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RESEARCH HIGHLIGHT

Mitochondrial ROS fuel the inflammasome

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IL-1β and IL-18 are pro-inflammatory cytokines that play a critical role in the response to a diverse array of injuries and infections. Accordingly, the production of these potent cytokines is tightly regulated at transcriptional and post-translational levels through control of both maturation and secretion. The maturation of these cytokines is regulated by caspase-1 inflammasomes. Several members of the Nod-like receptor (NLR) family of intracellular sensors, including NLRP3, NLRC4 and NLRP1, play critical roles in inflammasome regulation. However, the nature of the physiological cues that trigger inflammasome activation remain incompletely understood.

A recent study by Zhou *et al.* examined this important question in the context of activation of the NLRP3 inflammasome [1]. Zhou *et al*. demonstrated that generation of mitochondrial reactive oxygen species (ROS) led to NLRP3 inflammasome activation, and treatment of macrophages with NLRP3 activators resulted in the recruitment of NLRP3 to mitochondria-associated ER membranes (MAMs) where the protein recruited the ASC adaptor critical for inflammasome formation (Figure 1). Furthermore, autophagy limited NLRP3 inflammasome activation by targeting ROS-producing mitochondria (Figure 1). Therefore, this study provides a fundamental link between inflammasome activation and mitochondrial function.

NLRP3 can be activated by host-derived danger signals, such as monosodium urate (MSU) crystals or extracellular ATP, bacterial or viral infection, as well as environmental stimuli including asbestos and UVB radiation [2]. Several models of NLRP3 activation have been proposed. In the case of large particles or crystalline structures, such as asbestos, MSU and amyloid-β fibers, uptake may result in "frustrated phagocytosis" [3] or damage to phagosome and lysosome vesicles, leading to release of cathepsin B and NLRP3 activation [4]. A role for K^+ efflux in inflammasome activation has also been proposed. Interfering with K^+ efflux through the addition of extracellular K^+ or chemical inhibitors, such as glyenclamide, blocks NLRP3 inflammasome activation in response to several stimuli, including extracellular ATP, MSU and nigericin [5]. Finally, despite the diversity of signals recognized by NLRP3, the generation of ROS appears to be a common cellular response critical for NLRP3 activation since ROS scavengers attenuate NLRP3 activation [4]. However, the source of ROS and the mechanism by which NLRP3 senses ROS generation are currently unclear. Indeed, while several studies have suggested a role for NADPH oxidases in NLRP3 activation [4], macrophages lacking functional NOX1, NOX2 and NOX4 respond normally to NLRP3 stimulation [1].

Oxidative phosphorylation generates the largest source of ATP in eukaryotic cells and occurs through the reduction of O_2 to H_2O at the mitochondria. The reduction of O_2 by a series of protein complexes (I to IV) is used to generate an H^+ gradient that powers ATP synthesis by complex V. This process leads to the formation of several types of ROS intermediates, including O_2^-, H_2O_2 and hydroxyl radical. Therefore, mitochondria constitute a major source of cellular ROS. Zhou *et al*. hypothesized that mitochondrial ROS may be involved in NLRP3 activation.

To investigate a role for mitochondrial ROS in NLRP3 activation, the authors first inhibited complex I function with rotenone. As previously described [6], complex I inhibition led to increased mitochondrial ROS production. Interestingly, this correlated with increased IL-1β secretion. The increased production of IL-1β was specific to and dependent on NLRP3 inflammasome activation since macrophages lacking NLRC4/IPAF responded similarly to wild-type cells, and enhanced IL-1β production was lost in $NLRP3^{-/-}$ macrophages.

