

Mitochondrial apoptosis is dispensable for NLRP3 inflammasome activation but non-apoptotic caspase-8 is required for inflammasome priming

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Abstract

A current paradigm proposes that mitochondrial damage is a critical determinant of NLRP3 inflammasome activation. Here, we genetically assess whether mitochondrial signalling represents a unified mechanism to explain how NLRP3 is activated by divergent stimuli. Neither co-deletion of the essential executioners of mitochondrial apoptosis BAK and BAX, nor removal of the mitochondrial permeability transition pore component cyclophilin D, nor loss of the mitophagy regulator Parkin, nor deficiency in MAVS affects NLRP3 inflammasome function. In contrast, caspase-8, a caspase essential for death-receptor-mediated apoptosis, is required for efficient Toll-like-receptor-induced inflammasome priming and cytokine production. Collectively, these results demonstrate that mitochondrial apoptosis is not required for NLRP3 activation, and highlight an important non-apoptotic role for caspase-8 in regulating inflammasome activation and pro-inflammatory cytokine levels.

Keywords apoptosis; caspase-8; inflammasome; mitochondria; NLRP3

Subject Categories Immunology; Autophagy & Cell Death

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Introduction

The NOD-like receptor protein 3 (NLRP3) inflammasome senses a variety of pathogen, host and environmentally derived molecular patterns to mediate caspase-1 activation, thereby promoting caspase-1 processing and secretion of the cytokines IL-1 β and IL-18.

Caspase-1 can also induce a lytic inflammatory form of cell death known as pyroptosis [1], which like necroptotic cell death [2], appears to involve ion flux and perturbations in cellular osmotic pressure.

NLRP3 and pro-IL-1 β are transcriptionally induced, but not functionally activated, following Toll-like receptor (TLR) stimulation, a process known as inflammasome priming. The NLRP3 inflammasome is only activated following detection of a second stimulus, which can be provided by bacterial pore-forming toxins (e.g. nigericin), environmental irritants (e.g. asbestos particles) and host danger molecules (e.g. ATP, monosodium urate [MSU]). The diversity and number of NLRP3 stimuli has led to several models for NLRP3 activation, including lysosomal rupture, induction of ion fluxes and the generation of reactive oxygen species (ROS). However, all these models have significant caveats [3], including a lack of convincing genetic evidence.

Recently, Shimada *et al* [4] proposed that NLRP3 activation requires its binding to oxidized mitochondrial DNA (mtDNA) that is released from mitochondria following activation of the 'mitochondrial' (also called 'BAK/BAX dependent', 'BCL-2 regulated' or 'intrinsic') apoptotic pathway in response to canonical NLRP3 activators, such as ATP and nigericin, or by potent inducers of mitochondrial apoptosis, such as staurosporine. This model would therefore explain how a plethora of divergent molecules and cellular stresses engage the same signalling pathway, namely mitochondrial apoptosis, to promote NLRP3-driven inflammatory responses.

It has also been observed that cyclosporin A, a potent inhibitor of the mitochondrial permeability transition (MPT) pore, blocks NLRP3 activation [5,6], suggesting that inflammasome-related mitochondrial damage may occur via MPT. Nakahira *et al* reported that NLRP3 is required for mitochondrial damage [5], and therefore, MPT may act to merely enhance NLRP3 activity. Alternatively, it

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has been proposed that cyclosporin A inhibition of MPT prevents the exposure of the mitochondrial lipid cardiolipin, which may also activate NLRP3 [6]. MPT is a known activator of Parkin-mediated mitophagy, a process used to target damaged mitochondria for degradation. Notably, mitophagy has been suggested to dampen excess NLRP3 activity [5,7].

These studies, and others, suggest that mitochondrial dysfunction and cell death pathways play an important role in the activation of NLRP3 [8]. However, there is a lack of genetic evidence to support these mitochondria-based models. In this study, we have therefore examined the role of mitochondrial apoptosis and homeostasis in NLRP3 activation using mice genetically deficient in key mitochondrial functions.

Results

BCL-2 over-expression has no impact on NLRP3-mediated IL-1 β secretion

Over-expression of BCL-2, the prototypical inhibitor of the mitochondrial apoptotic pathway, was reported to prevent NLRP3 activation [4]. In contrast, we observed that BCL-2 over-expression in macrophages, using two separate transgenic mouse lines expressing BCL-2 under the control of either the H2K promoter (active in major histocompatibility class I⁺ cells) or the Vav promoter (active in all haematopoietic cells) did not significantly impair NLRP3-dependent IL-1 β secretion following stimulation with ATP, nigericin or alum [9] (Fig 1A–C). Moreover, the BH3-mimetic ABT-737, a potent inhibitor of BCL-2, BCL-XL and BCL-W [10], had no significant impact on IL-1 β secretion from bone marrow-derived macrophages (BMDMs) in response to LPS priming and ATP treatment (Fig 1D).

