

SPECIAL FEATURE REVIEW

The rOX-stars of inflammation: links between the inflammasome and mitochondrial meltdown

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E-mail: k.schroder@imb.uq.edu.auReceived 4 August 2019;
Revised 21 January 2020;
Accepted 21 January 2020

doi: 10.1002/cti2.1109

Clinical & Translational Immunology
2020; 9: e1109**Abstract**

The nod-like receptor protein 3 (NLRP3) inflammasome drives inflammation in response to mitochondrial dysfunction. As metabolic powerhouses with prokaryotic ancestry, mitochondria are a cache for danger-associated molecular patterns and pathogen-associated molecular pattern-like molecules that elicit potent innate immune responses. Persistent mitochondrial damage caused by infection, or genetic or environmental factors, can lead to inappropriate or sustained inflammasome signalling. Here, we review the features of mitochondria that drive inflammatory signalling, with a particular focus on mitochondrial activation of the NLRP3 inflammasome. Given that mitochondrial network dynamics, metabolic activity and redox state are all intricately linked to each other and to NLRP3 inflammasome activity, we highlight the importance of a holistic approach to investigations of NLRP3 activation by dysfunctional mitochondria.

Keywords: infection, inflammasome, mitochondria, NLRP3, ROS**INTRODUCTION**

Mitochondria are hubs for metabolic activity, where they produce ATP required for cellular functions. Mitochondria are unique amongst cellular organelles, as they are ancestrally derived from a bacterial endosymbiont that integrated with an early eukaryote precursor.¹ Many components of mitochondria have their origins in the mitochondrion's bacterial ancestry, such as CpG-rich mitochondrial DNA (mtDNA) in the matrix and cardiolipin in the inner membrane. Such bacterially derived mitochondrial molecules can induce important immunomodulatory mechanisms.²

Host pattern recognition receptors (PRRs) are at the interface between mitochondria and innate

immunity. PRRs have evolved to recognise and respond to foreign agents (pathogen-associated molecular patterns; PAMPs) and specific 'self' molecules whose mislocalisation or modification indicates cell stress or infection (danger-associated molecular patterns; DAMPs). Nod-like receptor protein 3 (NLRP3) is a cytosolic PRR activated by a wide range of PAMPs (bacterial toxins, fungal and viral components) and DAMPs (e.g. extracellular ATP, lysosomal-disrupting crystals), including those of mitochondrial origin. While NLRP3 signalling is generally host-protective, inappropriate or sustained NLRP3 activation drives hereditary autoinflammatory (e.g. cryopyrin-associated periodic syndromes) and acquired (e.g. gouty arthritis, Alzheimer's and Parkinson's diseases) diseases.³

Nod-like receptor protein 3 signals by forming a caspase-1-activating complex called the NLRP3 inflammasome. Upon activation, NLRP3 self-associates and engages with the signal adaptor, ASC (apoptosis-associated speck-like protein containing a CARD), which then interacts with caspase-1 to form the inflammasome.⁴ Active caspase-1 then cleaves various substrates to induce inflammatory signalling, including the cytokines IL-1 β and IL-18, and the cell executioner gasdermin D (GSDMD), which induces a lytic form of cell death called pyroptosis.⁵ The subcellular location of NLRP3 activation remains controversial, with various reports locating active NLRP3 at cytosolic face of the outer mitochondrial membrane, the attachment points between the mitochondria and ER,⁶ or the dispersed trans-Golgi network.⁷ In murine *in vitro* systems, NLRP3 activation generally follows a 2-step model.⁸ Signal 1 is usually a Toll-like receptor (TLR) agonist. Signal 1 activates the nuclear factor κ -light-chain-enhancer of activated B cells (NF- κ B) pathway to transcriptionally upregulate NLRP3 and pro-IL-1 β , and induce NLRP3 post-translational modifications that poise NLRP3 for signalling. A plethora of PAMPs and DAMPs can provide the 2nd signal, which is the 'trigger' for NLRP3 inflammasome assembly. These PAMPs and DAMPs induce one or more key features of disrupted cytosolic homeostasis – K⁺ efflux, lysosomal rupture or the production of reactive oxygen species (ROS) – to induce NLRP3 inflammasome assembly and signalling.

While there is currently no universal or unifying activation model for the NLRP3 inflammasome, NLRP3 is a sensor for disrupted homeostasis, including perturbed mitochondrial function. For example, mitochondrial dysfunction triggers the generation of ROS (mitoROS), which can elicit a K⁺-independent NLRP3 activation pathway.⁹ The TLR7 agonist imiquimod (R837) and the related molecule CL097 inhibit the mitochondrial quinone oxidoreductase NQO2 (N-ribosylidihydronicotinamide:quinone reductase 2) and complex I. In dysregulating mitochondrial respiration, these compounds trigger the production of mitoROS, and resultant NLRP3 inflammasome signalling.⁹ Apoptotic Bcl-2-associated X protein (BAX)/Bcl-2 homologous killer (BAK) pores on the outer mitochondrial membrane also trigger caspase-3/7-dependent NLRP3 activation.¹⁰ The mitochondrial antiviral signalling protein (MAVS) may also promote

mitochondrial inflammasome assembly by directly recruiting NLRP3 to the outer membrane.¹¹

