Mitochondrial damage activates the NLRP10 inflammasome

Upon detecting pathogens or cell stress, several NOD-like receptors (NLRs) form inflammasome complexes with the adapter ASC and caspase-1, inducing gasdermin D (GSDMD)-dependent cell death and maturation and release of IL-1β and IL-18. The triggers and activation mechanisms of several inflammasome-forming sensors are not well understood. Here we show that mitochondrial damage activates the NLRP10 inflammasome, leading to ASC speck formation and caspase-1-dependent cytokine release. While the AIM2 inflammasome can also sense mitochondrial demise by detecting mitochondrial DNA (mtDNA) in the cytosol, NLRP10 monitors mitochondrial integrity in an mtDNA-independent manner, suggesting the recognition of distinct molecular entities displayed by the damaged organelles. NLRP10 is highly expressed in differentiated human keratinocytes, in which it can also assemble an inflammasome. Our study shows that this inflammasome surveils mitochondrial integrity. These findings might also lead to a better understanding of mitochondria-linked inflammatory diseases.

Inflammasomes are protein complexes bridging the recognition of pathogens and sterile damage to GSDMD-mediated pyroptotic cell death and IL-1β-driven inflammation. Genetic factors, aging and metabolic dysfunction promote chronic inflammasome activation, contributing to multiple inflammatory diseases. Therefore, members of this class of innate immune receptors represent possible pharmacological intervention targets. Many inflammasome-forming sensors belong to the NACHT, leucine-rich repeat (LRR) and pyrin domain (PYD)-containing (NLRP) protein family. Their LRR domains participate in recognizing perturbations of key cellular processes and microbe- or stress-associated ligands, whereas the PYDs typically recruit the adapter ASC, providing a platform for pro-caspase-1 activation. The roles of several NLRP family members and their activating signals are incompletely characterized, impeding our understanding of pathophysiological processes and drug development.

The proximal events leading to the NLRP3 inflammasome activation remain a matter of debate. Several mechanisms are proposed to mediate this process, including ion fluxes, inducible protein–protein interactions, and ROS.
Fig. 1 | m-3M3FBS triggers mitochondrial damage and NLRP10 inflammasome activation. a, IL-1β secretion from WT, ASC-deficient or VX-765-pretreated (40 μM) BMDMs, primed with LPS (200 ng ml⁻¹) and stimulated with nigericin (10 μM), poly(dA:dT) dsDNA (2 μg ml⁻¹) or m-3M3FBS (85 μM) (n = 4). b, IL-1β secretion from WT or NLRP3-deficient BMDMs, stimulated as in a (n = 3). P-values were calculated using two-way analysis of variance (ANOVA) with Tukey’s (ns in a) or Šídák’s (ns in b) multiple comparison test. **P = 0.0016, ****P < 0.0001, NS (not significant) P = 0.2007. c, ASC speck formation of HEK cells stably expressing ASC-tagBFP and transfected with an empty vector (EV) or P (ANOVA) with Tukey's (as in a) m-agonist nigericin and the AIM2 agonist poly(dA:dT). However, we found no evidence that the response to m-3M3FBS is NLRP3 dependent (Fig. 1b), as NLRP3-proficient and -deficient cells released similar amounts of IL-1β following m-3M3FBS treatment. Further supporting the lack of NLRP3 involvement, m-3M3FBS-induced IL-1β secretion and ASC speck formation were not inhibited by increased extracellular potassium concentrations (Extended Data Fig. 1c,d), an NLRP3 activation inhibitor. In contrast to the NLRP3 activator nigericin, m-3M3FBS elicited ASC speck formation in macrophages without the requirement for LPS priming (Extended Data Fig. 1e, f), and the NLRP3 inhibitor CRID3 (also known as MCC950 or CP-456,773) did not affect IL-1β secretion or ASC speck formation in m-3M3FBS-stimulated cells (Extended Data Fig. 1f, g).

To identify which inflammasome sensor responds to m-3M3FBS, we performed an overexpression screen in HEK cells with a fluorescent ASC (ASC-tagBFP) reporter. Expression of NLRP10, but not other NLRPs (Fig. 1c), or other inflammasome-forming proteins (Extended Data Fig. 1h), enabled ASC speck formation in m-3M3FBS-treated HEK cells. This NLRP10-mediated inflammasome response was specific to m-3M3FBS, as nigericin and poly(dA:dT) did not cause ASC speck formation in NLRP10 reporter cells (Extended Data Fig. 1i).

To determine the upstream signal for NLRP10 activation, we tracked the subcellular localization of fluorescently tagged NLRP10 and protein–lipid interactions. Our initial investigation of the putative NLRP3 activator m-3M3FBS, a reported phospholipase C agonist, unexpectedly revealed that this molecule, in fact, triggers NLRP3-independent inflammasome activation.

Here, we discovered that m-3M3FBS and other molecules (thapsigargin and SMBAI) trigger mitochondrial damage leading to NLRP10 and AIM2 inflammasome activation in keratinocytes and macrophages, respectively. While AIM2 detected release of mitochondrial DNA, the novel NLRP10 inflammasome sensed DNA-independent factors in damaged mitochondria.

Our examination of inflammasome activation by m-3M3FBS confirmed that m-3M3FBS administration induces ASC- and caspase-1-dependent IL-1β secretion (Fig. 1a and Extended Data Fig. 1a) and ASC speck formation (Extended Data Fig. 1b). The extent of inflammasome activation was comparable with that elicited by the NLRP3 agonist nigericin and the AIM2 agonist poly(dA:dT). However, we found no evidence that the response to m-3M3FBS is NLRP3 dependent (Fig. 1b), as NLRP3-proficient and -deficient cells released similar amounts of IL-1β following m-3M3FBS treatment. Further supporting the lack of NLRP3 involvement, m-3M3FBS-induced IL-1β secretion and ASC speck formation were not inhibited by increased extracellular potassium concentrations (Extended Data Fig. 1c,d), an NLRP3 activation inhibitor. In contrast to the NLRP3 activator nigericin, m-3M3FBS elicited ASC speck formation in macrophages without the requirement for LPS priming (Extended Data Fig. 1e, f), and the NLRP3 inhibitor CRID3 (also known as MCC950 or CP-456,773) did not affect IL-1β secretion or ASC speck formation in m-3M3FBS-stimulated cells (Extended Data Fig. 1f, g).

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Fig. 2 Mitochondrial permeability transition activates NLRP10 and AIM2 inflammasomes. a, Representative micrographs from time-lapse recordings of iMacs expressing NLRP3, ASC\textsuperscript{Cerulean} and mCitrine targeted to the mitochondrial matrix (using cytochrome c oxidase subunit 8 mitochondrial targeting sequence), with or without m-3M3FBS (85 μM) treatment. Images of the same field at treatment onset (0 min) or indicated time points (n = 3). b, Quantification of mitochondrial fluorescence signal granularity and ASC specks per imaging field (n = 3). c, IL-1β secretion from WT or AIM2-deficient BMDMs, primed with LPS (200 ng ml\(^{-1}\)) and stimulated with poly(dA:dT) dsDNA (2 μg ml\(^{-1}\)), m-3M3FBS (85 μM) or thapsigargin (20 μM). d, e, IL-1β secretion from iMacs\textsuperscript{AIM2\/-} overexpressing AIM2 (d) or NLRP10 (e), with or without 2’3’-dideoxycytidine (ddC) (80 μg ml\(^{-1}\)) pretreatment to deplete mtDNA, LPS-primed and stimulated with nigericin (10 μM), poly(dA:dT) dsDNA (2 μg ml\(^{-1}\)), m-3M3FBS (85 μM) or thapsigargin (20 μM) (n = 3). P values were calculated using two-way ANOVA with Tukey’s (c) or Sidák’s (d,e) multiple comparison test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Individual data points (where applicable) are means of technical duplicates or triplicates; all error bars represent s.d.

We next examined the inflammasome response to mitochondrial rupture in mouse macrophages. These cells do not express NLRP10 (refs. 8,24) but nevertheless responded to m-3M3FBS (Fig. 2a,b), suggesting that another inflammasome-forming sensor is engaged in this cell type. We had already excluded a contribution of NLRP3 in the response to m-3M3FBS (Fig. 1b and Extended Data Fig. 1c–g), which was confirmed for thapsigargin and SMBAI (Extended Data Fig. 4a). As AIM2 is expressed in macrophages and was identified as a sensor for DNA leaking from damaged mitochondria\textsuperscript{28}, we tested whether IL-1β secretion induced by the mitochondria-damaging agents was inhibited in AIM2-deficient macrophages. Indeed, neither m-3M3FBS, thapsigargin (Fig. 2c) nor SMBAI (Extended Data Fig. 4b) triggered IL-1β release from AIM2-deficient macrophages. Similar to m-3M3FBS (Fig. 2a,b), thapsigargin and SMBAI induced ASC speck formation in macrophages (Extended Data Fig. 4c). These results suggest that these mitochondria-damaging stimuli activate the AIM2 inflammasome in macrophages.