Loss of BAK and BAX does not prevent caspase-1 and IL-1 β activation

Potent initiators of the mitochondrial apoptotic pathway may overcome the protective effects of BCL-2 over-expression. We therefore examined the impact of combined loss of the essential mediators of mitochondrial apoptosis, BAK and BAX [11], on NLRP3 activity. When activated by apoptotic stimuli, BAK and BAX form pores or channels in the outer mitochondrial membrane. This results in the release of cytochrome c into the cytosol to activate caspase-9 and initiate apoptotic cell death. Critically, it has yet to be determined whether loss of BAK/BAX, and thus the abrogation of mitochondrial apoptosis, influences NLRP3 activity.

Consistent with our observations using BCL-2 transgenic BMDMs, upon NLRP3 stimulation, *Bak*^{-/-}/*Bax*^{-/-} (double knockout, DKO) BMDMs showed similar caspase-1 activation and IL-1 β secretion when compared to wild-type (WT) BMDMs (Fig 1E–G). Furthermore, potent inducers of apoptosis, such as staurosporine, cycloheximide and UVB irradiation, caused comparable IL-1 β secretion in LPS-primed *Bak*^{-/-}/*Bax*^{-/-} and WT BMDMs (Fig 1E and G). Finally, loss of BOK, which shows extensive sequence similarity to BAK and BAX and is thought to function in a BAK/BAX-like pro-apoptotic manner [12,13], also had no impact on NLRP3 activity (Fig 1H).

Inconsistent with the notion that mitochondrial apoptosis is a general NLRP3 stimulus, staurosporine-induced IL-1 β activation occurred independent of NLRP3 [14,15] (Fig 2A). Further, the mitochondrial apoptosis initiator caspase, caspase-9, was not required for canonical NLRP3 activation, nor apoptotic stimulus induced IL-1 β secretion (Fig 2B). Loss of NLRP3, ASC (encoded by *Pycard*) or caspase-1 significantly delayed cell death triggered by ATP and nigericin, indicating that these NLRP3 stimuli primarily induce death by pyroptosis in this time frame (Fig 2C and D), rather than mitochondrial apoptosis. While NLRP3 stimuli can induce apoptosis, this occurs independently of mitochondria via ASC recruitment of caspase-8 and is critically dependent on upstream NLRP3 activation [16].

Collectively, these data provide strong evidence that neither NLRP3 stimuli nor agents known to induce mitochondrial apoptosis require the mitochondrial apoptotic pathway to activate IL-1 β .

Cyclophilin D-dependent mitochondrial permeability transition (MPT) is not required for NLRP3 activation

