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Mitochondria in innate immune signaling

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Abstract

Mitochondria are functionally versatile organelles. In addition to their conventional role of meeting the cell's energy requirements, mitochondria also actively regulate innate immune responses against infectious and sterile insults. Components of mitochondria, when released or exposed in response to dysfunction or damage, can be directly recognized by receptors of the innate immune system and trigger an immune response. In addition, despite initiation that may be independent from mitochondria, numerous innate immune responses are still subject to mitochondrial regulation as discrete steps of their signaling cascades occur on mitochondria or require mitochondrial components. Finally, mitochondrial metabolites and the metabolic state of the mitochondria within an innate immune cell modulate the precise immune response and shape the direction and character of that cell's response to stimuli. Together, these pathways result in a nuanced and very specific regulation of innate immune responses by mitochondria

Introduction:

Theories that mitochondria evolved from an independent prokaryotic organism to a symbiont residing within the cytosol of the eukaryotic cell suggest that this affiliation was to mutual benefit, with the mitochondrion generating energy for the cell and the cell providing reagents and security for the mitochondrion (1,2). This theory of a bacterial origin for mitochondria fits nicely with findings that the unique components of mitochondria, when exposed, reveal their prokaryotic history and are recognized as foreign by innate immune receptors triggering an inflammatory response. Intriguingly, more recent studies suggest that the relevance of mitochondria to the innate immune response extends beyond their identification as invading bacteria and instead profoundly impact many separate aspects of innate immune responses.

Mitochondria are dynamic organelles with inner and outer membranes and an internal negatively charged matrix. Their most described function is to provide energy for the cell as the site of oxidative phosphorylation, generating 32 molecules of ATP per molecule of glucose. The vast majority of mitochondrial proteins are encoded by the nuclear DNA, transcribed and translated by the eukaryotic machinery, and then transported to their functional sites in the mitochondrion (3). Mitochondria do have their own circular DNA that encodes thirteen proteins necessary for oxidative phosphorylation along with the ribosomal

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and transfer RNAs needed for their translation (4). While a basic view of mitochondria may be to regard them as simply a source of ATP, the paths by which ATP is made as well as the other functions and activities of mitochondria are more complex with significant impacts upon the cell and the organism. Mitochondria are neither static nor discrete structures. In response to the conditions within the cell and both the state of and the demands being placed on the mitochondrion, mitochondria undergo fusion to combine with other mitochondria or fission to separate and form new mitochondria (5, 6). The balance of fusion and fission events has relevance beyond just determining the number of mitochondria to a cell as these processes also impact calcium regulation, generation of reactive oxygen species, and impact oxidative phosphorylation (7).

Generation of ATP by mitochondria requires the negatively charged matrix that allows the passage of electrons over the electron transport chain, which consists of specialized complexes arranged on the inner mitochondrial membrane. Disruption of the negative charge of the matrix occurs in response to a number of stressors including antioxidants, oxidative phosphorylation substrates, and membrane uncoupling agents. This loss of negative potential results in a failure of ATP production and the generation and release of reactive oxygen species (ROS) that have the potential to cause widespread damage. Further, dysfunctional mitochondria can lose membrane integrity, allowing previously sequestered mitochondrial components to leak into the cytosol or out of damaged cells to the circulation (8–10). To limit the negative effects of ROS, damaged and dysfunctional mitochondria are removed through a process known as mitophagy (11).

The innate immune response has a critical role in the detection and correction of both infectious and sterile insults. The response begins with the recognition of the insult, commonly through germline encoded receptors termed pattern recognition receptors (PRRs). These receptors bind to conserved features of microbes that identify them as foreign, or to endogenous molecules with specific modifications or in locations that reveal tissue or cellular injury (12, 13). This recognition of the insult, with the PRR bound by its specific activating ligand, triggers the immune response. While the precise characteristic of that immune response reflects both the type of innate immune cell being activated and the specific receptor and ligand, in general these innate responses are initiated quickly and also quickly escalate and alert additional cells and tissues of the disorder. These early signals of the initial innate immune response have effects throughout the organism, driving recruitment of additional innate immune cells to the site, alerting and activating the more specific adaptive immune response, and triggering the production of molecules needed for inflammation and repair by numerous tissues (14–18).

Both structural and functional aspects of the mitochondria can impact the innate immune response. There are two broad categories by which this occurs: first, by directly activating the immune response and second, by modulating a response. Direct activation commonly reflects mitochondrial damage or pathology while modulation can occur as a byproduct of normal mitochondrial functions and processes. In this review, we will discuss the current literature that defines the interactions between mitochondria and the innate immune response.

Mitochondria derived alarmins of innate immunity:

Pathogen associated molecular patterns, or PAMPs, are conserved features of invading organisms that serve to identify these organisms as foreign, while damage associated molecular patterns, or DAMPs, are endogenous molecules released or modified by sterile insults. Both DAMPs and PAMPs are specifically recognized as alarmins by discrete receptors of the innate immune system and trigger the appropriate immune response (12, 13). Their release by mitochondria is in response to cell stress and loss of homeostasis, similar to more typical DAMPs, but the recognition of these alarmins by PRR depends on their structural similarity to the PAMPs of invading microbes. In this review, we will refer to these alarmins as DAMPs to emphasize their pivotal function as markers of cell stress. The DAMPs that are released by mitochondria include the structural phospholipid cardiolipin, n-formyl peptides (n-fp), reactive oxygen species (ROS) and mitochondrial DNA (mtDNA). While the exact mechanism by which these mitochondrial alarmins are released has not been elucidated completely, studies have suggested loss of mitochondrial membrane integrity results in the escape of mitochondrial components to the cytosol. There are discrete pathways by which the inner and outer mitochondrial membranes are disrupted, through sustained opening of a mitochondrial permeability transition (MPT) pore and mitochondrial outer membrane permeabilization (MOMP), respectively (19, 20). Despite being recognized very early, the details of the structure of the MPT pore as well as the triggers that lead to its formation and opening are not yet clear (21). In general, the MPT pore opens and remains open after insults to the mitochondrion that are associated with disruption of calcium levels or oxidative stress, consistent with the insults believed to be associated with release of mitochondrial DAMPs (22). Similar gaps exist in our knowledge of the structure and regulation of MOMP. MOMP has been studied extensively as a trigger for apoptosis, when BCL-2 family members induce the formation of pores in the outer mitochondrial membrane in response to either death receptors or various cellular or mitochondrial stresses (23). However, MOMP has been reported to occur in the absence of cell death, providing a potential pathway by which MOMP could be associated with the release of mitochondrial contents leading to an innate immune response (24, 25). A recent breakthrough shows the large pore induced in the outer mitochondrial membrane during intrinsic apoptosis is associated with a herniation of the inner mitochondrial membrane. In the cases where this herniated membrane ruptures, mitochondrial DAMPs were released to the cytosol where they could be sensed by various PRRs (24). While the exact pathways and pores or channels that are associated with the release of mitochondrial alarmins are only beginning to be defined, it is clear that after escaping the mitochondrion these molecules are recognized by separate receptors and trigger discrete inflammatory pathways that culminate in the restoration of normal cellular function.