To identify the mechanism of AIM2 activation by m-3M3FBS and thapsigargin, we depleted mtDNA using 2’,3’-dideoxyctydine (ddC) in AIM2-deficient immortalized macrophages overexpressing AIM2. The mtDNA depletion efficiency was confirmed by quantitative PCR (qPCR) (Extended Data Fig. 4d). We found that m-3M3FBS- and thapsigargin-mediated AIM2 activation depended on mtDNA (Fig.
2d). Since AIM2-deficient immortalized macrophages no longer responded to mitochondrial damage, we overexpressed NLRP10 in these cells and found that this was sufficient to enable IL-1β release upon treatment with m-3M3FBS and thapsigargin (Fig. 2e).

We next assessed the mechanism by which m-3M3FBS, thapsigargin and SMAB1 damage mitochondria. We determined that the mPT inhibitor cyclosporin A (CsA) \( K_{179M} \) and B (D249N) selectively blocked mitochondrial rupture and NLRP10 inflammasome activation in thapsigargin- but not m-3M3FBS-treated HEK cells (Fig. 3a,b and Extended Data Fig. 5a).

Similarly, CsA blocked mitochondrial damage, and AIM2-mediated ASC speck formation and IL-1β release in macrophages stimulated with thapsigargin but not with m-3M3FBS (Fig. 3c–e and Extended Data Fig. 5b). SMAB1 showed the same CsA sensitivity profile as thapsigargin, with CsA treatment entirely preventing NLRP10-mediated ASC speck formation in HEK cells (Extended Data Fig. 6a,b) and AIM2-mediated ASC speck formation in macrophages (Extended Data Fig. 6c,d).

CSA inhibits both mPT and calcineurin/nuclear factor of activated T cells. Nonimmunosuppressive analogs of CsA, Deboi025 and Nim811 are, in contrast, selective mPT blockers. We observed that, similar to CsA, Deboi025 and Nim811 inhibited NLRP10 and AIM2 inflammasome responses to thapsigargin but not to m-3M3FBS (Extended Data Fig. 7a–f). Together, these data confirm that mitochondrial damage is upstream of NLRP10 and AIM2, but the activation mechanisms of these inflammasomes likely differ.

Whereas some models suggest that mPT is initiated by mitochondrial Ca\(^{2+}\) fluxes, we did not observe a strong link between Ca\(^{2+}\) signaling and NLRP10 activation. m-3M3FBS still elicited ASC speck formation in the presence of the intracellular Ca\(^{2+}\) chelator BAPTA-AM (Extended Data Fig. 8a) and of the ionostil, a 4,5-triphosphate receptor blocker 2-APB (Extended Data Fig. 8b). Administration of ionomycin, globally increasing the cytosolic Ca\(^{2+}\) concentration, did not damage the mitochondria, as measured by the mito-mCherry reporter (Extended Data Fig. 8c,d), and did not cause NLRP10-driven ASC speck formation (Extended Data Fig. 8c–e). Conversely, m-3M3FBS remained capable of eliciting ASC speck formation in NLRP10 reporter HEK cells pretreated with ionomycin (Extended Data Fig. 8f), indicating that cytosolic Ca\(^{2+}\) does not inhibit NLRP10 under these conditions.

Since our work suggested that NLRP10 functions as an inflammasome sensor, we tested the requirement for the different NLRP10 domains in inflammasome formation. We expressed full-length NLRP10 or the individual NLRP10 PYD and NACHT domains (NLRP10\(^{10PYD}\), NLRP10\(^{NACHT}\)) in AIM2-deficient macrophages and found that neither individually expressed NLRP10\(^{10PYD}\) nor NLRP10\(^{NACHT}\) enabled inflammasome activation (Fig. 3f), demonstrating the requirement for the full-length protein. Furthermore, we profiled Walker A (K179M) and B (D249N) NLRP10 mutants and found that they cannot assemble inflammasomes (Fig. 3j), which is in accordance with the reports that NLRP3 Walker A/B mutants are inactive and that NLRP10 Walker A mutant is deficient in NF-kB induction in response to Shigella. These data show that full-length NLRP10 with active Walker A/B sites is required for sensing mitochondrial damage and inflammasome function.

We next tested whether NLRP10 recruits ASC. We thus monitored the localization of NLRP10\(^{mCitrine}\) and ASC\(^{TagBFP}\) in untreated and m-3M3FBS- or thapsigargin-stimulated HEK cells. Indeed, NLRP10 colocalized with ASC following m-3M3FBS and thapsigargin challenge but not under resting conditions, suggesting that NLRP10 recruits ASC upon sensing mitochondrial damage (Fig. 3g). Consistently, NLRP10\(^{mCitrine}\) communoprecipitated with ASC\(^{TagBFP}\) after stimulation with m-3M3FBS or thapsigargin but not under basal conditions (Fig. 3h), confirming the inducibility of the NLRP10-ASC interaction.

To interrogate the requirement of caspase-1 for the release of IL-1β by the mitochondrial damage-sensing inflammasomes, we used the caspase-1 inhibitor VX-765 (ref. 40) and the pan-caspase inhibitor emricasan. VX-765 and emricasan blocked IL-1β secretion driven by both NLRP10 (Fig. 3i) and AIM2 (Fig. 3j) and Extended Data Fig. 9a, suggesting that these inflammasomes engage caspase-1. Importantly, VX-765 and emricasan did not block NLRP10- (Fig. 3k and Extended Data Fig. 9b) or AIM2-mediated (Fig. 3l and Extended Data Fig. 9c) ASC speck formation, indicating that ASC speck assembly does not require upstream caspase activity. Instead, caspase-1 is likely engaged by the inflammasome sensors and ASC following mitochondrial damage.

NLRP10 is highly expressed in the epidermis and immortalized keratinocytes, representing the stem cell/progenitor epidermal compartment, did not display pyroptotic cell death morphology upon m-3M3FBS challenge (Fig. 4b, left). This is consistent with low endogenous NLRP10 expression. NLRP10 overexpression in N/TERT keratinocytes was sufficient to induce pyroptosis following m-3M3FBS treatment (Fig. 4b, right). Our assessment of further inflammasome activation hallmarks in response to m-3M3FBS, including ASC complex formation (oligomerization), GSDMD cleavage and release of mature IL-1β in N/TERT cells (Fig. 4c) demonstrated that NLRP10 overexpression enables inflammasome activation in undifferentiated N/TERT cells treated with m-3M3FBS. Similar to the observations in HEK cells, the NLRP10-driven inflammasome complexes, including NLRP10 and ASC, colocalized with mitochondria in m-3M3FBS-treated N/TERT keratinocytes (Fig. 4d).
Since NLRP10 expression is enriched in terminally differentiated keratinocytes in the stratum granulosum of human epidermis (Fig. 4a), we next differentiated normal primary human epidermal keratinocytes (NHEKs) using CaCl₂, which induced an increase in NLRP10 mRNA expression (Extended Data Fig. 10a). Correspondingly, immunoblotting analysis confirmed that N/TERT keratinocyte...
differential cytochrome c release (Fig. 4f). Further analyses using caspase-1 activity assays also supported our findings (Extended Data Fig. 10e). Collectively, these data indicate that NLRP10 drives inflammasome formation upon mitochondrial damage, leading to IL-1β maturation and release from differentiated keratinocytes.

NLRP10 variants have been linked with an increased risk of atopic dermatitis (AD). Intriguingly, one of these reported AD-associated variants produces a missense R243W mutation near the NLRP10 Walker A site. In the NLRP10 reporter system, we found that the R243W variant had a loss-of-function phenotype (Extended Data Fig. 10f), similar to the D249N NLRP10 Walker B mutant (Fig. 3f). Thus, the NLRP10 ability to assemble an inflammasome might protect against skin inflammation, suggesting that NLRP10 could liberate factors promoting skin homeostasis upon sensing physiological triggers in the stratum granulosum.

Our study demonstrates that NLRP10 nucleates inflammasomes in cells treated with m-3M3FBS, thapsigargin or SMAL. In contrast to earlier reports suggesting an inflammasome-inhibitory role for NLRP10 (refs. 45,46), the only NLRP family member lacking the LRR domain47, we found that NLRP10 is a mitochondrial-damage sensor. Our results also confirm the capacity of AIM2 to recognize mitochondrial disruption and mtDNA cytotoxic leakage, which was previously shown using other mitochondria-disrupting agents48. While this manuscript was under review, an unrelated study confirmed the mitochondria-rupturing m-3M3FBS activity49. Mitochondrial disruption described in this report was independent of most known factors mediating mitochondrial membrane permeabilization. In contrast to our observations, this mitochondrial permeabilization mechanism relied on intracellular Ca2+ fluxes. Future studies may reconcile these models, as the apparent differences may be due to subtle divergences in inhibitor or activator concentrations, administration modes or analyzed cell types.