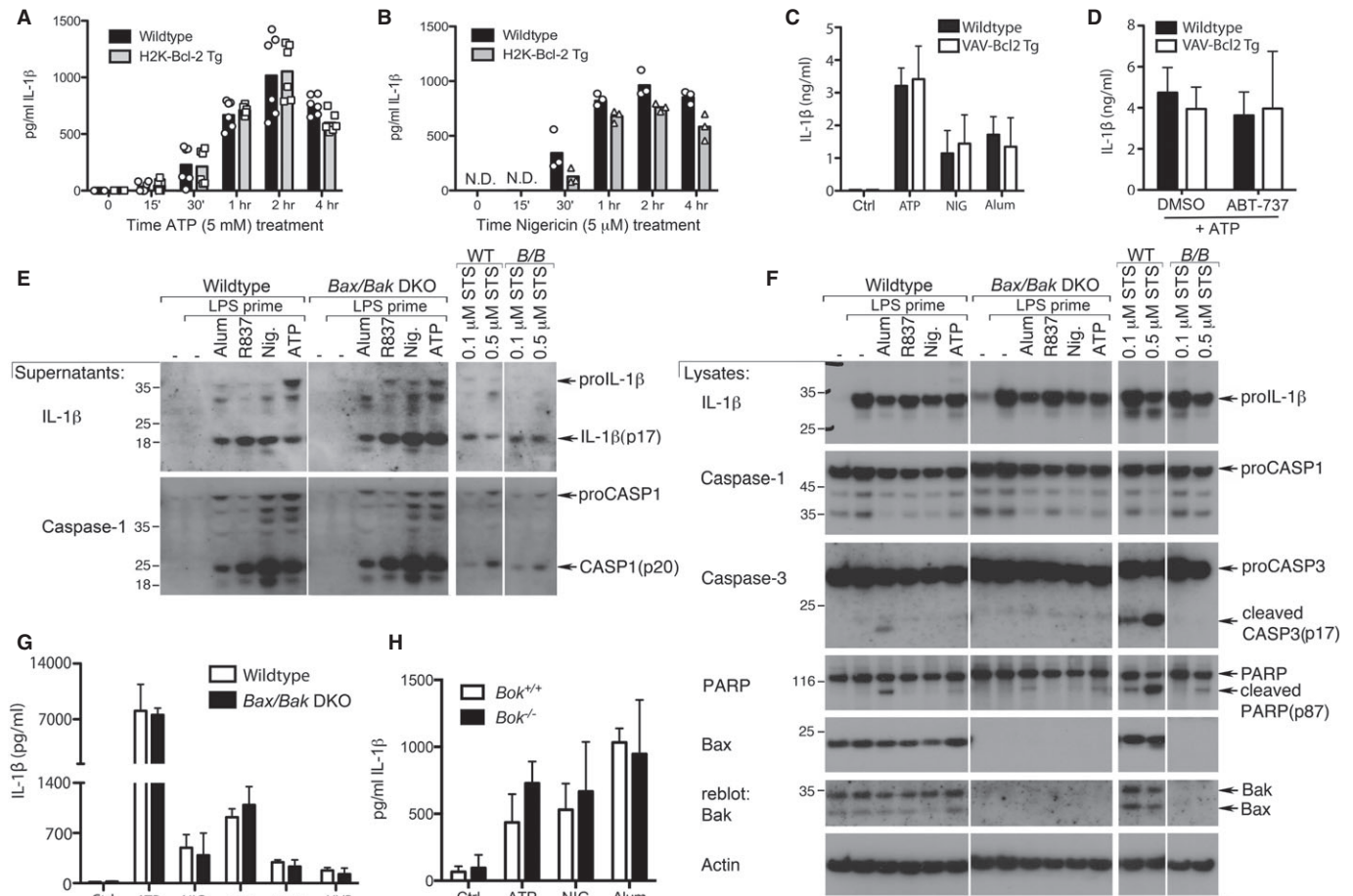
Mitochondrial permeability transition pore formation represents an alternate pathway that may cause mitochondrial damage and DAMP release to promote inflammasome formation. To investigate this possibility, we measured MPT following stimulation with ATP and nigericin. In the absence of LPS priming, treatment of BMDMs with ATP or nigericin for 30–40 min did not induce MPT (Fig 2E and Supplementary Fig S1A). Although LPS priming and ATP stimulation can induce modest MPT, this has been reported to occur downstream of NLRP3-caspase-1 activation [5]. Moreover, biochemical fractionation of LPS-primed BMDMs treated with ATP, nigericin or alum did not cause significant release of Smac/DIABLO or cytochrome c into the cytosol, nor BAX translocation onto mitochondrial membranes, unlike the mitochondrial apoptosis inducer staurosporine (Fig 2F and G, Supplementary Fig S1B and C).

MPT pore formation is putatively a requisite for NLRP3 activation largely based on the observation that the MPT pore inhibitor, cyclosporin A, blocks NLRP3 activation and IL-1 β secretion [5,6]. Consistent with this, we observed a dose-dependent decrease in IL-1 β secretion when LPS-primed BMDMs were pre-treated with cyclosporin A prior to stimulation with ATP or nigericin (Fig 2H).

Cyclophilin D is a component of the MPT pore complex involved in pore opening that is targeted by cyclosporin A and is required for efficient MPT [17–19]. To test whether cyclosporin A inhibits NLRP3 by targeting cyclophilin D, we derived macrophages from bone marrow or foetal liver of cyclophilin D-deficient mice (*CycD*^{-/-}; Fig 2I), primed them with LPS, and stimulated with ATP, nigericin and alum. In contrast to cyclosporin A-treated WT cells, *CycD*^{-/-} macrophages responded normally to NLRP3 stimuli (Fig 2J–L). Furthermore, cyclosporin A treatment still inhibited ATP and nigericin-induced caspase-1 and IL-1 β activation in *CycD*^{-/-} cells (Fig 2J–L). This demonstrates that cyclosporin A inhibits NLRP3 activation by a cyclophilin D/MPT-independent mechanism.