Recognition of mitochondrial molecules by host PRRs is a nascent field with emerging relevance to inflammasome biology and disease. When translocated to the cytosol during mitochondrial damage, several mitochondrial components are reported to trigger NLRP3 activation, including cardiolipin and mtDNA,^{12–15} although these remain controversial. Mechanistically, it is unclear how these components may become liberated from the mitochondrial inner membrane (cardiolipin) or matrix (mtDNA) for participation in NLRP3 signalling in the cytosol. Mitochondrial network dynamics, metabolic state and mitoROS can all influence NLRP3 activation and the inflammatory signalling capacity of a cell. It is thus possible that mitochondria, mitoROS or mtDNA promote NLRP3 inflammasome assembly and cytokine processing through an indirect mechanism. As mitochondria may stimulate NLRP3 activity through mechanisms other than membrane rupture and release of mitochondrial components, it is important to consider the mitochondrial network holistically, as a complex and dynamic organelle. Aside from NLRP3, other PRRs sense mitochondrial molecules to induce inflammatory signalling, amongst which TLR9, AIM2 and cGAS will be discussed herein. Here, we review mitochondrial functions in innate immunity and speculate how mitochondria may influence PRR activity during infection and disease. The mechanisms by which mitochondria influence PRR activity and drive inflammatory responses are important research foci for understanding protective innate immune functions during host defence against infection, as well as innate immune pathology in disease.

MITOCHONDRIAL NETWORK DYNAMICS DURING INFECTION

The dynamics of fission and fusion

Mitochondrial morphology is governed by ancient and highly conserved fission and fusion processes. There is always a dynamic equilibrium between fission and fusion. These competing processes are balanced to maintain tubular networks of mitochondria in healthy cells. Under stress, the balance is tipped in favor of fission, to enable rapid amputation and degradation of damaged sections of the mitochondrion.¹⁶ Asymmetric

fission yields daughter mitochondria with varying membrane potential.¹⁷ Daughter mitochondria with regions of decreased membrane potential are then selectively removed by a specialised form of autophagy called mitophagy.^{17,18}

The GTPase DRP1 is the primary mediator of mitochondrial fission. DRP1 self-assembles into oligomers, coiling around the mitochondrial outer membrane to 'pinch' the tubular network and fragment it into smaller, discrete segments^{19,20} (Figure 1). It is currently unclear how DRP1 constriction sites are specified; however, this may involve ER mitochondria-associated membranes (MAMs)²¹ and the actin cytoskeleton.²² NLRP3 activation by K⁺ efflux-inducing agents (ATP, MSU, silica, nigericin) is reported to be partially dependent on phosphoglycerate mutase family member 5 (PGAM5), a serine/threonine phosphatase located on the mitochondrial outer membrane.²³ PGAM5 may recruit and dephosphorylate DRP1, activating DRP1 and promoting mitochondrial fission.²⁴ The inner mitochondrial membrane has its own fission machinery that operates in parallel to the outer membrane. Another GTPase, OPA1, maintains the integrity of the matrix. OPA1 loss or functional downregulation leads to inner membrane fission.^{25–27} How DRP1 and OPA1 coordinate events at both membranes to execute mitochondrial fission is unclear, and yet they do so seamlessly. Both proteins are required for effective mitophagy, and inhibition of either is sufficient to induce the accumulation of damaged mitochondria.¹⁷ The reverse process of mitochondrial fusion is induced by suppression of the fission machinery or upregulation of fusion machinery. In mammals, mitofusins (MFNs) 1 and 2 (Figure 1) participate in multiprotein complexes to fuse fragmented mitochondria to form a tubular network.^{28,29} This dynamic regulation of mitochondrial morphology is important for repair, equal distribution of mitochondrial mass during replication, and starvation-induced metabolic responses.³⁰ Mitochondria are labile and responsive organelles that modulate immune signalling, as is highlighted by mitochondrial dynamics during microbial infection.

Viral infection

Some viruses influence mitochondrial mass by interfering with mitochondrial network fusion/fission dynamics, to create a favorable environment

for viral replication. For example, dengue virus,^{31,32} severe acute respiratory syndrome-related coronavirus (SARS-CoV)³³ and human immunodeficiency virus (HIV)³⁴ all downregulate DRP1 to promote mitochondrial fusion. In addition to downregulating DRP1, the HIV gp120 protein upregulates MFN1, while another HIV protein, Vpr, damages the mitochondrial outer membrane, which can lead to loss of membrane potential, mitochondrial dysfunction and apoptosis.³⁴ HIV induces expression of NLRP3 inflammasome components in patient brains.^{35,36} Given that NLRP3 inflammasome activity in the brain is linked to neurodegeneration,^{37,38} viral promotion of NLRP3 signalling may contribute to neurological disorders that are often a comorbidity of chronic HIV infection.^{35,36}