Having established a role for mitochondrial ROS in NLRP3 inflammasome activation, the authors examined how NLRP3 senses ROS. The subcellular localization of NLRP3 was examined using Flag-tagged versions of NLRP3 and the adaptor ASC. In unstimulated conditions, NLRP3 localized to the endoplasmic reticulum, as determined by colocalization with the ER marker

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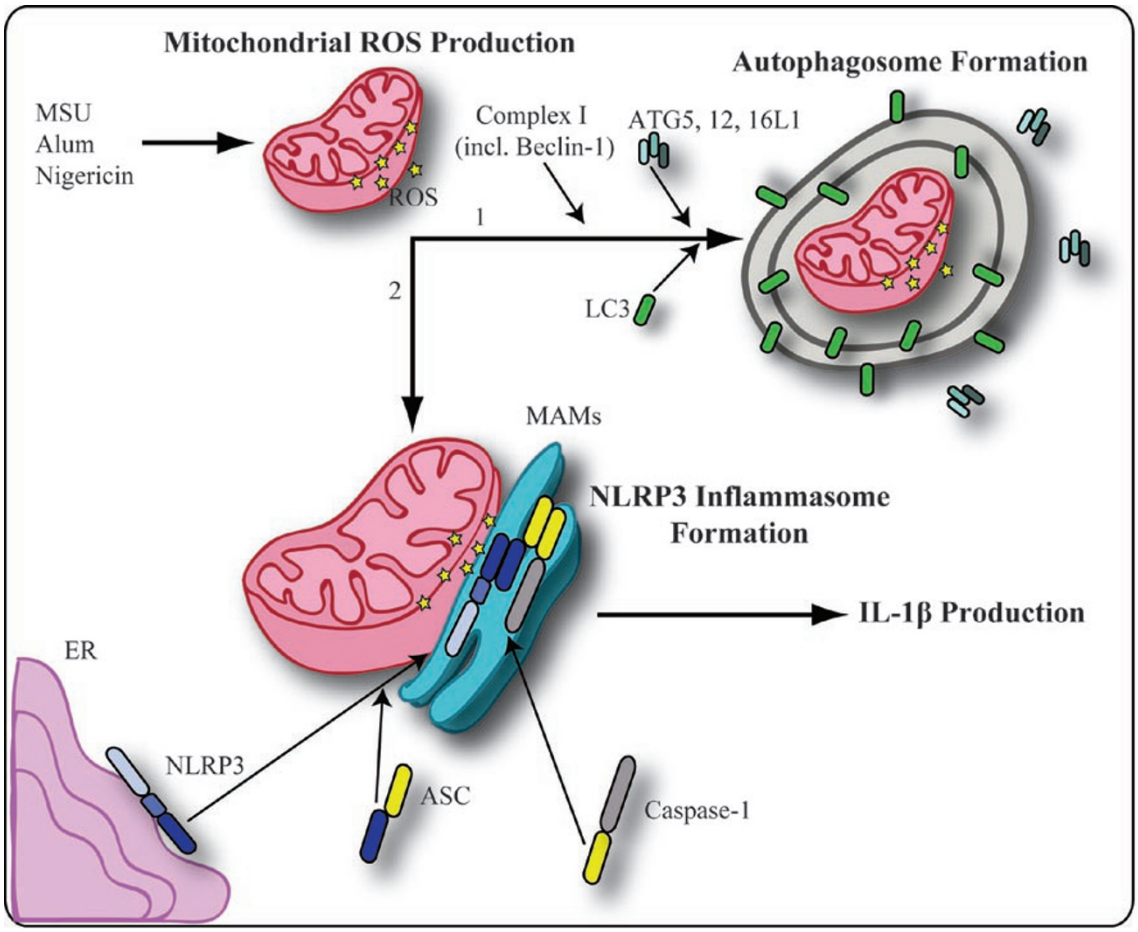

Figure 1 Mitochondrial ROS promote NLRP3 inflammasome formation. Treatment of macrophages with MSU, alum or nigericin leads to the production of mitochondrial ROS. In (1), this triggers the induction of mitophagy and the formation of an LC3+ autophagosome to remove the damaged mitochondria. In (2), the induction of mitochondrial ROS induces the recruitment of NLRP3 to MAMs. NLRP3 recruits ASC through PYD-PYD interaction (shown in dark blue), which then recruits pro-caspase-1 through CARD-CARD interactions (shown in yellow). This results in the activation of caspase-1 and the production of IL-1β.

calreticulin, while the ASC adaptor was primarily cytosolic. Following treatment with MSU, alum or nigericin, both NLRP3 and ASC localized to MAMs. ASC recruitment to MAMs depended on NLRP3 and did not occur in cells silenced for NLRP3. MAMs are important for mitochondrial function, as they contribute to phospholipid transfer and synthesis, steroidogenesis and regulate mitochondrial Ca^{2+} levels [7].