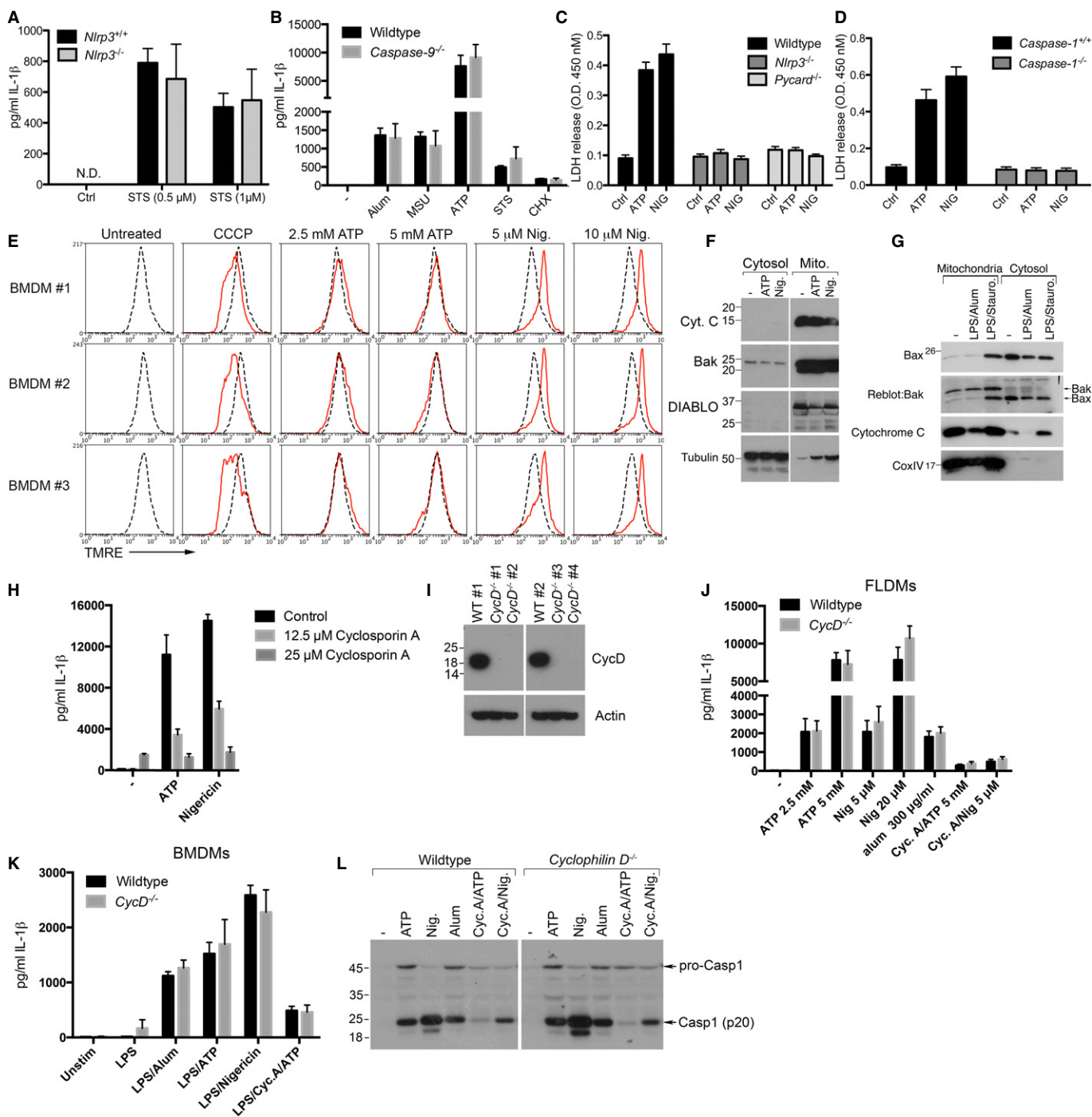
Mitophagy mediated by Parkin does not act as a negative regulator of NLRP3 activity

Following a loss of mitochondrial membrane potential, PINK1 and Parkin (encoded by *Park2*) can induce the selective autophagic



removal of damaged mitochondria, a process known as mitophagy [20]. Loss of genes required for autophagy enhances IL-1 β secretion in response to NLRP3 stimuli [5,21]. It has been proposed that this is because a loss of autophagy increases the number of damaged mitochondria that fail to be cleared by mitophagy [5,7]. However, the analysis of NLRP3 activity in mitophagy-specific-deficient animals or cells has yet to be examined. Chemicals that depolarize mitochondria to induce Parkin-dependent mitophagy, such as carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) [22], have also been reported to activate NLRP3 [7]. To establish whether Parkin-mediated mitophagy prevents excessive NLRP3 signalling, we

examined Parkin-deficient BMDMs. LPS-primed *Parkin*^{-/-} BMDMs contained more mitochondria than WT cells (Figs 3A and B and Supplementary Fig S2). However, despite the increased mitochondrial mass of *Parkin*^{-/-} BMDMs, no differences in IL-1 β or caspase-1 activation were observed between WT and *Parkin*-deficient cells stimulated with either NLRP3 activating or apoptotic stimuli (Fig 3C and D). It remains possible that Parkin-independent mitophagy regulates NLRP3 activity. However, because autophagy targets specific inflammasome components for degradation [23–25], autophagic pathways may also regulate inflammasome activity independent of mitochondrial clearance.



The anti-viral mitochondrial protein MAVS is dispensable for canonical NLRP3 activation

A recent study demonstrated that NLRP3 interacts with MAVS to facilitate NLRP3 localization to mitochondrial membranes [26]. This appeared important for NLRP3 activation since MAVS-deficient cells were defective in caspase-1 and IL-1β activation and secretion following stimulation with ATP or nigericin (but not alum) [26].