Mitochondria are signalling platforms for antiviral signalling via MAVS and NLRP3, so viral modulation of mitochondria may be a mechanism to suppress antiviral responses. MAVS is a signalling protein activated downstream of viral double-stranded (ds) RNA recognition by retinoic acid-inducible gene I (RIGI) and melanoma differentiation-associated protein 5 (MDA5). Upon activation, MAVS induces NF- κ B and interferon regulatory factor-3 (IRF3)-dependent interferon- β (IFN- β) production.³⁹ NLRP3 is reported to relocate to mitochondria for activation, where MAVS may promote NLRP3 signalling.^{11,40} NLRP3 can also sense mitochondrial dysfunction.⁹ Influenza A, hepatitis B (HBV) and hepatitis C (HCV) manipulate the mitochondrial inner membrane potential (influenza A) and mitophagy (HBV, HCV) to degrade the mitochondrial network, drive mitophagy and suppress antiviral signalling.^{41–43} The influenza A peptide PB1-F2 induces loss of mitochondrial membrane potential by disrupting the ADP/ATP translocase and voltage-dependent anion channel 1 (VDAC1) on the inner and outer membranes, respectively.⁴¹ HBV triggered DRP1 recruitment to the mitochondrial outer membrane, and both HBV and HCV stimulate PINK1 and parkin to upregulate mitophagy.^{42,43} In reducing the overall mitochondrial volume, this virally induced fission mechanism is presumed to decrease the cell's capacity for MAVS and IFN-dependent antiviral signalling and therefore advantage the virus.³⁴ By blocking mitochondrial viral recognition pathways, these viral offensive tactics boost viral replication.

Many such viruses that manipulate mitochondrial dynamics activate the NLRP3

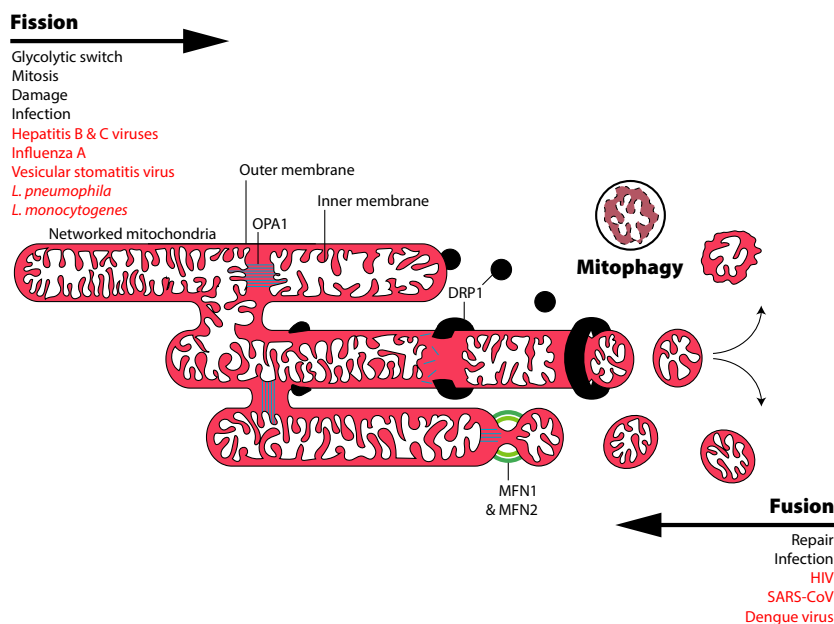


Figure 1. Mitochondria are dynamic organelles in constant flux between fission and fusion. While mechanisms controlling mitochondrial fission and fusion are not fully characterised, several key proteins exert key functions in these processes. *Upper:* Fission of an individual mitochondrion from the network is mediated by the GTPase DRP1, which operates in a ‘kiss and run’ manner. Asymmetric fission is driven by changes in metabolism, damage, mitotic division or infection by specific viral and bacterial agents, and occurs when DRP1 oligomerises on the outer membrane and coils around the section to be ‘pinched’ off. In a parallel fission process, OPA1 is downregulated at the inner membrane portions targeted for fission. It is currently unclear whether DRP1-mediated outer membrane fission occurs at sites where the mitochondrial matrix is already separated (as shown above), or requires the inner membrane to be pinched off in a manner similar to the outer membrane. If the mitochondrion is damaged, it is degraded by mitophagy. *Lower:* Fusion counters fission. An individual mitochondrion ‘docks’ to the mitochondrial network via MFN1 and MFN2, which anchor the opposing outer membranes and shape the lipid bilayer into extreme curvature. The membranes are forced to create a bridge and eventually reincorporate the mitochondrion back into the network, where, presumably, OPA1 reattaches between the inner membranes to support these as discrete compartments. It is currently unclear whether OPA1 drives true fusion of the inner membranes as DRP1 does for the outer membrane, or if it anchors two discrete matrix compartments to one another. Fusion is presumed to allow mitochondria to maintain an even distribution of matrix components across the network and may also be reparative. Fusion is promoted by cell infection with specific viruses.

