energy in the form of lipids [86]. These changes result in the dramatic upregulation of mitochondrial ROS levels. ROS is a potent inducer of NLRP3-inflammasome activation [24, 25, 59] and ROS is believed to be a prominent inducer of proinflammatory responses that are mediated by adipose tissue infiltrating macrophages. The buildup of damaged mitochondria that results from defective autophagy in obese individuals can also spur NLRP3-dependent inflammasome activation and associated cytokine production. In a recent study, it was shown that the excess saturated fatty acid palmitate causes the buildup of defective mitochondria as a result of impaired autophagy [82]. In this model, palmitate-mediated disruption of AMP-activated kinase (AMPK) activity caused impaired autophagy and the resulting accumulation of damaged mitochondria was responsible for inciting downstream NLRP3 inflammasome-induced inflammatory signaling.

NLRP3 inflammasome-induced cytokine production contributes to impaired insulin signaling and glucose homeostasis by inducing apoptosis in insulin-producing  $\beta$ -cells in the pancreas [87] and by promoting insulin resistance in adipocytes [83]. The release of IL-1 $\beta$  and IL-18 also results in potent recruitment of inflammatory macrophages and T cells into

adipose tissue. The mobilization of activated immune cells into metabolic organs provokes the release of additional obesity-associated danger signals from damaged tissue and spurs the development of a perpetual state of low-grade inflammation. Importantly, genetic ablation of critical NLRP3 inflammasome components and pharmacological inhibition of caspase-1 in high fat diet-fed mice markedly limited the development of obesity and resulted in improved insulin sensitivity and glucose tolerance [83].

In recent clinical trials, blockade of IL-1 signaling with IL-1R antagonist treatment was found to stabilize blood glucose levels and improve pancreatic  $\beta$ -cell function [88]. The efficacy of IL-1R antagonists in the treatment of T2DM underscores the central importance of inflammasome-derived cytokines in driving metabolic disease, however complete abrogation of IL-1 signaling can pose serious side effects including increased susceptibility to infections and some forms of cancer. Thus, improved strategies are needed to target the specific inflammatory pathways that instigate metabolic disease and obesity. Recent studies in animal models, which identify critical roles for NLRP3 inflammasome-mediated cytokine production in driving obesity-associated inflammation and metabolic dysfunction, suggest that therapeutics that target inflammasome activation may hold great promise in the treatment of obesity and associated metabolic disorders.

## Concluding remarks

Mitochondria have diverse functions that range from generating and storing energy for the cell, controlling cell death, inflammation and regulating NLRP3 inflammasome activation. Thus it is not surprising that defects in either mitochondrial function or NLRP3 inflammasome drive similar metabolic disorders such as obesity-associated diseases. While recent studies have provided an improved insight into how mitochondria regulate NLRP3 inflammasome activation, there are still multiple important questions that remain (Box 1). One of the limitations in understanding the role of mitochondrial function *in vivo* has been the generation of mice with deficiencies in mitochondrial proteins. This is mostly due to the critical roles played by these molecules in development and cell death. With the development of newer technologies such as the Crispr/Cas9 gene targeting system and conditional deletion of genes, several of these questions will hopefully be answered in coming years. Such future studies will result in a more complete understanding of how mitochondria are involved in regulating these diverse functions within the cell.

### Box 1

#### Outstanding Questions

1. How do mitochondria decide the fate of the cell between apoptotic and pyroptotic cell death?
2. Are mitochondria required for NLRP3 inflammasome activation?
3. Several mitochondrial components promote NLRP3 inflammasome activation following cellular stress, injury or infection. Is there a common pathway where these mitochondrial components converge to activate the NLRP3 inflammasome?

4. Why is mitochondrial-dependent regulation of inflammasome activation specific to the NLRP3 inflammasome and not to other types of inflammasomes such as the NLRC4, AIM2 and NLRP1b inflammasomes?
5. What comes first: inflammasome activation or mitochondrial dysfunction?
6. What are the common metabolic pathways triggered by mitochondrial and NLRP3 dysfunction that could be targeted to generate novel therapeutics?