As expected, transfection with poly(I:C) to activate MAVS failed to induce IFN-β production in MAVS-deficient BMDMs (Fig 3E). However, in contrast to the findings of Subramanian *et al*, we did not detect any defect in caspase-1 activation or IL-1β processing in MAVS-deficient BMDMs following stimulation with ATP, nigericin, MSU or silica (Fig 3F and G). Therefore, our findings question the conclusion that MAVS is necessary to tether NLRP3 to the mitochondria in order to facilitate canonical NLRP3 activity. These data support a recent study which also failed to detect a role for MAVS in

Figure 2. NLRP3 activation can occur in the absence of MPT and cyclophilin D.

- A LPS-primed BMDMs from control (WT) and *Nlrp3*-deficient mice were stimulated for 4 h with staurosporine (STS) at the indicated concentrations. Cell supernatants were collected and analysed for IL-1 β content by ELISA. Error bars represent the SEM from 3 independent experiments, each performed in triplicate.
- B BMDMs from WT and *Caspase-9*^{-/-} mice were primed with LPS and stimulated with alum, staurosporine (STS) and cycloheximide (CHX) for 6 h or ATP for 1 h and cell supernatants analysed for IL-1 β by ELISA. Error bars represent the SD of measurements from cells derived from 3 mice of each genotype.
- C, D LPS-primed BMDMs from control (WT) mice or mice lacking *Nlrp3*, *Pycard* (C) or *Caspase-1* (D) were stimulated for 2 h with ATP (5 mM) or nigericin (5 μ M). Cell supernatants were collected and analysed for LDH release (a measure of plasma membrane rupture). Error bars represent the SEM from 3 independent experiments, each performed in triplicate.
- E BMDMs were pre-treated with the indicated stimuli for 10–15 min and then stained with TMRE (tetramethylrhodamine, ethyl ester) for a further 20–25 min and cells analysed by flow cytometry. Dashed line represents non-stimulated background controls. Data derived from 3 different mice (numbered) are shown.
- F, G BMDMs were primed with LPS and then stimulated with ATP (5 mM) or nigericin (10 μ M) for 40 min (F) or alum (300 μ g/ml) and staurosporine (500 nM) (G) for 5 h. Mitochondria containing membranes and cytosolic content were separated and analysed by Western blot.
- H BMDMs were primed with LPS and then treated as indicated in the presence or absence of cyclosporin A. Cell supernatants were analysed for IL-1 β content by ELISA. Error bars represent the SD of measurements from cells derived from 3 mice of each genotype.
- I Macrophages from foetal liver of independent (numbered) WT and cyclophilin D-deficient mice were analysed by Western blotting for cyclophilin D expression as indicated.
- J, K Foetal liver (J) or bone marrow (K)-derived macrophages of the indicated genotypes were primed with LPS and treated as indicated for 1 h (ATP, Nigericin) or 6 h (alum). Error bars represent the SD of measurements from cells derived from 3–5 mice or embryos of each genotype.
- L Cell supernatants from (J) were analysed by Western blot for caspase-1 processing and secretion.

Source data are available online for this figure.

promoting ATP or nigericin-induced NLRP3 activation, and only observed MAVS-NLRP3-induced IL-1 β secretion when MAVS was directly engaged [27].

Loss of RIPK3 and caspase-8 limits TLR-induced inflammasome priming and cytokine production

We next investigated whether components of the extrinsic apoptotic pathway could regulate inflammasome activity. We focused on caspase-8, which has previously been shown to cleave IL-1 β similar to caspase-1 [14,28]. We examined BMDMs from *Ripk3*^{-/-}*Casp8*^{-/-} mice, which unlike *Casp8*^{-/-} mice, are viable and fertile (Fig 4A). Interestingly, *Ripk3*^{-/-}*Casp8*^{-/-} BMDMs produced less pro-IL- β and TNF upon LPS (TLR4), poly(I:C) (TLR3) and Pam3Cys4 (TLR1/2) priming when compared to WT or *Ripk3*^{-/-} BMDMs (Fig 4B and C). Accordingly, *Ripk3*^{-/-}*Casp8*^{-/-} BMDMs showed decreased NLRP3 activation (Fig 4D). Caspase-8 has been implicated in NF- κ B activation [29–31], which is required for proper TLR-induced transcriptional responses. However, *Ripk3*^{-/-}*Casp8*^{-/-} BMDMs displayed no defect in LPS-induced NF- κ B activation (Fig 4E). Notably, LPS injection caused lower levels of cytokine production in *Ripk3*^{-/-}*Casp8*^{-/-} mice when compared to WT and *Ripk3*^{-/-} mice, or mice lacking the RIPK3 substrate MLKL (Fig 4F–H) [32]. These results, together with other recent reports [33,34], reveal an unexpected role for caspase-8 in inflammasome priming and TLR-driven cytokine production, both *in vitro* and *in vivo*, that is independent of apoptosis.