inflammasome or evade detection by this immune sentinel. It is possible that these observations are linked, that is that NLRP3 may sense virally induced mitochondrial dysfunction, while other viruses that can manipulate mitochondrial dynamics without causing cell stress may evade detection. Dengue virus, influenza, HCV, encephalomyocarditis virus (EMCV), vesicular stomatitis virus (VSV), herpes simplex virus-1 (HSV-1) and SARS-CoV activate NLRP3,^{44–47} while HBV suppresses NLRP3 activation by inhibiting NF- κ B and the generation of ROS.⁴⁸ MFN2, part of the mitochondrial fusion machinery, associates with NLRP3 in LPS-primed bone marrow-derived macrophages (BMDMs) upon infection with influenza A or EMCV.⁴⁵ mtROS is also crucial for NLRP3 activation during influenza A infection in mouse BMDMs and human peripheral blood

mononuclear cells (PBMCs).⁴⁹ There are conflicting reports regarding the requirement for DRP1-dependent fission for NLRP3 activation during VSV infection.^{46,50} Inhibition of mitophagy because of parkin deficiency enhanced virus-induced IL-1 β /18 secretion during VSV and HSV-1 infection.⁴⁷ Mitochondrial dynamics during viral infection and their consequences for NLRP3-dependent detection are varied and complex, highlighting a poorly understood yet intriguing research area within the host–pathogen evolutionary arms race.

Bacterial infection

Bacteria also modulate mitochondrial function to create favorable environments for intracellular replication, or to evade host innate immune

recognition.⁵¹ Here, we focus on those bacteria that affect mitochondrial morphology, and speculate as to how this may influence inflammation.

The intracellular pathogen *Listeria monocytogenes* secretes a toxin, listeriolysin (LLO), which activates the NLRP3 inflammasome via K⁺ efflux.^{52,53} LLO also induces DRP1- and OPA1-independent fission by modulating the membranes shared between the ER and mitochondria (MAMs),⁵⁴ hinting at a novel ER-dependent fission mechanism. MAMs are emerging as important signalling hubs between the ER and mitochondria and are a possible site of NLRP3 inflammasome assembly.⁵⁵ It is possible that LLO-driven MAM disruption and mitochondrial fission removes a key site of inflammasome assembly to dampen K⁺ efflux-dependent NLRP3 signalling and thereby allows the bacterium to partially evade immune recognition.

Another intracellular bacterial parasite, *Legionella pneumophila*, secretes the toxin MitF to upregulate the fission agent DRP1 at the mitochondrial outer membrane.⁵⁶ MitF thereby induces mitochondrial fragmentation, which appears to be a strategy to reprogramme host macrophage metabolism⁵⁶ to limit oxidative phosphorylation (OXPHOS) and create the glycolytic environment favored by *L. pneumophila*. *L. pneumophila* indirectly activates NLRP3 through the caspase-11 and absent in melanoma 2 (AIM2) inflammasomes.^{57,58} Bacterially-induced metabolic changes may also indirectly affect NLRP3 activation and innate immune function more broadly.

MITOCHONDRIAL IMMUNOMETABOLISM IN INNATE IMMUNITY

Mitochondria are important for energy production, providing much of the cellular pool of ATP for cellular functions. During OXPHOS, the four complexes of the electron transport chain (ETC) cooperate with ATP synthase to produce ATP at the inner mitochondrial membrane.⁵⁹ The ETC generates a proton motive force that drives ATP synthase and results in the net production of 34 ATP molecules per molecule of glucose, outcompeting glycolysis and the Krebs cycle (each 2 ATP molecules) for the efficient generation of energy currency in resting cells. Many pathogen-

derived or sterile immune triggers can disrupt the mitochondrial membrane potential leading to suppressed OXPHOS, so mitochondria are exquisitely sensitive to the inflammatory status of the immune cells they inhabit. The emerging field of 'immunometabolism' examines alterations in cell metabolism during inflammation.⁶⁰ Because of the enormous scope of immunometabolism and its pervasive roles in an ever-growing list of immunological contexts, here we specifically focus on mitochondrial metabolic fluctuations in inflammatory macrophages, and how changes in cell metabolism influence NLRP3 activation.

Macrophages undergo metabolic reprogramming during their activation, switching from OXPHOS to glycolysis as the primary route for ATP synthesis,⁶¹ in a process known as the Warburg effect.⁶² Signalling through TLRs 2, 3, 4 and 9 induces this glycolytic switch during macrophage activation,⁶³ as a means to upregulate ATP production for inflammatory and antimicrobial functions, albeit at the cost of energy efficiency. Inflammatory, or classically activated, macrophages promote glycolysis by upregulating hexokinase and glucose-6-phosphate dehydrogenase (G6PD).⁶⁴ This glycolytic switch mechanism may contribute to the signal 1 step in NLRP3 activation, as hexokinase-dependent glycolysis is essential for NLRP3 inflammasome assembly and IL-1 β secretion.⁶⁵ NLRP3 signalling in microglia also upregulates glycolysis.⁶⁶ Complexes I and III of the electron transport chain generate mitoROS in response to alteration in the mitochondrial membrane potential.^{67,68} R837⁹ and likely other signal 2 stimuli^{9,69,70} disrupt mitochondrial OXPHOS, triggering mitoROS production and NLRP3 activation. This indicates that NLRP3 may function as a sentinel for mitochondrial dysfunction in specific circumstances.