Physiologically, NLRP10 is expressed in keratinocytes and cardiomyocytes50,51. These cell types are exposed to different sterile and microbial stimuli, suggesting that diverse upstream signals could converge on a common proximal event activating NLRP10. In the skin, commensal and invasive microbes, ultraviolet radiation and environmental chemicals could provide signals for mitochondrial damage and NLRP10 activation. In contrast, cardiomyocytes are shielded from these agents, but cell-intrinsic processes, including oxidative stress, may regulate their mitochondrial integrity and NLRP10 activation status.

The AD-associated NLRP10 variant46 was unable to initiate inflammasome activation. An essential future question is how the inflammasome-forming activity of NLRP10 is linked to its role in AD pathogenesis. As NLRP10-expressing keratinocytes in the stratum granulosum undergo physiological cell death producing a protective layer of cornified epithelium, it is tempting to speculate that this process involves NLRP10.

Further supporting the pathophysiological importance of NLRP10, a study by Zheng et al.22, cosubmitted with this study, uncovered a protective role of NLRP10 inflammasome in colitis. Loss of NLRP10 activity was linked with more severe disease. Collectively, it is plausible that NLRP10-mediated loss of damaged cells favors the epithelial barriers’ protective function. Given the reported inflammasome-independent involvement of NLRP10 in regulating innate immune signaling and inflammatory gene transcription22,47, it will be valuable to examine under which conditions NLRP10 behaves as an inflammasome and when it exerts other functions, and how this distinction is made. Understanding the partial redundancy between NLRP10 and AIM2 as mitochondrial damage sensors, especially in in vivo studies, will be vital for dissecting their roles in host defense against microbes or sterile inflammatory processes. Such studies will likely reveal disease mechanisms in which targeting NLRP10 could provide a therapeutic benefit.
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Methods
Immortalized cell lines
All stable cell lines (immortalized macrophages (iMacs) and HEK cells (HEK); see Supplementary Table 6) were grown under standard conditions (37 °C, 5% CO2) in DMEM with 4.5 g l−1 glucose and l-glutamine (DMEM; ThermoFisher Scientific, catalog no. 41965) supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin (100 units per ml or 100 μg ml−1, respectively; ThermoFisher Scientific, catalog no. 15140) (complete DMEM). Cells were passaged every 2–3 d by washing with DPBS (ThermoFisher Scientific, catalog no. 14190) and detachment from the growth surface with TrypLE Express Enzyme (ThermoFisher Scientific, catalog no. 12605), which was subsequently blocked with complete DMEM. Cells were screened for mycoplasma contamination.

Immortalized human keratinocytes (N/TERT) were a kind gift to SRIS, A*STAR by J. G. Rheinwald (MTA to SRIS) and cultured according to published protocols53. N/TERT keratinocytes constitutively expressing NLRP10-FLAG were generated using a lentiviral expression system (pCDH vectors; SystemBio).

Retroviral transduction and puromycin selection
To generate cell lines stably transduced with constructs of interest, retroviruses were used as a nucleic acid vector. HEK293T cells were used for the production of retroviruses. Briefly, ~5 × 10^6 HEK293T cells per well were seeded in 6-well plates in complete DMEM (~3 ml). After overnight incubation (37 °C, 5% CO2), the cells were transfected with combinations of three vectors: the vector of interest (carrying the insert to be stably integrated into the genome of the target cell line; 2 μg per well), the packaging vector (pCMV-gag-pol; 1 μg per well) and the entry vector (pCMV-vsv-g; 100 ng per well). To prepare transfection mixes (per one transfection well) GeneJuice transfection reagent (8 μl; or approx. 2.6 μl of GeneJuice per 1 μg of DNA) was combined with 100 μl of FBS- and antibiotic-free DMEM, and, in a separate tube, the three plasmids were mixed and filled up to 20 μl with FBS- and antibiotic-free DMEM. After 5 min, GeneJuice® and plasmids were mixed and left for 20 min to allow for the formation of transfection complexes. After this time, transfection complexes were transferred to the HEK cells, followed by an 18–20 h incubation (37 °C, 5% CO2). After this time, the tissue culture media were removed, discarded and replaced with ~2 ml of DMEM supplemented with 30% FBS. After 24 h of incubation under the high-FBS conditions (37 °C, 5% CO2), the retrovirus-containing tissue culture supernatants were collected. Briefly, the supernatants were collected in a Luer-Lok syringe using a blunt 18G needle. Then, the supernatants were filtered through a 0.45-μm membrane filter. Such supernatants were either directly used for stable transgene delivery into target cells or cryopreserved at −80 °C. For retrovirus-mediated transgene delivery (retroviral transduction), target cells were plated at ~5 × 10^4 cells per well (one day before transduction) or ~10^5 cells per well (on the day of transduction) in 24-well plates. Next, the cells were subjected to several doses of retrovirus-containing supernatants (typically 5–500 μl). After 24–48 h of retroviral transduction (37 °C, 5% CO2), the transduction efficiency was assessed using an epifluorescence microscope and, if necessary, the wells containing populations of positive cells were subjected to antibiotic selection with puromycin (~10 μg ml−1 in complete DMEM, at 37 °C and in the presence of 5% CO2). Puromycin was from ThermoFisher Scientific (catalog no. A11138). After successful transduction, the resulting cell lines were expanded and used for experiments at passages 4–20 following retroviral transduction and/or cryopreserved at −150 °C.

Cell transfection for protein overexpression
For transient protein overexpression, GeneJuice transfection reagent was used for DNA delivery into HEK cells. Typically, the transfection reagent was combined with plasmid DNA at the ratio of 2.3–2.8 μl of GeneJuice per 1 μg of plasmid DNA. GeneJuice was first mixed with serum- and antibiotic-free OptiMEM. In a separate tube, the plasmid of interest was mixed with the same volume of serum- and antibiotic-free OptiMEM. After a 5–10 min incubation at room temperature (RT), the contents of both tubes were combined, and the resulting mixture was incubated at RT for 15–20 min to allow for the formation of transfection complexes. Thus formed, transfection complexes were transferred to HEK cells plated 6–24 h in advance in complete DMEM medium in either uncoated 6-well tissue culture-treated plates or in poly-l-lysine-coated 96-well tissue culture-treated microscropy-adapted plates (Perkin Elmer, catalog no. 6055300). The typical plating densities were between ~10^4 and 2.2 × 10^4 cells per well (or ~1.6 × 10^4–6.9 × 10^4 cells/cm²) in 96-well plates, or ~5 × 10^4 cells per well (or ~5.3 × 10^4 cells/cm²) in 6-well plates. The final volume of the transfection mix was generally kept under 100 μl per well for cells transfected in 96-well plates or under 200 μl per well for cells transfected in 6-well plates. The addition of DNA transfection complexes was followed by a centrifugation step (340g, 5 min at RT).

For the transfections performed in 96-well plates, the cells were subjected to wide-field or confocal fluorescence microscopy after 24–48 h (depending on the experiment), either after direct fixation (4% formaldehyde with the nuclear counterstain (5 μM DRAQ5)) or following treatment with inflammasome activators and subsequent fixation. For the transfections performed in 6-well plates, the cells were incubated with the transfection complexes for 24 h (37 °C, 5% CO2). Next, the cells were detached from the growth surface using TrypLE Express Enzyme and replated in poly-l-lysine-coated tissue culture-treated microscropy-adapted 96-well plates. After a 24-h ‘resting’ period (37 °C, 5% CO2), these cells were treated with inflammasome activators, fixed (4% formaldehyde with the nuclear counterstain (5 μM DRAQ5)) and inspected by fluorescence microscopy.

Molecular cloning
Cloning of all constructs was performed following standard procedures and according to manufacturers’ instructions for all the reagents. The inserts were amplified by PCR using the pFuII Ultra II Hotstart PCR Master Mix. The PCR reactions were performed in 50-μl final volumes, using 0.5 μM forward and reverse primers and ~100–500 ng of DNA per reaction. For site-directed mutagenesis, the overlap-extension PCR protocol was used54. Generally, PCR was initiated with a 1-min denaturation step (94 °C), followed by 35 or 40 cycles of: (1) denaturation (94 °C, 30 s), (2) primer annealing (56–65 °C, 30 s) and (3) elongation (72 °C, 1 min per kilobase (kb) of DNA). At the end of the reaction, a 5- or 10-min final elongation step at 72 °C was included. The PCR products were electrophoretically separated in an agarose (Biozym) gel (1–1.5%) with green DNA-DNA dye (PEQ-green, Peqlab/WVR; ~1.5 × 10^4 dilution) and isolated using PureLink Quick Gel Extraction kit. Next, sticky ends were generated by digestion with a pair of restriction enzymes (ThermoFisher Scientific) according to the manufacturer’s instructions in final volumes of 50 μl. Inserts generated in this fashion were re-run in an agarose gel (1–1.5%) and purified. The backbone vectors were linearized by restriction digest with the same pair of restriction enzymes as the insert and then isolated from a 1% agarose gel after electrophoretic digest.