Conclusions

Mitochondrial apoptosis has been reported as a mechanism to explain how multiple stimuli engage NLRP3 to generate inflammatory responses [4]. However, this study lacked important genetic evidence, such as the analysis of BAK- and BAX-deficient mice. Our data indicate that in the time frame required for efficient NLRP3 activation, mitochondrial apoptosis is not detectable and that abrogation of mitochondrial apoptotic function does not impede NLRP3 activity. Prior work shows that NLRP3 stimuli can induce apoptosis, but this occurs downstream of NLRP3 and ASC,

from activation of caspase-8 [16]. Although we have not excluded that mtDNA may promote NLRP3 activity when released, our data (Supplementary Fig S3), and that of others, show that mtDNA is preferentially sensed by other inflammasomes, such as AIM2 [4,5]. The generation of AIM2 and NLRP3 double-knockout mice will be required to resolve this issue.

In conclusion, together with other recent studies [3,35], our genetic analyses do not support the model that perturbations to mitochondrial homeostasis reflect a general requirement for NLRP3-driven inflammatory responses. However, our experiments do reveal an important role for non-apoptotic caspase-8 function in inflammasome priming and TLR-driven cytokine production.

Materials and Methods

Macrophage cultures

Bone marrow myeloid progenitor cells were isolated from femoral and tibial bone marrow. Foetal livers were harvested from E13.5 embryos, and single-cell suspensions obtained by passage through a 40- μ m sieve. Progenitor cells were differentiated into macrophages using the same conditions used to generate BMDMs, as previously described [14].

LPS challenge

For LPS *in vivo* challenge, mice were pre-bled retroorbitally 2 days prior to intra-peritoneal injection of 100 μ g ultra-pure LPS (InvivoGen). After 6 h, the experiment was ended and cardiac bleeds performed to obtain serum for cytokine analysis by ELISA (IL-1 β ; R&D Systems or eBioscience, TNF, IL-6; ebioscience, manufacturers protocol).

Inflammasome analysis

Following differentiation, BMDMs or FLDMs were counted and replated at 1–2 \times 10⁵ cells/well (96-well plate) or 1 \times 10⁶ cells/well