Immunometabolic flux, mitochondrial network dynamics and mitochondrial depolarisation are all tightly linked. Loss of mitochondrial membrane potential can trigger metabolic shifts and mitoROS generation, and may be a consequence of mitochondrial permeabilisation. Mitochondria contain many molecules that are similar to bacterial PAMPs and are usually shielded from immune recognition. Here, we review the consequences of liberation of one such component, mtDNA, for recognition by cytosolic and endosomal PRRs, and resultant inflammatory signalling.

MITOCHONDRIAL DNA: A DANGER SIGNAL FOR PRRS

DNA is usually rapidly removed from the cytosol in healthy eukaryotic cells. During infection or cell damage, the amount of DNA liberated from the nucleus or mitochondria overwhelms the capacity for cytosolic DNases to remove DNA, leading to immune recognition by PRRs. Cytosolic DNases are also transcriptionally downregulated in senescent cells,⁷¹ suggesting that PRR sensing of accumulated mislocalised DNA may contribute to age-related inflammation ('inflammaging'). The location of DNA at the time of recognition drives compartment-specific responses,⁷² and here, we review mtDNA sensing by several key DNA sensors in innate immunity (Figures 2 and 3).

NLRP3, a cytosolic sensor for mtDNA?

Nod-like receptor protein 3 is a well-characterised cytoplasmic sensor for cell stress. One reported mechanism of stress sensing involves NLRP3 recognition of mislocalised mtDNA during mitochondrial dysfunction. NLRP3 activation in mouse macrophages can be associated with loss of mitochondrial membrane potential or defective mitophagy, leading to mitoROS production and mtDNA release from mitochondria to the cytosol.^{69,73–75} Oxidised mtDNA was reported to promote NLRP3 activation.¹⁴ In this study, the

authors observed direct interaction between transfected bromodeoxyuridine (BrdU)-labelled mtDNA and ectopically expressed NLRP3 in HEK293 cells, suggestive of a ligand–receptor relationship. In wild-type BMDMs, endogenous mtDNA labelled with BrdU co-immunoprecipitated with NLRP3 after LPS priming and a signal 2 (nigericin or ATP); however, this study did not confirm that this interaction was upstream, rather than downstream, of NLRP3 signalling. Since this initial report that mtDNA may activate NLRP3, subsequent studies have disagreed over the sequence of events leading to mtDNA liberation and sensing by NLRP3. Several groups describe mtDNA release as a signal 2-dependent, NLRP3-independent process that is required for NLRP3 activation.^{74–76} In one report, mtDNA immunoprecipitated with the NLRP3 complex after mouse BMDMs were treated with palmitic acid.⁷⁵ In another, TLR4 ligation by LPS (signal 1) upregulated mtDNA synthesis, while NLRP3 activators (signal 2; ATP, nigericin) promoted colocalisation of oxidised mtDNA with the NLRP3 inflammasome.⁷⁶ In all, these reports suggest a model in which mtDNA liberation from mitochondria occurs prior to, and is required for, activation of the NLRP3 inflammasome (Figure 2a). NLRP3 can also sense cytosolic DNA indirectly, via cell signalling through the cyclic GMP-AMP synthase (cGAS)/stimulator of IFN genes (STING) axis.⁷⁷ Many signal 2 stimuli induce

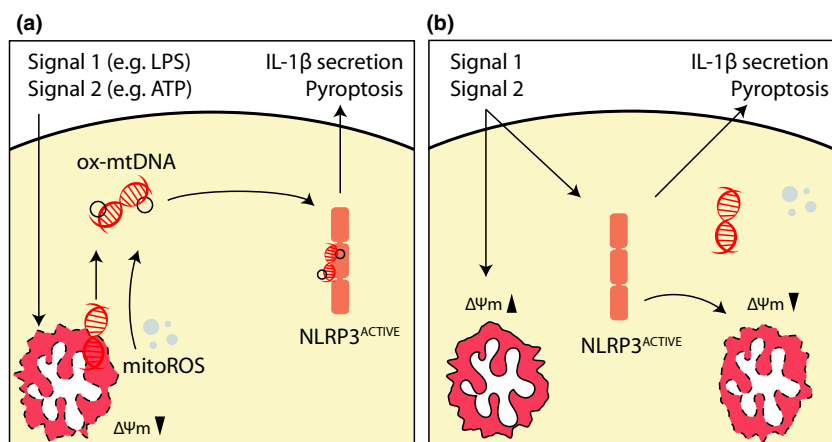


Figure 2. Models for mitochondrial disruption during signalling by the nod-like receptor protein 3 (NLRP3) inflammasome. **(a)** Loss of mitochondrial membrane potential ($\Delta\Psi_m$) via NLRP3 priming plus activation signals results in the liberation of mtDNA from the matrix to the cytosol. mitoROS, produced as a consequence of disrupted OXPHOS, oxidises mtDNA, converting it to an NLRP3 activator. Some studies report that NLRP3 binds directly to mtDNA, yet the domain of NLRP3 responsible for interacting with mtDNA is yet to be characterised. **(b)** One study reported that NLRP3 activation signal 2 induces transient membrane hyperpolarisation independent of NLRP3. Later loss of $\Delta\Psi_m$, and presumably permeabilisation, mtDNA release and mitoROS generation, occurs as a consequence of NLRP3 activation.