Ligation (20 μl final volume) was performed according to the manufacturer’s instructions. Briefly, the insert of interest was mixed with the backbone at the ratio of 3:1–9:1 and kept on ice (−4 °C) with T4 DNA ligase (ThermoFisher Scientific) in the presence of 1× ligation buffer. After preparation of this reaction mix, the tubes were shifted to 22 °C for 20 min. Then, the reaction mixes were returned to the ice bath. Vectors generated in this manner (1–5 μl) were used to transform chemically competent DH5α Escherichia coli cells (15 μl). The heat-shock transformation protocol was employed; briefly, the cells were incubated with the ligation products on ice (−4 °C) for 5 min, followed by a 45-s incubation at 42 °C and a 2-min ‘regeneration’ step on ice (−4 °C). Then, the transformed cells were diluted in LB broth without
antibiotics (-150 μl) and incubated for 30 min at 37 °C with shaking (400–600 r.p.m.). After this, the cells were plated in LB–agar (ThermoFisher Scientific) plates with ampicillin (100 μg ml⁻¹, Sigma/Merck) used as a selection antibiotic. Following overnight incubation at 37 °C, colonies were picked from agar plates. They were then used for inoculation of small bacterial cultures (6 ml LB broth with 100 μg ml⁻¹ ampicillin), which were grown for 16–18 h at 37 °C with shaking (~360 r.p.m.). After this time, bacterial cells from these cultures were collected, and plasmid DNA was isolated using the PureLink Quick Plasmid Miniprep kit, following the manufacturer’s instructions. Complete sequences of the inserts from the purified plasmids were obtained by Sanger sequencing performed by GATC or Eurofins Genomics. Of the positive clones, one was selected to prepare a cryopreserved stock (bacterial suspension in LB broth with 100 μg ml⁻¹ ampicillin supplemented with 25% glycerol (Sigma/Merck); stored at −80 °C). The glycerol stocks were used for inoculation of larger scale bacterial cultures (120–150 ml grown in LB broth with 100 μg ml⁻¹ ampicillin for 16–18 h at 37 °C with shaking (~340 r.p.m.)), from which plasmid DNA was isolated using the PureLink Quick Plasmid Maxiprep kit. For all subsequent applications, plasmid DNA from those validated Maxiprep preparations was used.

**Primary macrophage culture**

Femurs and tibias were typically obtained from 6–24-week-old female mice (see Supplementary Table 7 for details regarding sex, strain and genetic background of the animals). After isolation, the bones were briefly washed in DPBS in a tissue culture dish, followed by a 30-s incubation in 70% ethanol. Then, the bones were transferred to DPBS in a fresh tissue culture dish and kept there until the bone marrow extraction step (~30 min). The bones were opened using scissors and flushed (using a syringe) with FBS- and antibiotic-free DMEM (bones from one mouse were flushed ~10 ml of medium) in a fresh tissue culture dish. The bone marrow suspensions in DMEM were centrifuged (340g, 5 min) and either cryopreserved at −150 °C or resuspended in complete DMEM supplemented with 15–30% L929 cell-conditioned medium.

Bone marrow-derived macrophage (BMDM) differentiation was conducted for 7 d in a tissue culture incubator (37 °C, 5% CO₂). On the last day of differentiation, the L929 cell-conditioned medium-containing differentiation media was discarded, and the cells were washed with DPBS and detached from the growth surfaces using a cell scraper. The BMDM suspensions in DPBS were spun down (340g, 8 min), the DPBS supernatants were discarded and BMDMs were resuspended in complete DMEM supplemented with 1–5% L929 cell-conditioned medium. For further experimentation, BMDMs were plated in 96-well plates at the density of 5 × 10⁵ cells per well (or 1.56 × 10⁶ cells cm⁻²). All steps of this procedure were performed at RT. Experiments using BMDMs were performed after a 16–18-h resting period in a tissue culture incubator (37 °C, 5% CO₂), following cell plating.

**Differentiation of N/TERT keratinocyte cultures**

N/TERT keratinocytes were cultured to 80–90% confluence (referred to as undifferentiated) before the media was switched to high-CaCl₂ media, which were replenished every 2 d. The cultures were collected at day 6 after the high-CaCl₂ switch (referred to as undifferentiated) before the media was switched to high-CaCl₂ differentiating media were discarded, and the cells were washed with DPBS and detached from the growth surfaces using a cell scraper. The BMDM suspensions in DPBS were spun down (340g, 8 min), the DPBS supernatants were discarded and BMDMs were resuspended in complete DMEM supplemented with 1–5% L929 cell-conditioned medium. For further experimentation, BMDMs were plated in 96-well plates at the density of 5 × 10⁵ cells per well (or 1.56 × 10⁶ cells cm⁻²). All steps of this procedure were performed at RT. Experiments using BMDMs were performed after a 16–18-h resting period in a tissue culture incubator (37 °C, 5% CO₂), following cell plating.

**Generation of NLRP10 reporter N/TERT cell line**

The NLRP10 reporter line described in the manuscript was created using N/TERT cells that had NLRP1 and ASC knocked out before constitutive expression of GFP-tagged NLRP10 and mScarlet-tagged ASC. Briefly, CRISPR–Cas9 NLRP1 knockout N/TERTs were generated as above using the lentilCRISPRv2. ASC was knocked out using the Integrated DNA Technologies Alt-R CRISPR–Cas9 guide system according to the manufacturer’s instructions, and then delivery of CRISPR–Cas9 ribonucleoprotein (RNP) complex into NLRP1 knockout (KO) N/TERT using electroporation with the Nucleofector system from Lonza. Constitutive lentiviral expression of GFP-tagged NLRP10 and mScarlet-tagged ASC was performed using pCDH vector constructs (System Biosciences) and packaged using third-generation packaging plasmids.

**Generation of CRISPR–Cas9 knockout N/TERT**

Lentiviral Cas9 and guide RNA plasmid (LentiCRISPR-V2, Addgene, plasmid no. 52961) was used to create stable deletions in N/TERT-1 immortalized keratinocytes (provided by H. Rheinwald under MTA from Brigham Young Hospital) (51). Constitutive lentiviral expression was performed using pCDH vector constructs (System Biosciences) and packaged using third-generation packaging plasmids. The single guide (sg) RNA target sequences (5’ to 3’) for N/TERT keratinocytes are: CASP1 (ACAGACAAAGGTGCTGAACA), NLRP1 (GCCTGGGACTACAGATCGC), NLRP3 (CAGATCCGATTGACGCG), Cas9 control (GGATTATATCCGGAAGACCC). Note that Cas9- and NLRP10-deficient N/TERT keratinocytes used in Fig. 4 were previously reported in ref. (52). Knockout efficiency was tested by immunoblot. Alternatively, Sanger sequencing of genomic DNA and overall editing efficiency were determined using the Synthego ICE tool (Synthego Performance Analysis, ICE Analysis 2019, v.2.0. Synthego, https://ice.synthego.com/).
**Assessment of whole-cell mtDNA content by qPCR**

Total cell DNA from mtDNA-depleted and control cells was isolated using QIAamp DNA micro kit, following the manufacturer’s instructions. Per condition, material from nine wells of a 96-well plate was collected. The total cellular mtDNA content was analyzed by qPCR using sequences from nuclear DNA as a reference (‘housekeeping’) whose level is not strongly affected by the addition of the mtDNA-depleting agent 

For the qPCR reaction mixes (20 μl), 100 ng DNA per sample was analyzed. The primers were used at the final concentrations of 500 nM per primer. The qPCR master mix (Maxima SYBR Green/ROX 2× qPCR Master Mix) was based on SYBR Green DNA detection, and it was supplied as a 2× concentrated working solution. A 10-μl portion of the qPCR master mix was used per reaction, and nuclelease-free water was added to a final volume of 20 μl. qPCR was run in technical duplicates according to the standard two-step cycling protocol. Briefly, the DNA samples were denatured for 10 min at 95°C, followed by 40 cycles of: (1) denaturation (95°C, 15 s) and (2) annealing and elongation (60°C, 1 min). The fluorescence signal was acquired during the annealing and elongation step, following the manufacturer’s instruction. The fold changes in the mtDNA content values were calculated using the 2^(-ΔΔCt) method.

**qPCR for NLRP10 in primary human keratinocytes**

RNA of 2 × 10^3 cells seeded into a well of a 96-well plate was isolated using the RNeasy mini kit (Qiagen, catalog no. 74004) according to the manufacturer’s instructions. Cells were lysed with 350 μl of RLT lysis buffer containing 1% β-mercaptoethanol. RNA was eluted in 30 μl of nuclelease-free water and the RNA concentrations were assessed by measuring absorbance at 260 nm.