n-FP:

In a mann r to the initiation of protein translation in prokaryotes, mitochondrial initiation of protein translation requires N-formylated methionine (fMet), as mitochondrial translational initiation factor 2 can utilize only the formylated form of methionine, while unformylated methionine is used specifically for protein elongation (26). This unique characteristic of bacterial and mitochondrial proteins was proposed as a potential immune target well before the receptors and signaling pathways had been defined (27, 28). In the absence of tissue

stress or injury these bacteria-like n-formyl peptides (n-FP) are sequestered within the mitochondria. However, during traumatic injury and cell death associated with infection, n-FP are released and bind specific receptors, formyl peptide receptors (FPRs) that in turn recruit immune cells and trigger an extensive inflammatory response as discussed below (29–31).

Cardiolipin:

Cardiolipin is a unique phospholipid that was first identified in animal heart tissue and thus the family is known as cardiolipins. While the structure of cardiolipin varies, in general cardiolipin contains two phosphatidyl groups linked through a glycerol moiety. Cardiolipins are found in many prokaryotic membranes but in eukaryotic cells are limited to the mitochondrial membranes, primarily the inner mitochondrial membrane during normal mitochondrial function (32, 33). Cardiolipin contributes to the structural composition and integrity of the mitochondria membrane and constitutes about 20% of the phospholipid content of the mitochondrial inner membrane (34–36). In addition to its structural role, cardiolipin has numerous non-redundant roles in the mitochondria that reflect its remarkable ability to interact via non-covalent bonds with a wide variety of unrelated molecules. This unique binding capability allows cardiolipin to serve as a discretely controlled regulator of numerous otherwise separate mitochondrial pathways, including mitochondrial dynamics (fission and fusion), the import of cargo from cytosol, metabolic functions, innate immune responses, ROS generation, and apoptotic signaling (37, 38). These functions of cardiolipin occur in its normal environment within mitochondria, while other functions of cardiolipin, in particular its ability to drive innate immune responses, occur after its release from or externalization on the surface of the mitochondrion during conditions of mitochondrial dysfunction, stress, or damage (39). It is in this context that cardiolipin may play a role in activation of the NLRP3 inflammasome (40, 41). In a separate pathway, following ischemia/reperfusion injury, cells release oxidized phospholipids, including cardiolipin, that serve as DAMPs and trigger multiple types of innate immune responses (42). In contrast to these pro-inflammatory roles for exposed or released cardiolipin, externalized cardiolipin has been reported to downregulate innate cytokine responses by upregulating mitophagy pathways (43, 44).

mtDNA:

Mitochondrial DNA is a circular double strand of approximately 17Kbp that contains 13 mRNAs that encode the unique proteins of oxidative phosphorylation as well as related ribosomal RNAs and tRNAs. Similar to the mitochondrial use of bacterial-like machinery for protein translation discussed above, mitochondria DNA has characteristics consistent with prokaryotic nucleic acid. Mitochondrial DNA is a small molecule with methylation patterns discrete from nuclear DNA and is present at hundreds of copies per cell. Under normal conditions, mtDNA is contained within the mitochondrial matrix where mitochondria have unique mechanisms to repair DNA damage as well as to preserve genetic integrity through the selective amplification of intact copies of mtDNA (45, 46). These repair pathways may also modify the mtDNA in such a way to mark it as non-self to innate immune sensors. During cell death or mitochondrial damage the mtDNA can be released into the cytosol or the circulation where it can be sensed by a number of innate immune

receptors and trigger inflammatory responses as detailed below. This release of mtDNA associated with inflammation is supported by the finding of elevated levels of circulating mtDNA in patients suffering from trauma, rheumatoid arthritis, and femur fracture as well as in animal models of trauma and shock (47–51).

mROS:

Mitochondrial reactive oxygen species (mROS) generation occurs via the electron transport chain (ETC) in response to altered substrate availability, hypoxia, or other abnormal mitochondrial or cellular conditions (52, 53). Electrons that leak from the matrix ETC chain at complex I-III react with oxygen to result in superoxide radicals. While it has long been held that these reactive molecules augment the immune response by attacking intracellular pathogens (54, 55), it is now established that their relevance to the immune response is significantly more extensive, as mROS can modify innate cellular responses both directly and indirectly. mROS has been shown to directly modify cellular functions through their interaction with the pivotal transcription factors HIF-1α (hypoxia-inducible factor-1α) and NFKB (nuclear factor KB) (56, 57). Their indirect effects are through their ability to interact with and modify other molecules, including mtDNA, that results in heightened sensing by innate immune receptors.

Just as the production of mROS is tightly regulated, so is the removal of mROS. Scavenging enzymes of the superoxide dismutase family convert superoxide to hydrogen peroxide, which is then further reduced to water by catalase, glutathione peroxidases, and peroxidredoxins (58–60). Uncoupling proteins downregulate mROS production by decreasing the mitochondrial membrane potential (61). Finally, removal of mROS-producing damaged mitochondria by mitophagy reduces mROS and downregulates innate immune responses, while blockade of mitophagy is associated with an increase in inflammation (62).