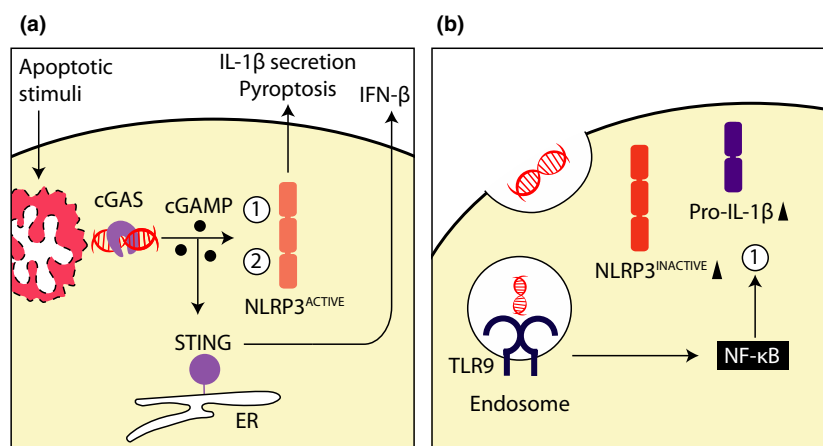


Figure 3. Signals 1 and 2 for nod-like receptor protein 3 (NLRP3) activation may be delivered by cGAS or TLR9. **(a)** The dsDNA sensor cGAS senses cytosolic mtDNA upon mitochondrial permeabilisation induced by apoptosis or other mitochondrial damage events. cGAS activates STING via a second messenger, cGAMP, to drive type I IFN signalling. Signalling through this cGAS/cGAMP pathway primes and activates NLRP3, functioning as both signal 1 and signal 2 for NLRP3 inflammasome signalling. **(b)** mtDNA is released to the extracellular environment during cell lysis. mtDNA may be recognised by TLR9, an endosome-localised sensor for CpG-rich unmethylated DNA. TLR9 ligation provides signal 1 for NLRP3 activity, for example by transcriptionally upregulating NF- κ B target genes in the inflammasome pathway (NLRP3, pro-IL-1 β).

cellular K⁺ efflux to activate NLRP3; however, it is unclear whether ionic flux drives NLRP3 activation in parallel to, or up- or downstream of, mitochondrial disruption and mtDNA release. Additionally, nigericin can integrate directly into the mitochondrial outer membrane to induce hyperpolarisation,⁷⁸ and so some signal 2 stimuli may directly perturb mitochondria in parallel to inducing ionic changes in the cytosol.

Other studies suggest an alternative model, in which mitochondrial permeabilisation is not upstream of NLRP3, but is instead a partial or full consequence of NLRP3 signalling (Figure 2b). Disabling autophagy (and by extension mitophagy) in mouse BMDMs resulted in mitoROS- and NLRP3-dependent liberation of mtDNA from the mitochondrial matrix.⁷³ Here, retention of dysfunctional or damaged mitochondria was alone insufficient for mtDNA translocation to the cytosol; the latter required NLRP3 signalling. Another study reported the loss of mitochondrial membrane potential as NLRP3-dependent, yet caspase-1-independent.⁶⁹ In keeping with the assertion that loss of membrane potential is a consequence rather than cause of NLRP3 activation, another study reported that NLRP3 deficiency in renal tubular cells protected mitochondria against depolarisation during hypoxia.⁷⁹ The above models may not be mutually exclusive; it is possible that an early loss of mitochondrial membrane integrity results in just

enough externalised mtDNA to participate in NLRP3 signalling, which then drives further mitochondrial damage. In line with this, one study examining temporal changes in mitochondrial stability during NLRP3 activation reported that LPS-primed BMDMs treated with ATP or nigericin exhibited some inflammasome-independent mitochondrial hyperpolarisation at early time points (up to 15 min post treatment). At later time points (0.5–1 h), mitochondria underwent depolarisation, and this was largely driven by NLRP3 and caspase-1.⁸⁰ The active N-terminal fragment of GSDMD has affinity for the mitochondrial lipid cardiolipin,⁵ and so it is possible that GSDMD perforates mitochondria downstream of NLRP3 activation. Sterile-alpha and armadillo motif-containing protein (SARM), a mitochondrially localised TLR adaptor, is also required for mitochondrial membrane depolarisation and pyroptosis downstream of NLRP3 activation in immortalised mouse macrophages (iBMDMs),⁸¹ through yet-undetermined mechanisms.

Regardless of which of the above models is accurate, mitochondrial permeabilisation must precede the liberation of mtDNA to the cytosol. Currently, the field is yet to reach a consensus on mechanisms mediating the permeabilisation of the two mitochondrial membranes that envelop mtDNA. We pose several hypotheses for mechanisms by which mtDNA may directly or

indirectly drive NLRP3 signalling in Figures 2 and 3. Until mitochondrial perforation mechanisms are discovered, the true biology may remain elusive.