Complementary DNA was synthesized on the template of messenger RNA using oligo-dT primers and SuperScript III Reverse Transcriptase (ThermoFisher Scientific, catalog no. 18080093), following the manufacturer’s instructions. Approximately 100–500 ng of RNA was mixed with nuclelease-free water to a volume of 12.9 μl, and 1 μl of oligo-dT primer (50 μM) per sample was added. The mix was first incubated at 65°C for 5 min, and then cooled on ice for 1 min. Next, 4 μl of 5× first-strand buffer, 1 μl of 10 mM dNTPs, 1 μl of 0.1 M dithiothreitol and 0.1 μl of SuperScript III Reverse Transcriptase per sample were added to the mix, which was subsequently incubated for 50 min at 50°C and then for 5 min at 85°C. Last, cDNA was diluted 1:5 by addition of nuclelease-free water.

The qPCR reaction was performed as described in the previous section, with HPRT primers targeting the housekeeping control reference gene and NLRP10 qPCR primers to assess the relative NLRP10 mRNA levels.

**RNAscope in situ hybridization**

Waste surgical skin tissues from abdomen and breast were collected with appropriate informed consent of the patients and sent to the Asian Skin Biobank (ASB) at the Skin Research Institute of Singapore (SRIS) (under A*STAR IRB 2020-209). The microtome and blade were sprayed with 100% ethanol before sectioning FFPE-embedded human skin biopsies at 15 μm. Freshly cut sections were allowed to fully adhere onto the slide for 1 h before being dewaxed and then dehydrated through an increasing ethanol gradient: 50%, 70%, 100%. Sections were then dried and demarcated using a hydrophobic barrier pen. Hydrogen peroxide was applied to each section for 10 min at RT before incubation with protease IV for 30 min at RT. Sections were then processed according to the RNAscope 2.5 HD RED chromogenic assay (ACDbio, catalog no. 322350) protocol provided by the manufacturer. Briefly, sections were incubated for 2 h at 40°C with the appropriate probe: NLRP10, PP1B (positive control) or dapB (negative control). Slides were then washed twice for 2 min at RT with RNAscope Wash Buffer. This was then followed by sequential hybridizations with six amplification probes, alternating between 30 min and 15 min of incubation at 40°C. After washing in RNAscope Wash Buffer, sections were incubated for 10 min at RT with RNAscope RED Working solution. This was then washed off with tap water and the sections were counterstained with 50% Gill’s haematoxylin I staining solution for 3 min at RT. Nuclei were then blued using 0.02% ammonia water before drying at 60°C for 15 min.

**Immunoblotting**

Protein concentrations in lysates were determined using the Bradford assay (Thermo Scientific, catalog no. 23200), and 20 μg of protein was loaded per lane of a gel, with the exception of cleaved GSDMD-NT visualization, where 40 μg of protein was used. All primary antibodies were used at 250 ng/ml. Visualization of ASC oligomerization was as previously described. For analysis of IL-1β in the media by immunoblotting, samples were concentrated using filtered centrifugation (Merck, Amicon Ultra, catalog no. UFC503936). Protein samples were electrophoretically separated and immunoblotted, and then visualized using a ChemiDoc Imaging system (BioRad).

**NLRP10-ASC coimmunoprecipitation**

HEK293T cells overexpressing hASC-TagBFP and hNLRP10-mCitrine were plated in poly-t-lysine (0.01%) coated 6-well tissue culture plates at 5 × 10^3 cells per well (three wells per condition) in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and rested overnight. On the next day, the culture medium was removed and discarded, and it was replaced with OptiMEM (900 μl per well). Then, 10× concentrated working solutions of the activators in OptiMEM were prepared (200 μM thapsigargin for the final concentration of final 20 μM; 850 μM m-3M3PBS for the final concentration of final 85 μM) and immediately used for stimulation of the cells. Untreated control was subjected to OptiMEM alone. The plates were swirled (figure of eight-shaped movements) to ensure equal distribution of the activator in the well and spun down at 340g for 3 min at RT. The plates were incubated for 45 min at 37°C, 5% CO2. After this time the plates were transferred to ice. The stimulation media were discarded and the cells were lysed with 150 μl per well of lysis buffer (Tris–HCl, pH 7.4, 100 mM NaCl, 1% glycerol, 0.5% NP-40, complete protease inhibitor cocktail). The cells were incubate with the lysis buffer for 5 min on ice, scraped and the resulting lysates were spun down at 1,000g for 5 min at 4°C. The resulting supernatants (also serving as input controls) were transferred to fresh tubes and the pellets were discarded. Following the centrifugation, the lysates were incubated with GFP-trap magnetic particles M-270 (Chromotek), according to the manufacturer’s instructions. At the end of this incubation, the beads were washed three times with the lysis buffer, after which the beads were resuspended in lithium dodecylsulfate lysis buffer with reducing agent (ThermoFisher Scientific) and boiled for 10 min at 95°C. In parallel, the input samples were also mixed with the lysis buffer and the reducing agent and boiled. The resulting samples were separated by SDS–PAGE and blotted for NLRP10- mCitrine (anti-human NLRP10 clone SH2, Merck Millipore) and ASC-TagBFP (anti-ASC AL177, Adipogen).

**Microscopy of N/TERT immortalized keratinocytes**

To assess pyroptotic cell morphology (Fig. 4b), N/TERT immortalized keratinocytes were seeded onto 12-well tissue culture plates and allowed to adhere overnight at 37°C, 5% CO2. The next day, cells were pretreated with a caspase-1 inhibitor (VX-765, 10 μM), to prevent pyroptotic cell detachment from the plate. After 1 h of incubation with the caspase-1 inhibitor, the cells were further treated with the indicated stimuli. Three images per treatment were then acquired using the EVOS microscope.

For the analysis of caspase-1 CARD domain aggregate formation or immunofluorescence staining (Extended Data Fig. 10d,e), N/TERT immortalized keratinocytes were plated in 96-well plates at a density of 1–1.5 × 10^4 cells per well, and kept in a tissue culture incubator (37°C, 5% CO2, 5% humid CO2).
5% CO₂ overnight (16–18 h). On the day of the experiment, the cells were treated with m-3M3FBS (85 μM). Per condition, 2–4 images were acquired using the Zeiss Observer microscope.

**Colocalization studies of NLRP10 with ASC and the mitochondria**

Before treatment with m-3M3FBS, cells were stained using MitoTracker Deep Red (ThermoFisher Scientific, catalog no. M2246) to visualize the mitochondria. The 1 mM MitoTracker stock was diluted in cell culture media to 100 μM and incubated for 15 min at 37 °C, 5% CO₂ incubator. Following MitoTracker staining, cells were treated with m-3M3FBS (80 μM) and imaged immediately without fixation using a confocal laser scanning microscope (Olympus, FV3000).

**Inflammasome stimulation**

One day before the experiment, the cells were detached from the growth surface using TrypLE Express Enzyme and plated in 96-well plates in complete DMEM. The plating densities were typically 5–6 × 10⁴ cells per well (-1.56–1.88 × 10⁶ cells cm⁻²) for immortalized macrophages, and 3.5–3.8 × 10⁶ cells per well (-1.1 × 10⁷–1.19 × 10⁶ cells cm⁻²) for HEK cells. The cells were kept in a tissue culture incubator (37 °C, 5% CO₂) overnight (16–18 h).

On the day of the experiment, the cells were primed with LPS (200 ng ml⁻¹, 2 h at 37 °C, 5% CO₂) in complete DMEM, or left unprimed. Of note, NLRP3/ASCmCerulean reporter iMac cells do not require the priming step to mount the NLRP3 inflammasome responses due to their constitutive overexpression of NLRP3.

For inflammasome activation, the cells were shifted to OptiMEM (ThermoFisher Scientific, catalog no. 31985070) or to an extracellular buffer consisting of (in mM) 123 NaCl, 5 KCl, 2 MgCl₂, 1 CaCl₂, 10 glucose, 10 HEPES (pH 7.4). For inflammasome stimulations under increased KCl concentrations, the buffer composition was modified (the KCl concentration was raised at the cost of an equimolar decrease in the NaCl concentration), and for stimulations under Ca²⁺-free conditions, CaCl₂ was not added. Typically, 90 μl per well of the extracellular buffer was added, followed by the addition of 10 μl of the following inflammasome activators: nigericin (final concentration 10 μM), thapsigargin (final concentration -20 μM), m-3M3FBS (final concentration -85 μM) or poly(dA:dT) (model double stranded (ds) DNA transfection complexes, administered at 200 ng per well and complexed with 0.5 μl of Lipofectamine 2000 per well, which corresponds to 2 μg ml⁻¹ poly(dA:dT) dsDNA complexed with 5 μl of Lipofectamine 2000). The addition of inflammasome agonists was followed by gently flicking the plate with a finger, a centrifugation step (340 × g, 5 min, at RT) and a 30–60-min incubation (37 °C, 5% CO₂). After this time, tissue culture supernatants were collected, or the cells were fixed (4% formaldehyde) and counterstained with a nuclear dye (5 μM DRAQ5).