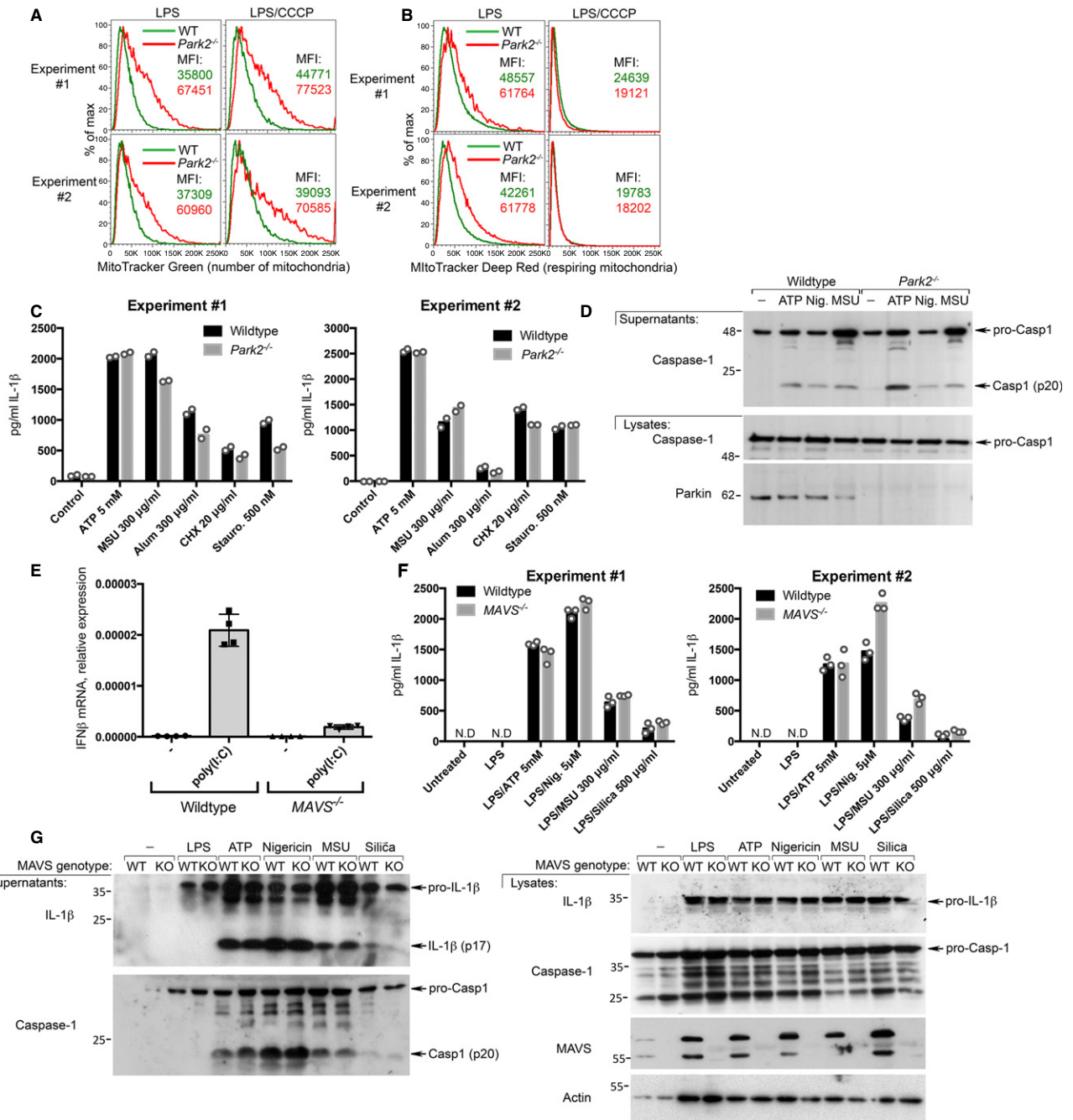


Figure 3. MAVS and Parkin are dispensable for canonical NLRP3 activation.

A, B BMDMs from WT and Parkin (*Park2*)-deficient mice were primed with LPS (3 h), then stimulated with or without CCCP (10 µM) for 1 h. Cells were subsequently stained with 200 nM MitoTracker Green (to measure mitochondrial mass) (A) or 200 nM MitoTracker Red (to measure respiring mitochondria) (B) for 20 min and analysed by flow cytometry.

C WT and Parkin (*Park2*)-deficient BMDMs were stimulated as indicated after LPS priming. Cell supernatants were analysed for IL-1β content by ELISA. Bars indicate the mean of duplicate experiments (symbols).

D BMDMs from WT and Parkin (*Park2*)-deficient mice were stimulated as indicated after LPS priming and cell supernatants and lysates examined by Western blot.

E BMDMs from WT and MAVS-deficient mice were transfected with poly(I:C) for 6 h. *IFN-β* mRNA was measured by RT-PCR and quantified relative to 18S rRNA expression. The mean and SD of two independent experiments are shown (each performed in duplicate; symbols).

F BMDMs from WT and MAVS-deficient mice were stimulated as indicated with ATP or nigericin for 1 h or with MSU or silica for 6 h. Cell supernatants were analysed for IL-1β content by ELISA. Bars indicate the mean of experiments performed in triplicate (symbols).

G BMDMs from WT and MAVS-deficient mice were stimulated as described in (F) and cell supernatants and lysates examined by Western blotting as indicated.

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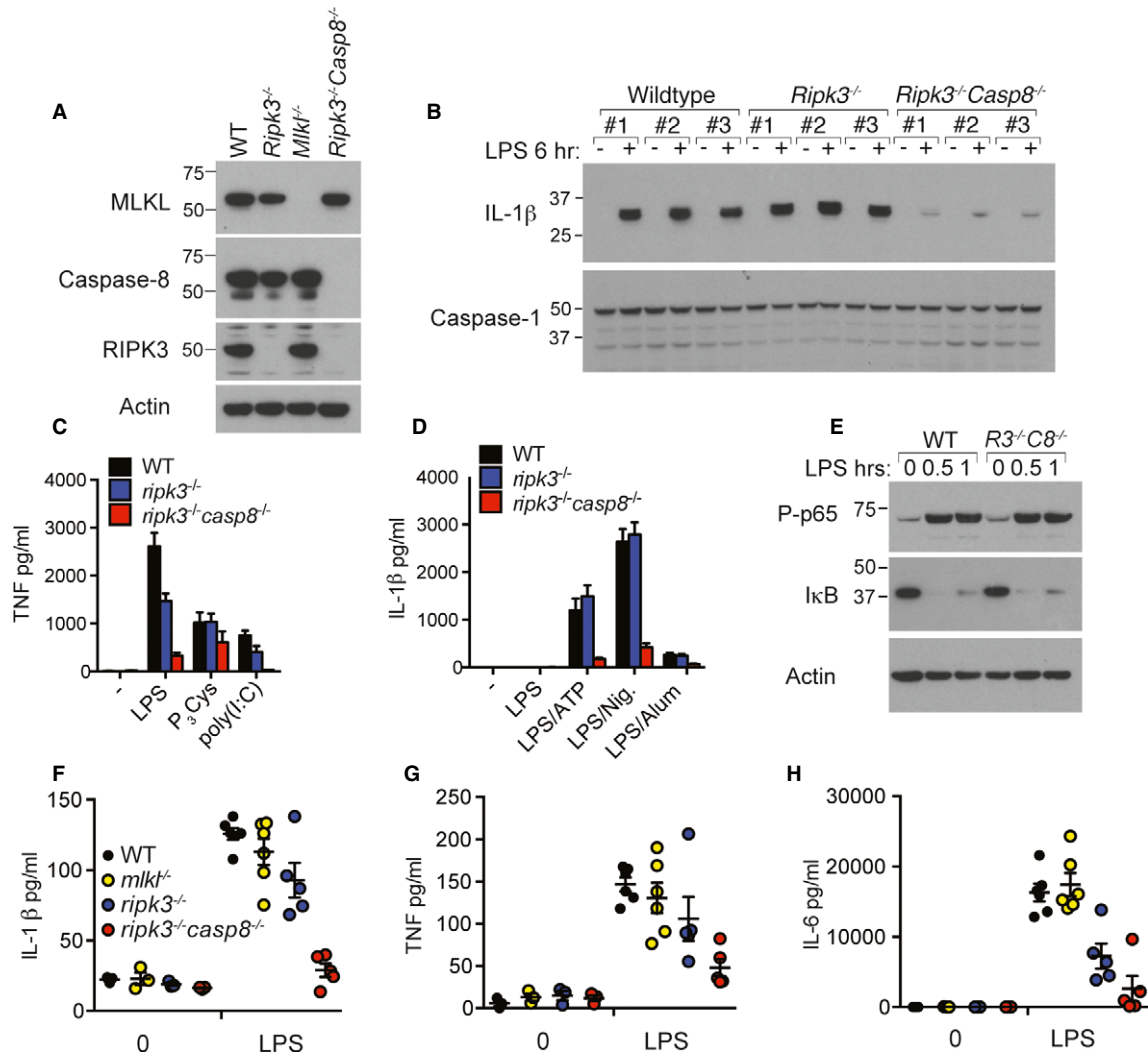