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## Glossary

<b>Apoptosis</b>	Programmed cell death that is executed by caspases. Apoptosis is commonly known as a silent cell death as this process does not induce inflammation
<b>Apoptosome</b>	During mitochondrial apoptosis (intrinsic cell death), cytochrome c release from the mitochondria prompts the formation of a multimeric protein complex containing APAF-1. The APAF-1 complex, known as the apoptosome then recruits and activates executioner caspases to initiate apoptosis
<b>Adenosine triphosphate (ATP)</b>	ATP is the cellular source of energy. Cells store energy in the form of ATP in the mitochondria. Energy is stored in the phosphate bonds of ATP, such that when ATP is broken down to ADP, energy is released
<b>Autophagy</b>	A highly conserved cellular mechanism that is responsible for recycling of damaged cellular organelles within the cytoplasm of the cell
<b>Cardiolipin</b>	Lipids present in the inner membrane of mitochondria. While cardiolipin is known for its role in mitochondrial oxidative phosphorylation and induction of apoptosis, recent work has shown that cardiolipin recruits and activates the NLRP3 inflammasome
<b>Crispr/Cas9</b>	A novel gene editing system that allows very efficient and targeted gene editing in both cells and mice <i>in vivo</i>
<b>Danger associated molecular patterns (DAMPs)</b>	DAMPs are cues that inform a cell about a possible invasion or danger. Some common examples of DAMPs include DNA in the cytosol, ATP, heat shock proteins and HMGB1

<b>Electron transport chain</b>	The electron transport chain present in the inner-membrane of the mitochondria is the major site for oxidative phosphorylation, where electrons from NADH and FADH <sub>2</sub> generated in glycolysis and the TCA cycle are used to generate ATP
<b>Glycolysis</b>	Glucose is converted into pyruvate by a metabolic pathway known as glycolysis. This is the first critical step for conversion of glucose molecules into ATP, as products generated in the glycolysis step are fed into the TCA cycle. Glycolysis also generates ATP, although it is not as efficient
<b>Inflammasome</b>	The inflammasome is a multimeric protein complex that consists of a Nod-like receptor sensor, bipartite adaptor ASC and caspase-1. Assembly of the inflammasome complex results in the activation of caspase-1. Bioactive caspase-1 cleaves pro-IL-1 $\beta$ and pro-IL-18 into their active forms and also provokes an inflammatory form of cell death that is referred to as pyroptosis
<b>Mitochondrial antiviral signaling protein (MAVS)</b>	MAVs is an adaptor protein localized in the outer-membrane of the mitochondria. MAVS is required for interferon signaling downstream of nucleic acid sensors such as RIG-I and MDA-5 and thus MAVS is a critical initiator of antiviral immune responses
<b>Metabolic disorders</b>	Disorders that arise as a result of disruption in the process of metabolism which is required to generate energy for the cell
<b>Mitophagy</b>	A process whereby damaged mitochondria are targeted for degradation and recycling via autophagy
<b>Mitofusins</b>	Mitofusins are GTPases present in the outer-membrane of mitochondria and mediate mitochondrial fusion. Mitofusins can interact with NLRP3 and regulate inflammasome activation
<b>Mitochondrial outer membrane permeabilization (MOMP)</b>	MOMP is a result of series of events that leads to pore formation in the mitochondrial membrane. MOMP results in the release of mitochondrial contents into the cytoplasm, which activates caspases to induce apoptosis
<b>Nod-like receptors (NLRs)</b>	NLRs are cytoplasmic pattern recognition receptors that are characterized by the presence of a C-terminal leucine rich repeat, a mid nucleotide oligomerization domain (NOD) [32] and an N-terminal activation domain. NLRs detect PAMPs and DAMPs in the cytoplasm and initiate immune responses by instructing inflammatory signaling and inflammasome formation
<b>Pathogen associated</b>	PAMPs can be recognized by cells to initiate immune responses against pathogens. Some common pathogen associated molecular patterns that are recognized by our immune cells include