**Mouse IL-1β concentration assessment by HTRF**

IL-1β concentrations in tissue culture supernatants were assessed by a homogenous time-resolved fluorescence (HTRF) ‘sandwich’ antibody-based assay, following the manufacturer’s instructions. The supernatants were analyzed either directly upon completion of the experiment or stored at 4 °C for up to 24 h.

Briefly, the anti-mouse IL-1β solutions were mixed at a 1:1 ratio. A portion of 4 μl per well of this mixture was distributed in white low-volume medium-binding HTRF-adapted 384-well assay plates (Greiner Bio-One, catalog no. 784075). This was followed by the addition of the samples (tissue culture supernatants; 16 μl per well). The plates were centrifuged at RT, 1,000 g for 5 min, followed by a 2–6-h incubation at RT or a 16–18-h incubation at 4 °C. After this time, the HTRF signals were measured using Spectramax 13 with an HTRF cartridge.

**Human IL-1β ELISA**

Secreted IL-1β cytokine levels were assessed by enzyme linked immunosorbent assay (ELISA) kit (BD), according to manufacturer’s instructions.

**Immunofluorescence staining and analysis of primary keratinocytes**

Keratinocytes were fixed with 4% formaldehyde for 30 min at RT. This was followed by a quenching step (50 mM NH₄Cl, 50 mM glucose in PBS) for 90 min, and a permeabilization/blocking step (10% goat serum and 0.1% Triton X-100 in PBS). After that, cells were incubated with the NLRP10 primary antibody (1:100) (clone 8H2, MABC293, Sigma) overnight at 4 °C and subsequently stained with the Alexa-488-labeled secondary antibody for 1 h at RT. The nuclei were stained with DAPI (1 μg ml⁻¹) for 15 min, and the samples were stored in PBS at 4 °C for no more than 7 d until further processing.

The imaging was performed using a Leica SP8 point-scanning confocal microscope equipped with the ×40 water-immersion objective (numerical aperture (NA) 1.1). The images were acquired using LAS X software (v.3.5.5) as z-stacks at 2,048 × 2,048 resolution and with a line averaging of 4. A total of 6–8 stacks were acquired per condition.

The image processing and analysis were performed using Fiji (https://imagej.net/software/fiji/). The final images were obtained using maximum intensity projection of z-stack, and only the linear adjustment of the brightness and contrast were performed to aid visibility.

**Endpoint wide-field fluorescence microscopy**

Formaldehyde-fixed cell samples were analyzed by fluorescence microscopy no later than one week after the completion of the experiment. Until that time, the samples were stored at 4 °C. Samples were imaged at the Microscopy Core Facility (Medical Faculty, University of Bonn) using the Observer.Z1 fluorescence microscope (Zeiss) with a dry
All imaging assays were performed in 96-well plates. For most experiments, six images per well were acquired. The automated acquisition was set up in the microscope software; all fixed samples were counterstained with the nuclear dye DRAQ5, the DRAQ5 channel was used as the reference channel for software autofocus. Generally, a phase-contrast micrograph was acquired for every condition, with the DAPI filter set (Zeiss filter set no. 49) in the light path. For the fluorescent proteins and dyes, the following filter sets were used: TagBFP, Zeiss filter set no. 49 (DAPI); mCerulean, Zeiss filter set no. 47 HE (CFP); mCitrine, Zeiss filter set no. 46 HE (YFP); mCherry, Zeiss filter set no. 43 HE (DsRed); DRAQ5, Zeiss filter set no. 50 (Cy5).

After the acquisition, where applicable, sample images were exported as TIFF files using Zen Lite software. Only linear adjustments were applied to the images (adjustments to the lookup table’s lower and upper boundaries), and these adjustments were uniformly applied to all sample images within one experiment. No nonlinear adjustments were applied. TIFF files exported from Zen Lite software were directly imported into figures prepared using Ai Illustrator software. In Ai Illustrator, the images were cropped and, when necessary, the image dimensions were adjusted to the layout of the figure. For image quantification, raw imaging data were imported into Cell Profiler software.

**Time-lapse wide-field fluorescence microscopy**

Samples were imaged at the Microscopy Core Facility (Medical Faculty, University of Bonn) using the Observer.Z1 fluorescence microscope (Zeiss) with a dry ×20 LD Plan Neo Fluar objective (NA 0.4) or a dry ×20 Plan Apochromat objective (NA 0.8). The microscope was operated using Zen 2.3 Pro software. Image acquisition was performed at RT.

All imaging assays were performed in 96-well plates. For most experiments, six images per well were acquired. Automated acquisition with time-lapse recording was set up in the microscope software; the phase contrast was typically used as the reference channel for software autofocus, or the definitive focus option was used. The time interval between consecutive acquisitions of a given imaging field was 1 min or 4 min, depending on the activator (nigericin, m-3M3FBS, thapsigargin, ionomycin and untreated controls, 1 min; poly(dA:dT), 4 min).

Generally, a phase-contrast micrograph was acquired for every condition, with the DAPI filter set (Zeiss filter set no. 49) in the light path. For the fluorescent proteins, the following filter sets were used: TagBFP, Zeiss filter set no. 49 (DAPI); mCerulean, Zeiss filter set no. 47 HE (CFP); mCitrine, Zeiss filter set no. 46 HE (YFP); mCherry, Zeiss filter set no. 43 HE (DsRed); DRAQ5, Zeiss filter set no. 50 (Cy5).

After the acquisition, where applicable, sample images were exported as TIFF files using Zen Lite software. Only linear adjustments were applied to the images (adjustments to the lookup table’s lower and upper boundaries), and these adjustments were uniformly applied to all sample images within one experiment. No nonlinear adjustments were applied. TIFF files exported from Zen Lite software were directly imported into figures prepared using Ai Illustrator software. In Ai Illustrator, the images were cropped and, when necessary, the image dimensions were adjusted to the layout of the figure. For image quantification, raw imaging data were imported into Cell Profiler software.

**Image quantification and analysis**

For image quantification (obtaining nuclear and ASC speck counts from images acquired on the Zeiss Observer.Z1 wide-field fluorescence microscope), raw imaging data were imported into Cell Profiler software. The methods to distinguish between clumped objects were shape, and the definite focus option was used. The number of deviations was set to 0, threshold smoothing scale to 0.02. The averaging was global, and the thresholding method was robust background.

For identification of the nuclei, a typical diameter of 10–80 pixels was used (this parameter was adjusted between image sets), and objects outside this diameter range were discarded. Objects touching the border of the image were retained. The thresholding strategy was global, and the thresholding method was robust background. The lower and upper outlier fractions were set to 0.02. The averaging method was mode, and the variance method was standard deviation. The number of deviations was set to 0, threshold smoothing scale to 1.3488, threshold correction factor to 1.86 and the lower and upper bounds on threshold were set between -0.001 and -1 (this parameter was adjusted between image sets), and objects outside this diameter range were discarded. Objects touching the border of the image were retained. The thresholding strategy was global, and the thresholding method was robust background. The lower and upper outlier fractions were set to 0.02. The averaging method was mode, and the variance method was standard deviation. The number of deviations was set to 0, threshold smoothing scale to 1.3488, threshold correction factor to 1.97 and the lower and upper bounds on threshold were set between -0.01 and -0.05 for HEK cells and between -0.01 and -1 for macrophages (this parameter was adjusted between image sets, depending on the brightness of the ASC signal). The method to distinguish between clumped objects was shape, and...
the method to draw dividing lines between clumped objects was intensity. The size of the smoothing filter for declumping and the minimum allowed distance between local maxima were both automatically calculated. Lower resolution images were used to find local maxima. Holes in identified objects were filled after both thresholding and declumping.

Raw imaging data from time-lapse recordings of cells overexpressing fluorescent protein-based markers targeted to the mitochondrial matrix were imported into CellProfiler software. The granularity of the mitochondrial fluorescence signal was assessed in unprocessed images. The module used was 'MeasureGranularity', with the following parameter settings: measurement within objects was disabled, the subsampling factor for granularity measurements was 0.25, the subsampling factor for background reduction was 0.25, the radius of structuring element was 3 and the range of the granular spectrum was 1. To facilitate the comparisons between multiple recordings, the image granularity in the first frame of the recording was set to 100% and the values obtained from subsequently recorded frames were normalized to this initial value.

Data collection and statistical analysis
No statistical methods were used to predetermine sample sizes but our sample sizes are similar to those reported in previous publications (for examples, please see refs. [5, 15]). Data collection was not randomized. Data collection and analysis were not performed blind to the conditions of the experiments. Data distribution was assumed to be normal but this was not formally tested. No samples and data points have been excluded from analysis.

Data are presented as mean ± s.d.; the number of independent experiments is indicated for each panel in the figure legends. In most cases individual data points represent the mean of technical replicates in each independent experiment; this is specified in the figure legends, as are the numbers of technical replicates. Microsoft Excel 16 was used for data processing; GraphPad Prism 6, 7, 8 or 9 were used for plot preparation, s.d. calculation and statistical significance analysis. The specific tests used and $P$ value ranges are indicated in the figure legends.