Additional mitochondrial alarmins include ATP that is expelled extracellularly by apoptotic or necrotic cells and sensed through P2X7 purinergic receptors to trigger innate immune responses including the NLRP3 inflammasome (63, 64). Mitochondrial transcription factor A (TFAM) is structurally homologous to the nuclear alarmin molecule high mobility group box protein 1 (HMGB1), and is released from damaged mitochondria (65). TFAM have been reported to activate dendritic cell subsets, amplify TLR9 signaling, and trigger inflammatory cytokine release (66, 67).

Mitochondria and innate immune responses:

The many DAMPs of mitochondria can be released into the cytosol or extracellularly following infection, injury, or loss of cellular or mitochondrial homeostasis. These DAMPs can then be sensed by the numerous PRRs, which are germline encoded receptors that recognize the unique signatures of PAMPs and DAMPs and upon activation trigger the innate immune response (68). Based on their structures, locations, and functional specificities, PRRs are separated into discrete families that include the membrane bound Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) and the cytosolic NOD (nucleotide-binding oligomerization domain)-like receptors (NLRs) and RIG (retinoic acid-inducible gene)-l-like receptors (RLRs). Upon activation, these receptors trigger the release

of cytokines and chemokines that recruit and activate other immune cells as well as regulate organism-wide responses to the specific insult that lead to (69).

Mitochondria and TLR activation and signaling:

The TLRs are a PRR, first identified in Drosophila, that are activated by ligand binding to their carboxy-terminal leucine rich repeat (LRR). Ten TLRs have been identified in humans, with TLRs 1,2, 4, 5, 6, and 10 found on the cell surface and TRLs 3, 7, 8, and 9 spanning the endosomal membrane. Activation of TLRs results in signaling through the p38 and MAPK pathways with the resultant activation and nuclear localization of NFKB triggering the expression of pro-inflammatory genes. While TLRs are activated by pathogens and non-mitochondrial DAMPs, a number have been shown to be activated by mitochondrial components. For example, mtDNA, with its prokaryote like structure, can be recognized by TLR9 **(Figure 1)** (47). However, mitochondria are implicated in TLR signaling beyond acting as direct activators. Activated TLRs can signal through TRAF6 (tumor necrosis factor receptor-associated factor 6), which translocates to the mitochondrion and ubiquitinates the mitochondrial complex l-associated protein ECSIT (evolutionarily conserved signaling intermediate in Toll pathways). This causes the mitochondrion to both move to the phagosome and enhance mROS production, resulting in direct antimicrobial killing (55, 70). Additional studies have shown relevance for mitochondria in the signaling pathways downstream of TLR activation. Early studies linked LPS- (lipopolysaccharide) induced ROS to TLR4 signaling and dsRNA-induced ROS to TLR3 signaling, with loss of ROS associated with downregulation of NFKB activation (71). Subsequent studies linked ROS to p38 signaling activation, with the ROS generated by TLR4 activation necessary for TRAF6 and ASK1 (apoptosis signal-regulating kinase 1) to trigger p38, but not NFκB signals (72). More recently, NFκB signaling following TLR4 activation has been confirmed to be dependent upon mitochondria, although not specifically ROS, with TLR4 activation drives the interaction of mitochondrial ECSIT with both TRAF6 and TAK1 (transforming growth factor- β-activated kinase 1), and that this interaction is required for downstream NFKB signaling (73). TLR7 signaling by viral nucleic acid is also modulated by mitochondrial components as the mitochondrial outer membrane ubiquitin ligase MARCH5 (membrane-associated ring finger (C3HC4) 5) has been shown to interact with and ubiquitinate TANK (TRAF family member- associated NFκB activator) in response to TLR7 stimuli. This interaction prevents TANK from inhibiting TRAF6 and leads to enhanced TLR7-induced responses (74).

The interactions between TLR signals and mitochondria is bidirectional, as TLR activation impacts mitochondrial characteristics as well. Mitochondrial gene expression is upregulated downstream of both TLR2 and TLR4 through activity of members of the PPAR-γ coactivator family (75, 76). Separate studies have shown upregulation of mtDNA downstream of TLR2 and TLR4 activation through functions of the transcription factors NFκB, CREB (CAMP response element binding protein), NRF2 (nuclear factor erythroid 2-related factor 2), IRF (interferon regulatory factor)3, and IRF7 (77, 78). Thus, mitochondria can both directly activate TLR pathways and also modulate their signaling to regulate the resulting innate immune response.

Mitochondria and the NLR family:

Unlike the membrane-associated TLRs, the NLR family of pattern recognition receptors are localized in the cytoplasm of the cell where they are activated by PAMPs and/or DAMPs. Most members of the NLR family have a tripartite domain structure: carboxy-terminal LRR, a central nucleotide binding domain (NBD), and a variable amino- terminal domain that is involved in protein-protein interactions. NLRs are divided into subfamilies by the class of amino-terminal domain: an acidic transactivation domain (AD), a baculoviral inhibitory repeat (BIR)-like domain, a caspase recruitment domain (CARD), a pyrin domain, or a domain of unknown function. A unique feature of some NLRs is, upon activation, their formation of a multi-protein complex termed an “inflammasome”. The core structure of an inflammasome is the NLR as the sensory component, bound at the amino-terminal domain to the adaptor ASC (apoptosis-associated speck-like protein containing a CARD), that forms a bridge to the CARD domain of pro-caspase-1, which is the inactive pro-enzyme of the effector cysteine protease caspase-1. Inflammasome assembly triggers auto-catalysis of pro-caspase-1 to active caspase-1 that in turn cleaves inactive pro-IL (interleukin)- 1β and pro-IL-18 to their active, secreted forms that have broad pro-inflammatory effects. Active caspase-1 also cleaves gasdermin D that can introduce pores in the cell membrane through which IL-1 β and IL-18 are secreted and that can also induce inflammasome-associated pyroptotic cell death (79, 80). A number of NLRs have been suggested to form inflammasomes that culminate in caspase-1 activation, while some have been reported to have inflammasome-independent functions. Thus far, a defined role for mitochondria in NLR activation and function has only been confirmed for NLRP3 and NLRX1 as detailed below.