Cytosolic DNA recognition by cGAS

Cyclic GMP-AMP synthase recognises pathogen- and host-derived dsDNA and signals to promote antiviral, antitumor and cell senescence pathways.⁸² Activated cGAS produces the second messenger, cyclic GMP-AMP (cGAMP), that binds to STING. STING translocates from the ER to the Golgi to activate IRF3, TANK-binding kinase (TBK1) and NF- κ B to drive transcriptional upregulation of type I IFNs.⁸³ Host mtDNA is recognised by cGAS to drive cGAMP/STING-mediated IFN- β antiviral signalling.^{82,84–86} While AIM2 inflammasome activation inhibits cGAS signalling via GSDMD-dependent K⁺ efflux,⁸⁷ cGAS/STING-dependent NF- κ B activation may provide a signal 1 for NLRP3 inflammasome activation. cGAS/STING signalling may also be a downstream consequence of NLRP3 activation; for example, IL-1R signalling in the human THP-1 myeloid cell line drives loss of mitochondrial membrane potential and recognition of mtDNA by cGAS.⁸⁶ Deficiency in the mitochondrial transcription factor, TFAM, also drives mtDNA mislocalisation to the cytosol and resultant cGAS activation in mouse embryonic fibroblasts (MEFs) and BMDMs.⁸⁵ When caspases are inhibited in apoptotic MEFs, mtDNA is liberated from mitochondria by BAX/BAK macropores and is recognised by cGAS.⁸⁴ cGAS/STING is linked to pathology in *Prkn*^{-/-} mice (a model for human *PRKN* gene-associated Parkinson's disease), which exhibited higher circulating cell-free mtDNA (normalised to genomic DNA) than healthy wild-type mice, and STING-dependent elevations in serum IL-6 and IFN- β levels.⁸⁸ The cGAS/STING pathway also promotes NLRP3 and AIM2 activation in murine macrophages.⁸⁹ Cytosolic DNA-induced cGAS/STING signalling triggers lysosomal rupture (signal 2), leading to NLRP3 activation in human cells.⁷⁷ cGAS/STING-mediated recognition of mtDNA may thereby provide either signal 1, signal 2, or both for NLRP3 inflammasome activation (Figure 3a). In murine macrophages, active caspase-1 cleaves and inactivates cGAS,⁹⁰ possibly providing a mechanism for switching off mtDNA-induced cGAS/STING signalling once inflammasome pathways are engaged.

TLR9 senses endosomal CpG DNA

Unmethylated CpG DNA activates immune cells via TLR9.^{91,92} TLR9 is expressed in murine myeloid-derived cells and plasmacytoid dendritic cells (pDCs), but is primarily expressed by B cells and pDCs in humans.⁹³ TLR9 recognises unmethylated CpG in the endosomal system,^{94,95} including that of mitochondrial origin following mitochondrial disruption.^{96–99} CpG recognition by TLR9 provides a signal 1 for NLRP3 activation, transcriptionally upregulating inflammasome-required genes under NF- κ B control^{100,101} (Figure 3b). The mitochondrial dynamics that drive disruption and exposure of mtDNA to TLR9 are varied and unclear, yet some evidence implicates defective fission/fusion dynamics. OPA1 guides mitochondrial inner membrane fusion,²⁷ and its deficiency in mice drives increased NLRP3, ASC and IL-1 β expression and increased plasma IL-1 β .⁹⁶ This was cGAS-independent and may have been TLR-dependent.⁹⁶ Mitochondria in OPA1 knockdown cells appear more fragmented than the untreated cells⁹⁶ and may mimic the swing towards fission experienced during some microbial infections. Thus, some forms of mitochondrial disruption may deliver a TLR9-driven priming signal for the NLRP3 inflammasome.

Cytosolic DNA recognition by the AIM2 inflammasome

Absent in melanoma 2 is a DNA sensor that clusters on cytoplasmic bacterial, viral or host dsDNA to drive ASC-dependent caspase-1 activation, IL-1 β and IL-18 maturation and pyroptosis.^{102,103} mtDNA is approximately 17 kb in size,¹⁰⁴ and AIM2 clusters upon DNA fragments \geq 180bp¹⁰⁵; as such, cytoplasmic mtDNA meets AIM2 ligand requirements. While the available evidence is limited, it supports the hypothesis that cytoplasmic mtDNA triggers AIM2 inflammasome signalling. Transfected mtDNA induced AIM2-dependent IL-1 β secretion in BMDMs, and transfected mtDNA bound to ectopically expressed AIM2 in HEK293 cells.¹⁴ In addition, circulating cell-free mtDNA (ccf-mtDNA) from type 2 diabetic patients triggered AIM2 signalling in mouse BMDMs.¹⁰⁶ Thus, while the field awaits formal confirmation that mtDNA is a bona fide ligand for human AIM2, this seems likely.