Reporting summary
Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability
Representative images for all ASC speck formation quantifications are deposited in Mendeley Data using the following link: https://data.mendeley.com/datasets/42f5264kn5/1. Source data are provided with this paper. All other data are available in the article Supplementary files or from the corresponding author upon reasonable request.

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Extended Data Fig. 1 | m-3M3FBS activates the inflammasome independent of NLRP3. (a) IL-1β secretion from WT iMacs, LPS-primed (200 ng/ml) and stimulated with nigericin (10 μM), poly-(dA:dT) (2 μg/ml) or m-3M3FBS (80 μM) (n = 3). (b) ASC specks per nuclei in iMacs expressing NLRP3 and ASCmCerulean, stimulated as in (a) (n = 3). (c) IL-1β secretion from WT iMacs, LPS-primed (200 ng/ml) and stimulated with nigericin (10 μM), poly-(dA:dT) (2 μg/ml) or m-3M3FBS (85 μM), in the presence of increasing doses of KCl (0, 5, 10, 25, 50, 75, 100, 125 mM) (n = 3). (d) ASC specks per nuclei in iMacs expressing NLRP3 and ASCmCerulean, stimulated as in (c), in the presence of increasing doses of KCl (0, 5, 10, 25, 50, 75, 100, 125 mM) (n = 3). (e) ASC specks per nuclei in WT iMacs expressing ASCmCerulean, with or without LPS priming (200 ng/ml) (41, 42), stimulated as in (c). While nigericin-induced ASC speck formation depends on priming, m-3M3FBS and poly-(dA:dT) trigger ASC speck formation in both LPS-primed and -unprimed cells (n = 3). (f) IL-1β secretion from WT iMacs, LPS-primed (200 ng/ml) and stimulated as in (c), in the presence or absence of the NLRP3 inhibitor CRID3 (5 μM) (43-45) (n = 3). (g) ASC specks per nuclei in iMacs expressing NLRP3 and ASCmCerulean, stimulated as in (c), in the presence or absence of the NLRP3 inhibitor CRID3 (5 μM) (n = 3). P values were calculated by 2-way ANOVA with Sidák’s multiple comparisons test. **P = 0.0068, ****P < 0.0001, ns P = 0.5730-0.9999. (h) ASC speck formation in HEK cells stably expressing ASCmCerulean and transfected with an empty vector (EV) or human NLRC4, Pyrin or AIM2, stimulated with increasing doses of m-3M3FBS (40, 55, 70, or 85 μM); normalized to the unstimulated control (n = 3). (i) ASC specks per nuclei in HEK cells expressing human NLRP10 or mCitrine and ASCmCerulean, stimulated with nigericin (10 μM), poly-(dA:dT) (2 μg/ml) or m-3M3FBS (85 μM) (n = 4). Individual data points are means of technical triplicates (a, b, e, h, i) or duplicates (c, d, f, g); error bars represent SD.
Extended Data Fig. 2 | NLRP10 does not colocalize with the ER, endolysosomal or Golgi compartments. (a–c) Representative confocal micrographs of HEK cells stably expressing NLRP10mCherry and transfected with an endoplasmic reticulum marker (DDOSTmCitrine) (a), an endolysosomal compartment marker (LAMP1mCitrine) (b), or a Golgi apparatus marker (TGOLN2mCitrine) (c), stimulated with m-3M3FBS (85 μM) or nigericin (10 μM) (n = 4).
Extended Data Fig. 3 | NLRP10 colocalizes with mitochondria and nucleates ASC specks upon thapsigargin and SMBA1 treatments. (a) Representative confocal micrographs of HEK cells stably expressing NLRP10mCherry and transfected with TOMM20mCitrine mitochondrial marker, stimulated with thapsigargin (20 μM) or SMBA1 (50 μM) (n = 4). (b) ASC specks per nucleus in HEK cells expressing NLRP10 and ASCTagBFP, stimulated with m-3M3FBS (85 μM), thapsigargin (20 μM) or increasing doses of SMBA1 (5, 10, or 50 μM) (n = 3). Individual data points (where applicable) are means of technical duplicates (b); all error bars represent SD.
Extended Data Fig. 4 | Upon mitochondrial permeabilization, AIM2 but not NLRP10 activation requires mtDNA. (a) IL-1β secretion from WT iMacs, LPS-primed (200 ng/ml) and stimulated with thapsigargin (20 μM) (n = 3) or SMBA1 (50 μM) (n = 4), in the presence or absence of the NLRP3 inhibitor CRID3 (5 μM) (43-45). P values were calculated by unpaired two-tailed Student’s t-test. ns P = 0.8492–0.8931. (b) IL-1β secretion from WT or AIM2-deficient bone marrow-derived macrophages, primed with LPS (200 ng/ml) and stimulated with poly-(dA:dT) dsDNA (2 μg/ml) or SMBA1 (50 μM) (n = 3). P values were calculated by 2-way ANOVA with Šídák’s multiple comparison test. **** P < 0.0001. (c) ASC specks per nuclei in iMacs expressing NLRP3 and ASCmCerulean, stimulated with nigericin (10 μM), poly-(dA:dT) dsDNA (2 μg/ml), m-3M3FBS (85 μM), thapsigargin (20 μM) or SMBA1 (50 μM) (n = 4 for treatment with SMBA1 and 3 for other activators). (d, e) qPCR assessment of the whole-cell mtDNA content in WT iMacs (d) or HEK cells expressing NLRP10 and ASCTagBFP (e), cultured for 72–96 h with 2',3'-dideoxycytidine (ddC) (80 μg/ml) (n = 3). (f) ASC specks per nuclei in HEK cells expressing NLRP10 and ASCTagBFP, cultured in the presence or absence of ddC (80 μg/ml), and stimulated with m-3M3FBS (85 μM) or thapsigargin (20 μM) (n = 3). P values were calculated by 2-way ANOVA with Šídák’s multiple comparisons test. ns P = 0.1337–0.9909. Individual data points are values of technical monoplicates (d and e) or means of duplicates (a, c) or triplicates (b, f); all error bars represent SD.
Extended Data Fig. 5 | Differential sensitivity to cyclosporin A links mitochondrial damage to NLRP10 and AIM2 activation. (a, b) Representative micrographs from time-lapse microscopy of HEK cells expressing NLRP10, ASC\textsuperscript{Flp}, and mCherry targeted to the mitochondrial matrix (a) or iMacs expressing NLRP3, ASC\textsuperscript{Cerulean} and mCitrine targeted to the mitochondrial matrix (b) (using cytochrome c oxidase subunit 8 mitochondrial targeting sequence). Cells were treated with m-3M3FBS (85 μM) or thapsigargin (20 μM), with or without cyclosporin A (CsA; 15 μM) pre-treatment (n = 4). Images of the same field at treatment onset (0 min) or indicated time points.
Extended Data Fig. 6 | NLRP10 activation by SMBA1 depends on mitochondrial permeability transition. (a, c) ASC specks per nuclei in HEK cells expressing NLRP10 and ASC<sup>TagBFP</sup> (a) or iMacs expressing NLRP3 and ASC<sup>mCerulean</sup> (c), treated with SMBA1 (50 μM), in the presence or absence of cyclosporin A (CsA; 10 μM) (n = 3). P-values were calculated by unpaired two-tailed Student’s t-test. ***P = 0.0009, ****P < 0.0001. (b, d) Representative endpoint fluorescence micrographs of HEK cells expressing NLRP10 and ASC<sup>TagBFP</sup> (b) or iMacs expressing NLRP3 and ASC<sup>mCerulean</sup> (d) with stable expression of mCitrine targeted to the mitochondrial matrix (using mitochondrial targeting sequence from cytochrome c oxidase subunit 8), stimulated with SMBA1 (50 μM), in the presence or absence of CsA (10 μM). Note that in the presence but not in the absence of CsA pre-treatment, mCitrine retains the granular distribution pattern consistent with mitochondrial localization (n = 3). Individual data points (where applicable) are means of technical duplicates; all error bars represent SD.
Extended Data Fig. 7 | Debio025 and NIM811 inhibit thapsigargin- but not m-3M3FBS-induced NLRP10 activation. (a, d) ASC specks per nuclei in HEK cells expressing NLRP10 and ASC\textsuperscript{mCherry}, stimulated with m-3M3FBS (85 μM) or thapsigargin (20 μM), in the presence or absence of Debio025 (10 μM) (a) or NIM811 (10 μM) (d) (n = 3). P values were calculated by 2-way ANOVA with Šídák's multiple comparisons test. *P = 0.0276, **P = 0.0038, ns P = 0.0778-0.0792. (b, e) ASC specks per nuclei in iMacs expressing NLRP3 and ASC\textsuperscript{mCerulean}, stimulated with nigericin (10 μM), poly-(dA:dT) dsDNA (2 μg/ml), m-3M3FBS (85 μM) or thapsigargin (20 μM), in the presence or absence of Debio025 (10 μM) (b) or NIM811 (10 μM) (e) (n = 3). P values were calculated by 2-way ANOVA with Šídák's multiple comparisons test. ***P = 0.0002, ****P < 0.0001, ns P = 0.2813-0.9999. (c, f) IL-1β secretion from WT iMacs, primed with LPS (200 ng/ml) and stimulated as in (b, e), in the presence or absence of Debio025 (10 μM) (c) or NIM811 (10 μM) (f) (n = 3). P values were calculated by 2-way ANOVA with Šídák's multiple comparisons test. **P = 0.0027, ****P < 0.0001, ns P = 0.1174-0.9996. Individual data points are means of technical duplicates; all error bars represent SD.
Extended Data Fig. 8 | NLRP10 activation upon m-3M3FBS treatment is independent of calcium fluxes. (a, b) ASC specks per nuclei in HEK cells expressing NLRP10 and ASC\textsuperscript{TagBFP}, with or without m-3M3FBS (85 μM) treatment, in the presence or absence of BAPTA-AM (50 μM) (a) or 2-APB (50 μM) (b) in Ca\textsuperscript{2+}-free medium (n = 3). P values were calculated by 2-way ANOVA with Šídák’s multiple comparisons test. **P = 0.0019, ns P = 0.3923. (c) Representative micrographs from time-lapse microscopy of HEK cells expressing NLRP10, ASC\textsuperscript{TagBFP} and mCherry targeted to the mitochondrial matrix (using cytochrome c oxidase subunit 8 mitochondrial targeting sequence), treated with ionomycin (10 μM) in the presence of CaCl\textsubscript{2} (1 mM). Images of the same field at treatment onset (0 min) or indicated time point (30 min) (n = 4). (d) Quantification of mitochondrial fluorescence signal granularity and ASC specks per imaging field (n = 4). (e) ASC specks per nuclei in HEK cells expressing NLRP10 and ASC\textsuperscript{TagBFP}, stimulated with m-3M3FBS (85 μM) or with increasing doses of ionomycin (5, 10, 15 μM) in the presence of CaCl\textsubscript{2} (1 mM) (n = 3). (f) ASC specks per nuclei in HEK cells expressing NLRP10 and ASC\textsuperscript{TagBFP}, with or without m-3M3FBS (85 μM) treatment, in the presence or absence of ionomycin (15 μM) in Ca\textsuperscript{2+}-free medium (n = 3). P values were calculated by 2-way ANOVA with Šídák’s multiple comparisons test. *P = 0.0155. Individual data points are means of technical duplicates (a, b, e, and f) or means from 4 independent experiments (d); all error bars represent SD.
Extended Data Fig. 9 | Impact of emricasan on IL-1β secretion and ASC speck formation during mitochondrial rupture. (a) IL-1β secretion from WT iMacs, primed with LPS (200 ng/ml) and stimulated with nigericin (10 μM), poly-(dA:dT) dsDNA (2 μg/ml), m-3M3FBS (85 μM) or thapsigargin (20 μM), in the presence or absence of emricasan (5 μM) (n = 3). P values were calculated by 2-way ANOVA with Šídák’s multiple comparisons test. ****P < 0.0001. (b) ASC specks per nuclei in HEK cells expressing NLRP10 and ASCTagBFP, stimulated with m-3M3FBS (85 μM) or thapsigargin (20 μM), in the presence or absence of emricasan (5 μM) (n = 3). (c) ASC specks per nuclei in iMacs expressing NLRP3 and ASCmCerulean, stimulated as in (a), in the presence or absence of emricasan (5 μM) (n = 3). P values were calculated by 2-way ANOVA with Šídák’s multiple comparisons test. ns P = 0.4112-0.9999. Individual data points are means of technical duplicates; all error bars represent SD.
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | NLRP10 expression in primary and immortalized keratinocytes. (a) qPCR analysis of NLRP10 mRNA expression in primary human keratinocytes, undifferentiated or differentiated for 6 days in medium containing CaCl2 (0.1–1 mM) (n = 2). (b) Representative western blot analysis of the differentiation marker involucrin and NLRP10 in undifferentiated or differentiated immortalized N/TERT human keratinocytes (n = 3). (c) Representative western blot analysis of NLRP10 in WT, Cas9 control or NLRP10-deficient (NLRP10sg2, NLRP10sg3) immortalized N/TERT human keratinocytes (n = 3). (d) Representative widefield fluorescence microscopy analysis of caspase-1 CARD domain aggregate formation in NLRP10-overexpressing or NLRP10-deficient immortalized N/TERT human keratinocytes, expressing Casp1-CARDΔz, with or without m3M3FBS (85 μM) treatment (n = 1). (e) Representative immunofluorescence staining and widefield fluorescence microscopy of primary normal human keratinocytes, with or without m3M3FBS (85 μM) treatment. Shown as maximum intensity projection of the z-stack, from an orthogonal (f) or top (g) view (n = 3). (h) ASC specks per nuclei in HEK cells expressing NLRP10 (either WT or the R243W variant) and ASCTagBFP, stimulated with m3M3FBS (85 μM) or thapsigargin (20 μM) (n = 3). P values were calculated by 2-way ANOVA with Šidák’s multiple comparisons test. ***P = 0.0002-0.0005. Individual data points are means of technical duplicates (a, h); all error bars represent SD.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
- Biotek Gen5 Image+ v 3.03, Leica LAS AF v 2.7.3, Leica LAS X v 3.5.5, Thermo Fisher QuantStudio Software v 1.7.1
- Molecular Devices SoftMax Pro v 6.3, Zeiss Zen Blue v 3.1