NLRP3:

NLRP3 was first described when its mutation was found to be causative to a group of autoinflammatory disorders, now collectively known as CAPS (cryopyrin-associated periodic syndromes) (81–83). Subsequently, activation of the NLRP3 inflammasome has been linked to a wide array of infectious and sterile inflammatory disorders, including but not limited to bacterial, viral, and fungal infections, metabolic syndrome, ischemia-reperfusion injury, atherosclerosis, Alzheimer’s disease, and gout (39). NLRP3 is expressed in macrophages, monocytes, dendritic cells, neutrophils, as well as in numerous non-hematopoietic cells (84). Although NLRP3 is the most studied member of the NLR family, significant gaps remain in our understanding of its activation. NLRP3 activation occurs in response to two discrete steps, termed priming and activation. While it has been established both steps must occur, with priming preceding activation, the precise events that occur during each step to allow inflammasome activation to proceed are defined incompletely. In priming, an inflammatory stimulus such as TLR activation or binding of a cytokine to a receptor signal through the adaptor molecule MyD88 (myeloid differentiation primary response 88) or TRIF (TIR-domain-containing adapter- interferon β) with subsequent activation of NFKB (85, 86). This induces the upregulation of expression of both NLRP3 and pro-1 L-1 β, although increased protein levels are neither sufficient nor required for priming to occur (85, 87, 88). Additional steps implicated in priming include multiple post-translational modifications to inflammasome components, including the de-ubiquitination and phosphorylation of NLRP3 and the ubiquitination and phosphorylation of ASC (87, 89–

95). The activation step is induced by a wide array of structurally diverse molecules, including endogenous and exogenous crystals that require phagocytosis, ATP via the cell surface P2X7 receptor, and pathogen-derived toxins. As none of these activators has been shown to directly associate with NLRP3, studies have focused on the identification of a final common pathway downstream of these divergent stimuli to determine the mechanism by which the NLRP3 inflammasome is activated. These studies have led directly to the mitochondrion, showing that activated NLRP3 inflammasomes co-localize with mitochondria (96). Recent work by our group has shown the use of mitochondria as a scaffold for inflammasome assembly is initiated at priming, as both NLRP3 and pro-caspase-1 move to the mitochondrion at priming, with the movement of the adaptor molecule ASC following in response to the activating stimulus (97). While additional studies have confirmed the co-localization of the NLRP3 inflammasome with mitochondria, the mechanism by which it occurs is less clear, with studies separately linking it to MAVS (mitochondrial antiviral signaling protein), cardiolipin, or c-FLIP (c-FLICE-like inhibitory protein) (39, 40, 98–100). The role of MAVS is intriguing as instead of serving as a common mediator for all NLRP3 inflammasome activators, it appears to be necessary for only a subset of NLRP3 activators. The initial report describing a role for MAVS in NLRP3 activation showed that only the non-crystalline subset of NLRP3 activators required MAVS, as activation of NLRP3 by crystalline activators did not require or involve MAVS (98). A subsequent study confirmed a role for MAVS in NLRP3 activation by Sendai virus but did not explore further the type of agonist driving the response (99). Additional studies will be needed to determine what function MAVS provides specifically to the non-crystalline activators of NRLP3 and to determine if a separate agent serves a parallel role for the crystalline activators of NLRP3.

In the same study that first showed the co-localization of NLRP3 inflammasomes with mitochondria the authors also showed that the activation of NLRP3 was associated with both mitochondrial damage and the release of mROS, and that these were required for NLRP3 activation (96). Subsequent studies have confirmed mROS is induced by most but not all activators of NLRP3, although the induction of mitochondrial dysfunction has still been shown to be required for some of these ROS-independent activators (40, 101–106). This is also consistent with studies showing inhibition of mitophagy, the removal of damaged or dysfunctional mitochondria, augments activation of NLPR3 (62, 96). While the source of the mitochondrial damage has not been determined, one possible mechanism is that the elevated cytosolic calcium required during NLRP3 inflammasome activation is taken up by mitochondria, overloading the mitochondria causing loss of negative potential in the mitochondrial matrix and resulting in the mitochondrial dysfunction that drives NLRP3 activation.

The relevance of mitochondria to NLRP3 activation extends beyond this role for mROS and mitochondrial dysfunction as well as its function as a scaffold upon which the NLRP3 inflammasome assembles. In contrast to the various extra-cellular activators the result in NLRP3 activation, specific components of mitochondria have been suggested to directly activate NLRP3. An early study by Dr. Choi’s group showed mtDNA was released into the cytosol during NLRP3 activation downstream of mROS release (101). This finding was built on by subsequent work by Dr. Arditi’s group that showed mtDNA undergoes oxidation

during the mitochondrial dysfunction associated with NLRP3 activation and that this oxidized mtDNA bound to and activated the NLRP3 inflammasome, suggesting mtDNA may be the ligand that directly drives NLRP3 activation (102). Studies from our group showed a different mitochondrial component, cardiolipin, both tethers NLRP3 to the mitochondria and triggers the activation of NLRP3 (40). Future studies will be necessary to determine the relative roles of discrete mitochondrial factors in the subcellular localization and activation of NLRP3.

NLRX1:

That the NLR family member NLRX1 interacts with mitochondria was initially suggested based on its novel amino-terminal domain. Rather than the pyrin or CARD domains found in other NLR family members, the NLRX1 protein begins with a putative mitochondrial targeting sequence. NLRX1 was confirmed experimentally to localize to the mitochondria but its exact mitochondrial location and its function have been less straight forward to determine (107, 108). Initially, NLRX1 was shown to be a negative regulator of type I interferon production by binding to MAVS, preventing the interaction of MAVS and RIG-I (retinoic acid inducible gene-l), and thereby blocking the downstream activation of IRF3 and NFkB (107). This negative regulatory role was supported by two subsequent studies, the first of which showed NLRX1 blocked the inflammatory response to DNA viruses by binding to STING (stimulator of interferon gene) and preventing downstream cGAS (cyclic GAMP synthase) signaling (109). The second confirmatory study reported the loss of NLRX1 was associated with enhanced NFKB signals, consistent with NLRX1 being a negative regulator of inflammation (110). In contrast, a separate report using overexpressed NLRX1 showed that rather than inhibiting interferon signaling, NLRX1 increased NFKB signaling downstream of *Shigella flexneri* infection (108). In support of NLRX1 not downregulating the inflammatory response, NLRX1-deficient fibroblasts and macrophages had no defect in interferon responses to Sendai virus and NLRPXI-deficient mice had no identifiable defects in early signals to influenza A virus in vivo (111). A second study supported this independence of anti-viral signaling from NLRX1 (112). A recent study may have found the explanation for these seemingly conflicting functions, as NLRX1 was shown to simultaneously promote the upregulation of IRF1 but limit the formation of IRF3 dimers, consistent with a nuanced regulatory function for NLRX1 in innate immune responses with potentially both pro- and anti-inflammatory aspects (113). However, controversy relating to NLRX1 persists: while initial studies described the localization of NLRX1 on the outer mitochondrial membrane, NLRX1 has more recently been described as undergoing translocation into the mitochondrial matrix in a pathway dependent upon the negative potential within the mitochondrial matrix (107, 114). With this matrix localization NLRX1 was found to interact with a protein of the respiratory chain, potentially explaining the modulation of ROS described with NLRX1 overexpression, but was subsequently and separately shown instead to interact with mitochondrial TUFM (Tu translation elongation factor) to regulate viral-induced autophagy (108, 114–116). In addition to these conflicting reports as to the location of NLRX1 and its impact on interferon signals, the relevance of NLRX1 in regulating ROS is similarly unclear. While several studies have shown NLRX1 driving increased ROS in response to inflammatory stimuli, a conflicting report described the loss of NLRX1 was associated with an increase in oxygen consumption, consistent with a downregulatory rather

than upregulatory role for NLRX1 (108, 111, 115, 117, 118). One possible explanation for the divergent findings of NLRX1 in these studies is that NLRX1 may impact uncoupling of oxidative phosphorylation, resulting in a disconnect between oxygen consumption, ATP generation, and ROS production (119). Additional studies will be needed to confirm if a possible role in uncoupling explains these seemingly discrepant results, to expand on how NLRX1 balances up- and down-regulation of interferons, and also to determine how NLRX1 modulates outer mitochondrial membrane components like MAVS if its location is confirmed within the mitochondrial matrix.

Mitochondria and RLRs:

As viral genomes can undergo amplification in the cytoplasm of host cell, they are often inaccessible to detection by TLRs. Intact type I interferon responses to viral infections in cells lacking the sole TLR3 adaptor TRIF suggested a TLR-independent receptor pathway may exist that also generated these potent anti-viral cytokines (120). The quest to identify these sensors of viral infections lead to the identification of the RNA helicase retinoic acid inducible protein I (RIG-I). RIG-I, along with the other RLR (RIG-l-like receptor) family members, melanoma differentiation associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) are cytosolic proteins that sense viral RNA (121). The RLRs share a conserved domain structure with a pair of amino-terminal CARD domains (absent in LGP2), a central RNA helicase domain, and a carboxy-terminal regulatory domain. RIG-I and MDA5 bind cytosolic viral RNA and, through the adaptor MAVS, trigger the release of type I interferons (122–125). RIG-I recognizes the unique 5’-phosphorylated blunt ends of viral genomic RNA while MDA5 binds long dsRNA (126–128). The function of LGP2 seems to be to augment these RIG-I or MDA5-triggered responses (129–133).

As noted, RLR signaling require MAVS, a 540 amino acid protein with three domains: an amino-terminal CARD, a proline rich central domain, and a carboxy-terminal transmembrane portion. The transmembrane domain anchors MAVS primarily to the mitochondrial outer membrane, but MAVS is also inserted within, and can signal from, the mitochondrial associated membranes, the endoplasmic reticulum, and peroxisomes (134, 135). RIG-I or MDA5, activated by their nucleic acid ligands, bind to and activate MAVS via CARD-CARD interactions, triggering polymerization of MAVS into prion-like structures that are required for it to signal downstream (136, 137). MAVS then complexes the cytoplasmic kinases TBK1 (Tank binding kinase 1) and IKKε (IKB kinase-ε) resulting in activation of the transcription factors IRF3, IRF7 and NFKB and upregulating the transcription of type I interferons, interferon inducible genes and proinflammatory cytokines (136, 138). This activation pathway is regulated through extensive post-translational modifications, including ubiquitination and phosphorylation at each step of activation (139–143). The mitochondrion itself also significantly impacts and regulates the activation of MAVS. MFN2 (mitofusin 2), a mitochondrial GTPase that, along with MFN1 and OPA1, regulates mitochondrial fusion, was shown to inhibit RLR signaling by binding to MAVS and preventing CARD dimerization (144). Despite significant homology between MFN2 and MFN1, in contrast to MFN2, MFN1 was found to enhance MAVS signaling (145, 146). Additional proteins that have been suggested to serve as negative regulators of MAVS are NLRX1, gC1qR (globular head domain of complement component 1q receptor), and PLK1

(Polo-like kinase 1) (107, 147, 148). The negative membrane potential of the mitochondria has also been linked to MAVS signaling as dissipation of that potential abrogated RLR signaling (149). ROS have been shown in two studies to regulate RLR signaling, first that the increase in ROS resulting from the blockade of autophagy upregulates RLR signaling and subsequently that MAVS protein expression, and thereby MAVS signaling, is dependent upon ROS (150, 151). Despite these important findings, the mechanistic details as to the activation, regulation, and signaling of these pathways remain to be determined.

Mitochondria and cGAS-STING pathways:

Although DNA was hypothesized to be immunogenic over a century ago, the sensors and immunologic pathways activated by cytosolic DNA remain only partially characterized (152). STING (stimulator of interferon genes) was identified ten years ago as pivotal in the release of type I interferons (105, 153, 154). This activation of STING was found to be in response to cytosolic DNA, and while more commonly considered to be of nuclear or microbial sources, this activation is also induced by mitochondrial DNA that can be released during apoptosis or other conditions of mitochondrial damage and dysfunction (155–161). STING is not the sensor of cytoplasmic DNA but rather an adaptor that links the activated receptor to downstream inflammatory gene upregulation. Cytosolic DNA binds to cGAS (cyclic GMP (guanosine monophosphate) synthase) and induces a change in cGAS conformation that activates cGAS to catalyze GTP and ATP to the second messenger cGAMP (cyclic GMP-AMP) that then binds to and activates STING (162–167). This binding induces a conformational change in STING, triggering its translocation from the ER to the Golgi apparatus (153, 166, 168, 169). This movement to the Golgi activates STING, in turn activating TBK1 and IKK which phosphorylate and activate the transcription factors IRF3 and NFκB. These transcription factors move to the nucleus and drive type I interferon and proinflammatory cytokine production (170, 171). Thus, any mitochondrial event that results in the leaking of mtDNA can stimulate the cGAS-STING pathway, resulting in a potent innate inflammatory response.