Figure 4. Combined loss of RIPK3 and caspase-8 limits TLR-induced cytokine production.

- A BMDMs of the indicated genotypes were analysed by Western blot.
 B BMDMs of the indicated genotypes (3 mice each; numbered) were stimulated with LPS and pro-IL-1 β measured by Western blot.
 C BMDMs of the indicated genotypes were treated with the TLR-ligands LPS (20 ng/ml), Pam₃Cys₄ (2.5 μ g/ml) or poly(I:C) (5 μ g/ml) and TNF secretion into the supernatant determined by ELISA after 3 h. Error bars are the SEM from 3 mice of each genotype.
 D BMDMs of the indicated genotypes were primed with LPS (20 ng/ml) for 3 h and stimulated as indicated. IL-1 β secretion into the supernatant was measured by ELISA. Error bars are the SEM from 3 mice of each genotype.
 E BMDMs of the indicated genotypes were stimulated with LPS (50 ng/ml) as indicated and NF- κ B activation assessed by Western blot.
 F-H Mice of the indicated genotypes were injected i.p. with LPS (100 μ g). Serum cytokine levels (F, IL-1 β ; G, TNF; H, IL-6) were measured in mice before challenge (0) and 6 h post-LPS injection. Each symbol represents one mouse (5–6 per genotype). The mean and SEM are shown.

Source data are available online for this figure.

(48-well plate) and allowed to adhere. Cells were then stimulated as indicated in the figure legends. Unless noted otherwise, cells were primed with LPS (20 ng/ml, 3 h) and stimulated with ATP (5 mM, 1 h), nigericin (5 or 10 μ M, 1 h), staurosporine (500 nM or 1 μ M, 6 h), silica (500 μ g/ml, 6 h), alum (300 μ g/ml, 6 h), MSU (300 μ g/ml, 6 h), cycloheximide (20 μ g/ml, 6 h) or UVB irradiation (500 mJ/cm² followed by 6 h rest). Following such stimulation, cell supernatants were collected for ELISA (IL-1 β ; R&D Systems or eBioscience, TNF; eBioscience, manufacturers protocol), LDH assays (Pierce, manufacturer protocol) or Western blot analysis.

Measurements of mitochondrial permeability transition/membrane potential

BMDMs were treated as indicated in the figure legend, stained with either TMRE (tetramethylrhodamine, ethyl ester; 80 nM) or DiOC₆ (3,3'-dihexyloxycarbocyanine iodide; 10 nM) for 20–25 min at 37°C and analysed by flow cytometry. Please refer to Supplementary Methods for full experimental details.

Supplementary information for this article is available online: <http://embor.embopress.org>

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Author contributions

RA, KEL, EC-WY, RL, ALM, DMM, KDM, MJW and JEV performed experiments. BTK, RA, JEV, KDM, and LAO'R, FK, AS, WSA, KS and DLV contributed essential reagents and/or discussion. JEV co-wrote the manuscript with KEL, RA, LAO'R, MJW and BTK. RA and JEV conceived the project.

Conflict of interest

EC-WY is currently a F. Hoffmann-La Roche Pharmaceuticals Ltd. employee.

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