Data analysis
- Adobe Ai Illustrator CC2018 (22.1.0) and 2020 (24.1), Cell Profiler 3.1.8, 3.1.9, Geneious R9-Prime 2020, Leica LAS X Lite v 3, Microsoft Excel v 16, Graphpad Prism v 6, 7, 8, 9, Zen Pro v 2.3, Zen Lite v 2, Fiji v 2.9.0

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Source data are provided with this paper. Representative images for all ASC speck formation quantifications are provided on Mendeley Data, link: https://data.mendeley.com/datasets/42fsz64kn5/1.
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No statistical methods were used to pre-determine sample sizes; typically we performed the experiments as three biologically independent repeats to ensure reproducibility, which is a standard in the field as reported in previous publications (for examples, please see studies by Muñoz-Planillo et al. Immunity 2013, Chen and Chen Nature 2018, Lee et al. Nature 2012, Rosas et al. Nature Communications 2012, Fernandez-Alnemri et al. Nature 2009, Hornung et al. Nature 2009, Baerenheid et al. The Journal of Immunology 2009, Franchi et al. The Journal of Immunology 2009, Coll et al. Nature Medicine 2015, Dang et al. Cell 2017). In the instances where biological material availability was the limiting factor, we performed two biological repeats of the experiments. |
| Data exclusions | No data points were excluded from analysis. |
| Replication | All attempts at replication were successful. All experiments were performed two or more times to validate reproducibility. |
| Randomization | Randomization was not performed. No patient or experimental animal in vivo interventions were performed that would require randomization. In in vitro experiments on pools of cells (such as were performed in our study), randomization was achieved by stochastically dividing cells from the same initial population into distinct experimental conditions (different assay wells). |
| Blinding | No blinding was performed. No patient or experimental animal in vivo interventions were performed that would require blinding on the subject side. The researchers were not blinded to the conditions, because data analysis involved only objective assessments (measurements of cytokine concentrations, counting objects, determining protein localization, comparing fluorescence signal intensities), as opposed to subjective assessments such as histopathologic scoring, which were not performed in our study. |

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| Animals and other organisms | Animals and other organisms | | |
| Human research participants | Human research participants | | |
| Clinical data | Clinical data | | |
| Dual use research of concern | Dual use research of concern | | |

**Antibodies**

Anti-ASC, Adipogen, Catalog # AL-177, used at 250 ng/mL (Western blotting)

NLRP10, Clone 8H2, Sigma, Catalog # MABC293, used at 1:100 (immunostaining) or 1:500 (Western blotting)

GAPDH, Santa Cruz Biotecno, Catalog # 47724, used at 250 ng/mL (Western blotting)

IL-1β (total), R&D, Catalog # AF-401-NA, used at 250 ng/mL (Western blotting)

IL-1β (cleaved), Cell Signaling Technology, Catalog # 83186S, used at 250 ng/mL (Western blotting)

GSDMD (cleaved), Cell Signaling Technology, Catalog # 36425, used at 250 ng/mL (Western blotting)

Involutrin, abcam, Catalog # ab68, used at 1:500 (Western blotting)

AlexaFluor-488-conjugated goat anti-rat secondary antibody (Thermo Fisher Catalog # A-11006), used at 1:500 (immunostaining)
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