Mitochondria and FPR signaling:

Formyl peptide receptors (FPR) are a family of G protein coupled receptors expressed as transmembrane proteins on the surface of many hematopoietic as well as non-hematopoietic cells (172). They were first described on neutrophils and have been studied most extensively in regulating neutrophil migration and function (172, 173). These receptors are activated by n-FP, peptides produced by microbes or mitochondria that contain an amino-terminal formylated methionine (fMet) (27, 28). There are three family members in humans, FPR1, FPR2, and FPR3, with FPR1 the best characterized. FPR1 and FPR2 share significant sequence homology, particularly for the cytoplasmic signaling domains (174, 175). Both FPR1 and FPR2 bind n-FP, although with different binding specificity as FPR1 and FPR2 preferentially bind shorter and longer peptides, respectively (176, 177). While the other innate immune pathways induced by mitochondrial DAMPs discussed in this review predominantly result in inflammatory cytokine release, the interaction of n-FP with FPRs primarily triggers migration and neutrophil activation (178, 179). The relevance of FPRs to disease was confirmed by studies showing circulating mitochondrial DAMPs in patients with trauma-associated systemic inflammatory response syndrome with mechanistic animal

studies confirming neutrophil migration to mitochondrial DAMPs required FPR1 (49, 180, 181). The intracellular signaling downstream of FPR1 activation starts with G-protein dissociation of the α and βγ subunits. Activation of phospholipases drives the generation of diacylglycerol and inositol triphosphate, triggering the release of calcium from the ER to the cytosol that culminates in ROS production (182). Parallel activation of kinase pathways results in signals necessary for cytoskeletal organization and contribute to ROS production and neutrophil activation (183). FPR1 has been shown to function in other innate immune cells, stimulating inflammatory cytokine release from monocytes and regulating the maturation and migration of dendritic cells (184, 185).

Thus, numerous components of the mitochondria can be released and trigger an array of inflammatory receptors and signaling pathways. These are summarized in Table 1.

Mitochondrial metabolism and innate immunity:

The focus of this section is the specific regulation of innate immune pathways by mitochondrial metabolism. For a broader discussion on, and insight into, immune regulation by metabolism the reader is referred to a recent in-depth review (186).

Mitochondria use oxidative phosphorylation via the electron transport chain (ETC) and the tricarboxylic acid (TCA) cycle to generate ATP for cellular functions. The electron transport chain consists of five multiprotein complexes on the inner mitochondrial membrane. Electrons are donated from NADH to complex I or $FADH_2$ to complex II (also known as succinate dehydrogenase) and transferred through coenzyme Q, complex III, cytochrome C, complex IV, to complex V (also known as ATP synthase) which generates ATP from ADP. The TCA cycle, responsible for the majority of ATP production, progresses in concert, regenerating NADH and $FADH_2$ to maintain electron transport. Under normal conditions a small amount of electrons escape the ETC to combine with oxygen and form oxygen radicals, collectively known as reactive oxygen species (ROS) (Figure 2A) (187–189).

In the TCA cycle, fatty acids and pyruvate are brought into the mitochondrion and oxidized to acetyl-CoA which enters the TCA cycle through combining with oxaloacetate to form citrate. Citrate is oxidized to continue the cycle, leading to regeneration of NADH and FADH2 for the ETC and then back to oxaloacetate to continue the cycle (187–189). Perturbations to the ETC, TCA cycle, and TCA cycle metabolites have impacts upon wider immune cell function and respon.

Activated macrophanges manipulate the TCA cycle and ETC to modulate their immue function (190, 191). Inflammatory (M1), but not anti-inflammatory (M2) macrophages, have what is referred to as a “broken” TCA cycle (192). Down regulation of the TCA enzyme isocitrate dehydrogenase and upregulation of immune-responsive gene 1 protein (IRG1) blocks TCA progression and result in the conversion of accumulating citrate to itaconate (Figure 2B) (193, 194). Itaconate has direct antimicrobial functions that augment the innate immune response, but also inhibits complex II (also known as succinate dehydrogenase), preventing succinate oxidation (195–197). This results in reverse electron transport back down the ETC; associated increased ROS stabilizes the transcription factor HIF-1α that enhances the inflammatory response (198, 199). Interference with the TCA cycle in M1

macrophages blocks the production of ATP, consistent with the finding that M1 macrophages rely on glycolysis for energy production while M2 macrophages generate ATP with the TCA cycle (194, 200–202). Indeed, additional studies have shown that differential stimuli of innate immune cells trigger unique metabolic signatures necessary for subsequent immune function (203, 204).

Concluding remarks:

From their relatively humble beginnings as ancient bacteria, mitochondria have established themselves as regulators of sweeping aspects of mammalian function at both the cellular and organism level. This regulation extends well beyond simply providing critical bioenergetics and includes, but is not limited to, precise and nuanced control of innate immune activation and signaling. The advances in our understanding of the importance of mitochondria to the innate immune response along with technical advances in our ability to study the mitochondria are likely to reveal additional novel pathways through which these seemingly discrete systems are in fact intertwined.