To directly test the hypothesis that NLRP3 inflammasome activation depends on ROS production from respiring mitochondria, Zhou *et al*. inhibited mitochondrial respiration by knocking down expression of voltage-dependent anion channels (VDAC). Interestingly, in cells lacking VDACs, IL-1β production and caspase-1 activation were abrogated in response to NLRP3 activators. Similarly, cells overexpressing Bcl-2, which inhibits VDAC function, also displayed impaired production of IL-1β in response to MSU, alum or nigericin.

Previous studies have shown that macrophages lacking the autophagy proteins ATG16L1 or ATG7 produce elevated levels of IL-1β, suggesting that autophagy may regulate inflammasome activation [8]. Furthermore, autophagy plays an important role in clearing damaged ROS-producing mitochondria [9]. Therefore, Zhou *et al*. investigated a role for autophagy in attenuating NLRP3 activation. Inhibition of complex I function with rotenone led to the targeting of mitochondria by autophagy. Interestingly, inhibition of autophagy resulted in accumulation of damaged, ROS-producing mitochondria. In cells displaying impaired autophagy, either following treatment with 3-methyladenine or by knocking down ATG5 or Beclin-1, an NLRP3-dependent, NLRC4-independent enhancement of IL-1β was observed.

The findings of Zhou *et al*. are compatible with those of Nakahira *et al*. who also recently examined the interplay of

autophagy, mitochondria and NLRP3 inflammasome activation [10]. Nakahira *et al*. also found that autophagy limited caspase-1 activation by clearing ROS-producing mitochondria. Furthermore, the authors also demonstrated the necessity of respiring mitochondria for NLRP3 activation, in this case by depleting cells of mitochondria DNA (mtDNA) through ethidium bromide treatment. In Nakahira *et al*.'s model, treatment of macrophages with LPS plus ATP leads to the release of mtDNA. The release of mtDNA requires ROS formation and is enhanced in cells lacking autophagy. Furthermore, transfection of mtDNA enhanced the response to LPS and ATP, and cytosolic mtDNA levels correlated with IL-1β production. Notably, NLRP3 was required for mtDNA release following LPS+ATP stimulation and the loss of mitochondrial membrane potential, but not mitochondrial ROS production.

Taken together, the results by Zhou *et al*., associated with those by Nakahira *et al*., suggest a complex model, in which NLRP3 activators trigger mitochondrial ROS production that is limited by autophagic clearance of damaged mitochondria. ROS production leads to the relocation of NLRP3 to MAMs, where ASC is recruited, thereby promoting NLRP3 inflammasome activation. In addition, the NLRP3 inflammasome would promote the release of caspase-1-activating mtDNA. The link proposed between NLRP3 activation, mitochondrial ROS generation, mtDNA release and regulation of mitophagy holds great promise for future research in the field of inflammasome activation, and also opens up new questions. It will be interesting to dissect the role that NLRP3-driven mtDNA release has in caspase-1 activation and IL-1β secretion, and whether this release functions as a positive feedback through the NLRP3 inflammasome or another cytosolic, AIM2-independent sensor. It will also be important to determine whether mitochondrial ROS are ubiquitously induced by NLRP3 activators, and elucidate the pathways leading to mitochondrial stress in each case. The relative contribution of NADPH-triggered versus mitochondria-triggered ROS in NLRP3-dependent induction of the inflammasome will also need to be carefully evaluated. Nevertheless, the study by Zhou *et al*. adds to the emerging picture that the mitochondrion not only serves as critical organelle involved in cell metabolism, but also plays essential roles in controlling innate immune pathways.

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