<b>molecular patterns (PAMPs)</b>	lipopolysaccharides (LPS), peptidoglycans and double stranded RNA
<b>Pyroptosis</b>	Programmed cell death that is mediated specifically by activation of inflammasomes. Caspase-1 and caspase-11 have been shown to induce pyroptotic cell death, which results in the release of cellular contents and is inflammatory
<b>Reactive oxygen species (ROS)</b>	ROS are chemically reactive compounds that consist of oxygen ions and peroxidases. ROS are generated as byproducts of several metabolic pathways and are used by cells to combat infection
<b>TCA cycle</b>	The tricarboxylic acid cycle (also known as the citric acid cycle, or Krebs cycle; named after Hans Krebs) takes place in the mitochondrial matrix and is the second major metabolic pathway involved in the generation of ATP from glucose. TCA cycle accepts pyruvate generated from glycolysis to generate ATP and reducing agents NADH and FADH <sub>2</sub> .

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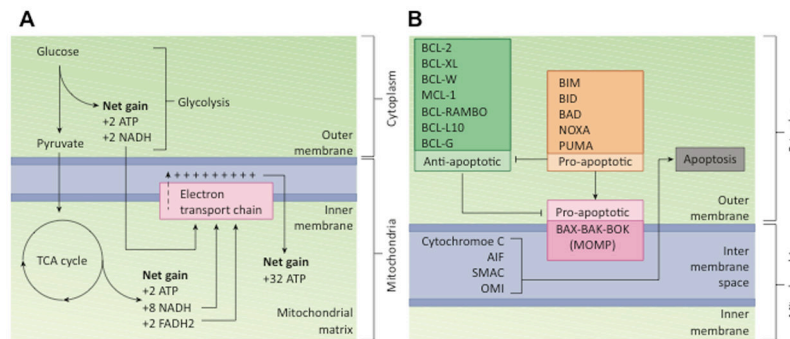
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### Highlights

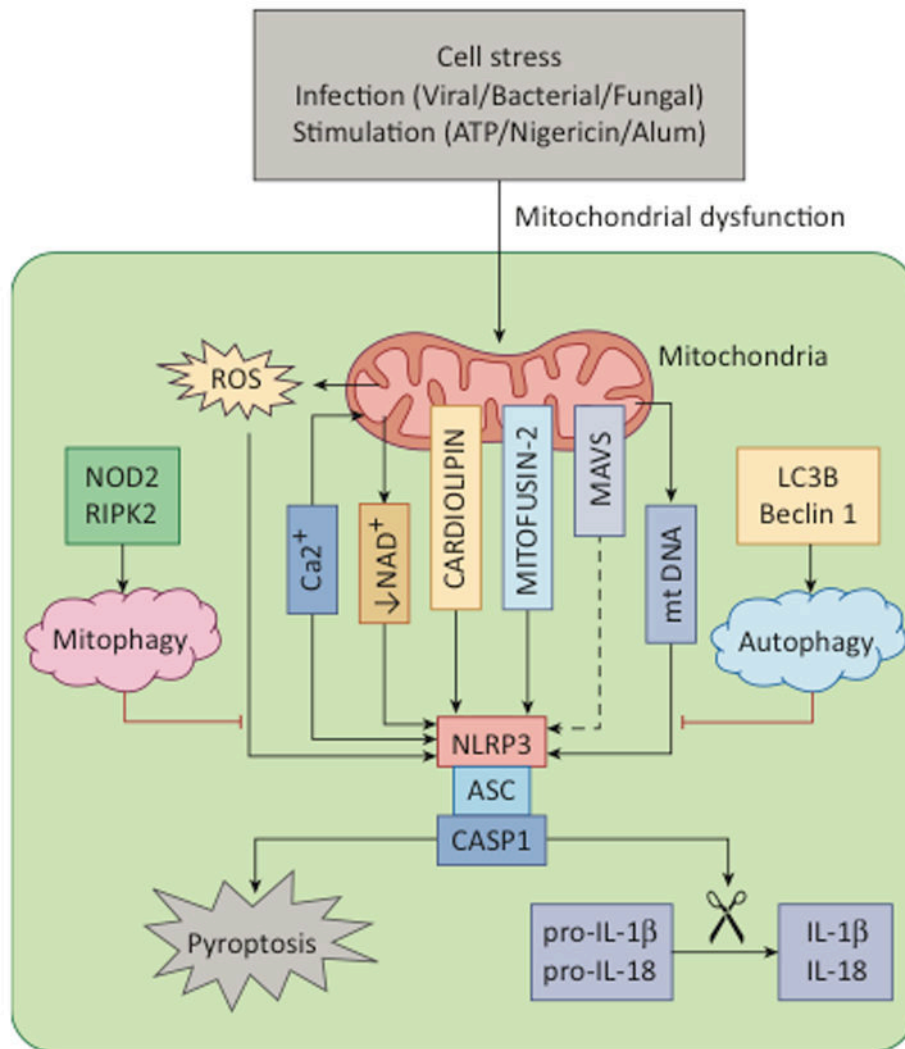
1. Mitochondrial dysfunction induces specific activation of NLRP3 inflammasome.
2. Mitochondria recruit NLRP3 and aid the assembly of NLRP3 inflammasome.
3. Autophagy/mitophagy clear damaged mitochondria and inflammasome activation.
4. Mitochondria and NLRP3 dysfunction promote metabolic diseases.