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Abbrevations:

PAMP	Pathogen Associated Molecular Pattern
DAMP	Damage Associated Molecular Pattern
PRRs	Pathogen Recognition Receptors
ROS	Reactive Oxygen Species
TLR	Toll Like Receptor
RLR	Retinoic acid Inducible Gene like Receptor
NOD	nucleotide-binding oligomerization domain
NLR	NOD like receptor
ATP	Adenosine Tri-Phosphate
MFN1/2	Mitofusin
RIG-I	Retinoic acid Inducible Gene I
MAVS	Mitochondrial Anti-Viral Signaling
ASC	Apoptosis associated Speck like protein containing CARD
ETC	Electron Transport Chain

ESCIT	Evolutionarily Conserved Signaling Intermediate in the Toll pathway
NFκB	Nuclear factor κ B
IRF	Interferon Regulatory Factor
IFN	Interferon
JNK	cJUN NH2- terminal kinase
TANK	TRAF family member-associated NFKB activator
TBK1	TANK Binding Kinase 1
cGAS	cyclic GMP-AMP Synthase
STING	Stimulator of Interferon Gene
CARD	Caspase Activation and Recruitment Domain
IL	Interleukin
CL	Cardiolipin
mROS	mitochondrial ROS
mtDNA	mitochondrial DNA
TCA	Tri-carboxylic acid
NADH	Nicotinamide adenine dinucleotide
TFAM	Mitochondrial transcription factor A
HMGB1	high mobility group box protein 1
fMet	N-formylated methionine
n-fp	n-formyl peptides
FPR	formyl peptide receptor
CLR	C-type lectin receptor
LRR	leucine rich repeat
MAPK	mitogen-activated protein kinase
TRAF6	tumor necrosis factor receptor-associated factor 6
LPS	lipopolysaccharide
ASK1	apoptosis signal-regulating kinase 1
TAK1	transforming growth factor-β-activated kinase 1
MARCH5	membrane-associated ring finger (C3HC4) 5

PPAR	peroxisome proliferator-accelerated receptor
HIF	hypoxia-inducible factor
CREB	cAMP response element binding protein
NRF2	nuclear factor erythroid 2-related factor 2
NBD	nucleotide binding domain
CAPS	cryopyrin associated periodic syndromes
MyD88	myeloid differentiation primary response 88
TRIF	TIR-domain-containing adapter-inducing interferon β
MAVS	mitochondrial antiviral signaling protein
TUFM	Tu translation elongation factor
MPT	mitochondrial permeability transition
MOMP	mitochondrial outer membrane permeabilization

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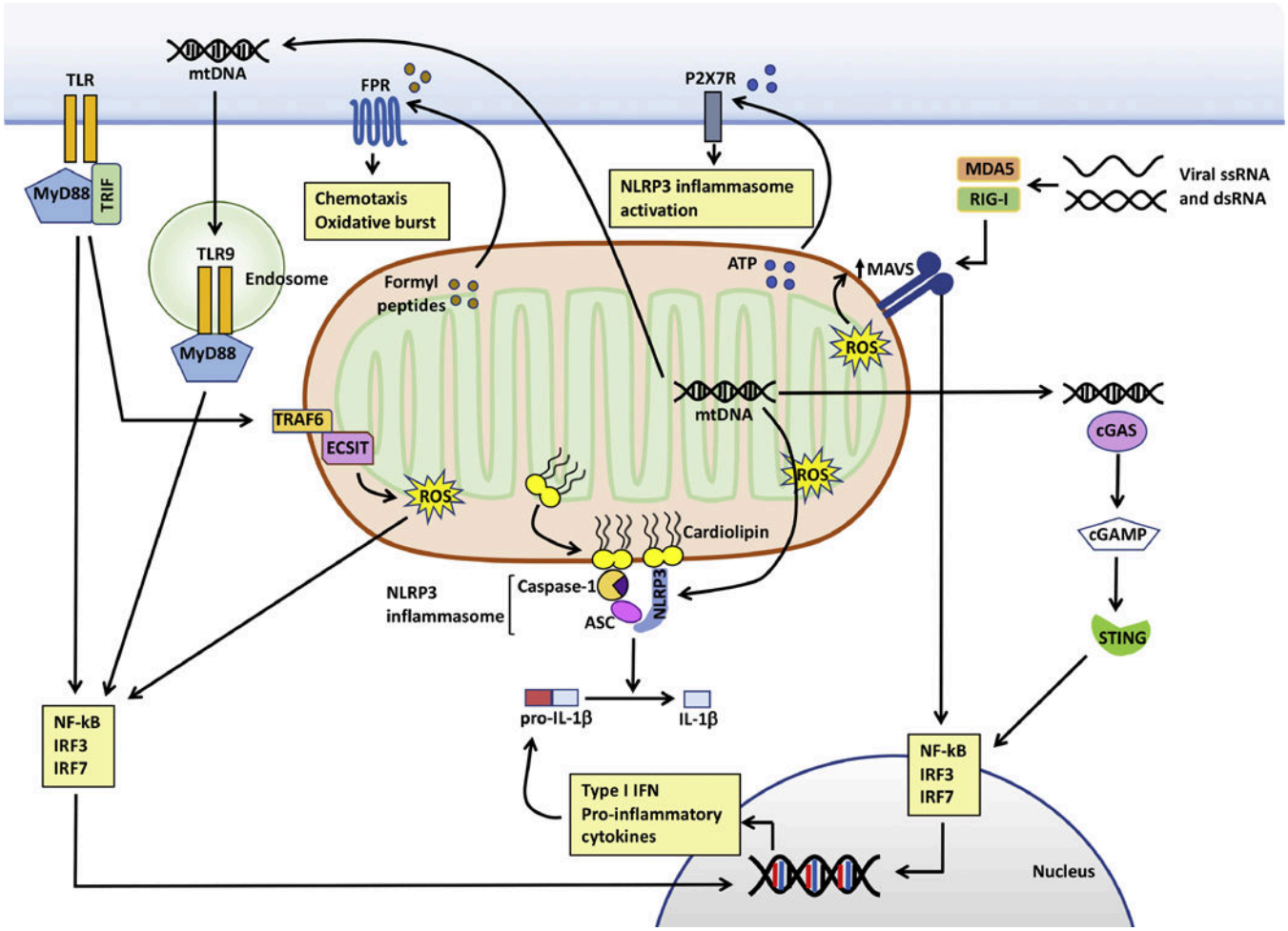

Figure 1. Mitochondrial regulation of innate immune responses.