MITOCHONDRIAL DISRUPTION AND NLRP3 INFLAMMASOME ACTIVITY IN HUMAN DISEASE

Nod-like receptor protein 3 drives a wide range of acquired and hereditary autoinflammatory diseases. As such, investigations into NLRP3 activation mechanisms have the potential to reveal novel anti-inflammatory strategies. Cytosolic sensors for mtDNA alert the cell to mitochondrial permeabilisation, a potentially catastrophic event induced by severe cell damage. While this damage is self-limiting in healthy individuals, it is possible that genetic or environmental factors that prolong mitochondrial disruption drive chronic NLRP3 activation and contribute to disease pathology. ccf-mtDNA is upregulated in patients with NLRP3-driven inflammation, and may contribute to disease severity in conditions such as cardiovascular disease¹⁰⁷ and chronic low-level age-related inflammation (inflammaging).¹⁰⁸ In murine models, ccf-mtDNA predisposes mice to type I diabetes.¹⁰⁹ Patients with type II diabetes and coronary heart disease exhibit significantly elevated levels of ccf-mtDNA compared to their healthy counterparts.¹¹⁰ In chronic obstructive pulmonary disease (COPD), ccf-mtDNA may activate TLR9 to drive inflammation¹¹¹ and may potentially provide signal 1 for NLRP3 activation, as NLRP3 contributes to disease pathology.¹¹²

Parkinson's disease (PD) is a neurodegenerative disorder that is strongly linked to mitochondrial dysfunction and NLRP3-driven pathology. PD is causally associated with mutations in genes involved in mitochondrial functions (e.g. OXPHOS) or maintenance (e.g. mitophagy).¹¹³ The NLRP3 inflammasome induces neuroinflammation and drives PD pathogenesis, as murine models of PD are protected from disease by *Nlrp3* deficiency or dosing with MCC950,³⁸ a small-molecule NLRP3 inhibitor.^{114,115} Excitingly, MCC950 suppressed disease in a murine model of PD in which dopaminergic neurons are deficient in TFAM (MitoPark model).³⁸ This highlights NLRP3 inhibition as a promising therapeutic strategy for the treatment of PD and other diseases involving mitochondrial dysfunction.

To determine how mitochondria may drive inflammatory and neurodegenerative diseases, it is important to understand how mitochondria participate in normal immune signalling. Sterile (host-derived) signals are generally less potent

than pathogen-derived signals such as signals 1 and 2 for NLRP3 inflammasome activation.¹¹⁶ This is desirable, as immunogenic host molecules could trigger rampant, unintended inflammation. As bacteria-like organelles, mitochondria provide a potent 'in-house' pseudo-pathogen signal that can boost the innate response to immune-evasive pathogens. Normally, mitochondrial immunogenic components are shielded from cytosolic and endosomal PRRs by the dual mitochondrial membranes. Mitochondrial permeabilisation, indicating mitochondrial damage, allows mitochondrial components to be liberated for immune recognition, thereby providing a strong cellular distress signal. How NLRP3 recognises mtDNA in a physiological context beyond *in vitro* assays remains to be seen and will no doubt be an important future addition to the inflammasome field.

CONCLUSION

Mitochondria are ATP factories, as well as reservoirs for PAMP-like molecules. Links between mitochondrial dysfunction and NLRP3 activation are now irrefutable, but raise several further questions. One such hot topic is the mechanism by which NLRP3 senses cytosolic mtDNA. Our understanding of this process is complicated by the many links between mitochondrial processes and inflammasome signalling, both up- and downstream of NLRP3 activation. Our emerging understanding of ER-mitochondria dynamics, including the important signalling functions of MAMs, suggests it may be prudent for future studies to consider the ER and mitochondria together, rather than in isolation. Continued advances in high-resolution spatiotemporal imaging techniques are likely to yield important insight into mitochondrial and ER dynamics prior to, and during, NLRP3 inflammasome signalling. As many current mitochondrial labelling strategies interfere with mitochondrial function to some degree, a particular challenge will be to develop techniques for live-cell monitoring of mitochondria without affecting their metabolic function, morphology or membrane potential.

We predict that future exciting discoveries will include the specific mechanisms underpinning mtDNA recognition by NLRP3, *in vivo* and *in vitro*, and in disease and health. Mechanisms and outcomes of signal integration by the suite of mtDNA-activated PRRs, such as cGAS, AIM2, NLRP3

and TLR9, will also be of prominent interest. Despite the technical challenges, this topic promises to be an exciting area in the coming years.

ACKNOWLEDGMENTS

The authors thank Dr Madhavi Maddugoda for editing and helpful comments. This work was supported by the National Health and Medical Research Council of Australia (Fellowship 1141131 to KS) and the Australian Research Council (Project Grant DP160102702 to KS). CLH is supported by the Australian Government Research Training Program Scholarship and a Yulgilbar Alzheimer's Research Program PhD Top-up Award from the Yulgilbar Foundation.

CONFLICT OF INTEREST

KS is a co-inventor on patent applications for NLRP3 inhibitors which have been licensed to Inflazome Ltd, a company headquartered in Dublin, Ireland. Inflazome is developing drugs that target the NLRP3 inflammasome to address unmet clinical needs in inflammatory diseases. KS served on the Scientific Advisory Board of Inflazome in 2016–2017. KS is a consultant for Quench Bio, USA. The authors have no further conflicts of interest to declare.

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