**Figure 1. Mitochondria are critical for generation of ATP and regulating apoptosis**

**A.** Energy is stored in the form of ATP in the mitochondria. Three major metabolic pathways convert glucose into ATP; Glycolysis, the TCA cycle and the Electron transport chain. Glycolysis is the first step in which one glucose molecule is converted into two pyruvate, two ATP and two NADH molecules in the cytoplasm. The TCA cycle takes place in the mitochondrial matrix, and generates two ATP, 8 NADH and two FADH<sub>2</sub> molecules. The Electron transport chain generates 32 ATP molecules by utilizing the NADH and FADH<sub>2</sub> generated in the previous steps.

**B.** BCL-2 family members are centrally involved in mitochondrial apoptosis. Activated BAX, BAK and BOK form pores in the mitochondrial membrane. Anti-apoptotic BCL-2 family members BCL-2, BCL-xL, BCL-W, MCL-1, BCL-RAMBO, BCL-L10 and BCL-G have also been shown to inhibit mitochondrial apoptosis by inhibiting BAX, BAK or BOK and subsequent mitochondrial outer membrane permeabilization (MOMP). Anti-apoptotic BCL family members BIM, BID, BAD, NOXA and PUMA on the other hand have been identified to promote apoptosis by either promoting translocation of BAX to the mitochondrial membrane or inhibiting the function of anti-apoptotic BCL proteins. Translocation of BAX, BAD and BOK results in MOMP and release of apoptotic effectors such as cytochrome C, AIF, SMAC and OMI, all of which induce apoptosis.



**Figure 2. Mitochondria at the center of NLRP3 inflammasome activation**

NLRP3 inflammasomes are activated by a myriad of stimuli that ranges from virus, bacteria, and fungi to danger associated molecular patterns (DAMPs) such as ATP, nigericin and alum. Once activated, NLRP3 forms a multimeric protein complex with associated speck-like protein containing a CARD (ASC) and caspase-1 (CASP1) termed the inflammasome. Caspase-1 is activated in the inflammasome complex, which cleaves pro-IL1 $\beta$  and pro-IL-18 into their bioactive mature forms. Mitochondria are central regulators of NLRP3 inflammasome activation. Mitochondrial reactive oxygen species (ROS), Ca<sup>2+</sup> overload, reduced NAD<sup>+</sup>, cardiolipin, mitofusin, mitochondrial antiviral signaling protein (MAVS) and mitochondrial DNA (mtDNA) have all been shown to promote NLRP3 inflammasome activation. NOD2/RIPK2-dependent mitophagy and LC3B/Beclin 1-mediated autophagy are involved in the clearance of damaged mitochondria, and thus negatively regulate NLRP3 inflammasome activation.