Mitochondrial DAMPs activate a number of innate immune pathways. Mitochondrial DNA that escapes to the cytosol from damaged mitochondria is recognized by cGAS and signals through cGAMP and STING to activate inflammatory gene transcription. Mitochondrial DNA leaked from a cell can be phagocytosed and bind endosomal TLR9, triggering MyD88-dependent signaling to interferons and pro-inflammatory cytokines. These inflammatory cascades also serve to prime the NLRP3 inflammasome and upregulate pro-lL-1 β. The NLRP3 inflammasome is then activated by oxidized mtDNA from dysfunctional mitochondria. Cardiolipin, which moves to the outer mitochondrial membrane in response to mitochondrial dysfunction, tethers the NLRP3 inflammasome to the mitochondrion and can also trigger its activation. ATP that leaks from a damaged cell can bind to the P2X7 receptor on adjacent cells, triggering NLRP3 inflammasome activation within those nearby cells. Similarly, formyl peptides are also released by damaged cells and are recognized by FPRs on neutrophils and result in neutrophil activation including chemotaxis and the respiratory burst. Modulation of innate immune signaling pathways also depend upon mitochondria. Mitochondria- independent activation of TLRs results in signals through the mitochondrial protein ECSIT, generating mROS and enhancing inflammatory gene output. Activation of the RLRs MDA5 and RIG-I by viral RNA is initiated in the cytosol but signaling depends

upon the mitochondria, as both MDA5 and RIG-I must bind MAVS on the outer mitochondrial membrane to activate their downstream signaling pathways. This results in upregulation of interferons and other inflammatory genes. Further, mROS can enhance RLR signaling by upregulating MAVS expression on the outer membrane.

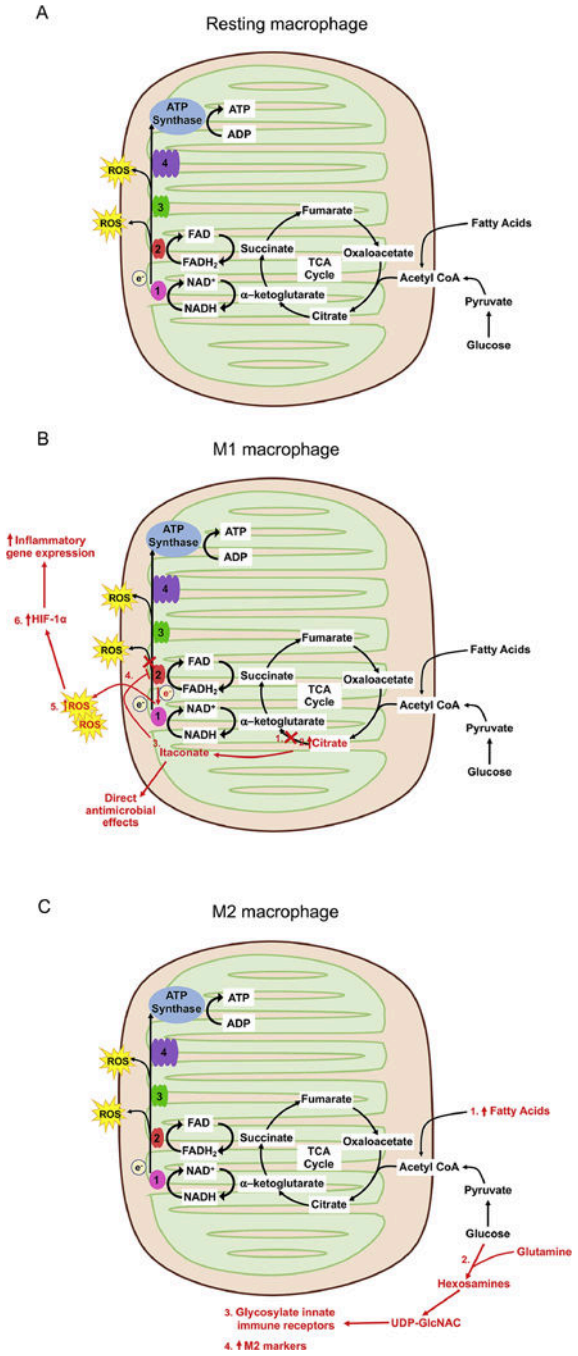

Figure 2. Mitochondrial metabolism in immune cell polarization and innate immune responses.

A. In resting macrophages, glucose and fatty acids are broken down to acetyl CoA that enters the TCA cycle. As the TCA cycle progresses, NADH and $FADH_2$ are regenerated as electron donors for the ETC. Most electrons in the ETC are used to generate ATP although some leak off and combine with oxygen to create ROS. **B.** Activated M1 macrophages have modifications in their metabolism that drives their pro-inflammatory characteristics. (1) M1 macrophages downregulate isocitrate dehydrogenase, resulting in a block in the cycle moving forward from citrate. (2) Citrate accumulates. (3) The metabolic products of citrate

are converted to itaconate by IRG1 (immune response gene 1 protein), which is markedly upregulated in M1 macrophages (4) In addition to direct antimicrobial effects, itaconate also inhibits complex II (also known as succinate dehydrogenase), preventing forward progression of the ETC. (5) Reverse transfer of electrons to complex 1 results in increased ROS generation. (6) increased ROS results in stabilization of the transcription factor HIF-1a with subsequent upregulation of inflammatory gene expression, including the potent inflammatory cytokine IL-1 β. C. The metabolic signature of activated M2 macrophages is necessary for their function. (1) M2 macrophages have enhanced fatty acid oxidation driving the TCA cycle to generate ATP. (2) M2 macrophages also require glycolysis, hydrolyzing glucose and using glutamine through the hexosamine pathway to generate uridine diphosphate (UDP)-N-acetylglucosamine (UDP- GIcNAc). This production of UDP-GIcNAc is necessary for (3) glycosolation of immune receptors and (4) the upregulation of expression of specific M2 markers.

Table 1.

Mitochondrial DAMP	Sensor	Result
n-formyl peptides	FPR1, FPR2	Chemotaxis, Oxidative burst
Cardiolipin	NLRP3	Inflammasome activation, pro-inflammatory cytokines
mtDNA	TLR9, NLRP3, cGAS-STING	Type I interferons, pro-inflammatory cytokines, inflammasome activation
ROS	NLRP3	Inflammasome activation, pro-inflammatory cytokines
ATP	NLRP3	Inflammasome activation, pro-inflammatory cytokines