Plasma membrane damage causes NLRP3 activation and pyroptosis during *Mycobacterium tuberculosis* infection

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*Mycobacterium tuberculosis* is a global health problem in part as a result of extensive cytotoxicity caused by the infection. Here, we show how *M. tuberculosis* causes caspase-1/NLRP3/gasdermin D-mediated pyroptosis of human monocytes and macrophages. A type VII secretion system (ESX-1) mediated, contact-induced plasma membrane damage response occurs during phagocytosis of bacteria. Alternatively, this can occur from the cytosolic side of the plasma membrane after phagosomal rupture in infected macrophages. This damage causes K<sup>+</sup> efflux and activation of NLRP3-dependent IL-1β release and pyroptosis, facilitating the spread of bacteria to neighbouring cells. A dynamic interplay of pyroptosis with ESCRT-mediated plasma membrane repair also occurs. This dual plasma membrane damage seems to be a common mechanism for NLRP3 activators that function through lysosomal damage.
**Results**

Mycobacterium tuberculosis (Mt) is a human pathogen, causing about 1.6 million deaths per year. A pathological hallmark of Mt infection is extensive necrosis in infected tissues. Necrosis has long been regarded as an unregulated type of cell death, but recently several programmed necrotic pathways have been identified. A highly inflammatory form of programmed necrosis is pyroptosis, occurring mainly in myeloid cells after pattern-recognition receptor activation. In the classical pathway, activation of nucleotide-binding oligomerisation domain-like receptors (NLRs) or absent in myeloma 2 (AIM2)-like receptors (ALRs) by pathogen- or self-ligands drives the assembly of an inflammasome consisting of oligomerised NLRs or ALRs, while the second signal is characterised by a range of roles for NLRP3 in vivo. The agonist of AIM2 (ref.39), and release of active cathepsin B from permeabilisation presumably allows the release of Mtb DNA and other molecules that Mt induces the assembly of one of the ASC-dependent inflammasomes, NLRP3 or AIM2, upon infection of macrophages. THP-1 macrophages expressing ASC-GFP were infected with Mt H37Rv constitutively expressing BFP (Mt-BFP) and imaged after 24 h of infection or over an entire 24 h infection time course by time-lapse microscopy. The cell-impermeable DNA dye DRAQ7 was present in the medium to assess cell death. Mt induced ASC-speck formation in infected macrophages (Fig. 1), and specks were exclusively localised to dead cells as indicated by DRAQ7-negative nuclei (Supplementary Fig. 1a). The number of dead cells, ASC specks and IL-1β secretion increased with increasing multiplicity of infection (MOI, Supplementary Fig. 1b). Treatment with the specific NLRP3 inhibitor MCC950 (ref. 40) or increased extracellular concentrations of KC inhibited the formation of ASC specks, demonstrating that Mt infection results in potassium efflux-driven activation of the NLRP3 inflammasome (Fig. 1a). Correspondingly, IL-1β secretion was inhibited by MCC950 and KC, as well as the caspase inhibitors Z-VAD-FMK (pan-caspase) or VX765 (caspase-1). Treatment with Z-VAD, VX765, MCC950 or KC all reduced cell death measured by DRAQ7 in infected cells, while the proportion of apoptotic cell death was noted the cumulative number of dead cells by pyroptosis, observed in infected cells, while the proportion of apoptotic cell death was larger in infected bystander cells (Fig. 1f). In addition, the

Thiopental- and correlative microscopy to spatially resolve Mt-induced inflammasome activation and cell death at the single-cell level. Our findings identify a common mechanism where plasma membrane (PM) damage caused by Mt or crystals triggers inflammasome activation, IL-1β release and pyroptosis, unless the membrane damage is balanced by repair mediated by the endosomal sorting complexes required for transport (ESCRT) machinery.
Fig. 1 Mtb H37Rv infection induces canonical NLRP3 activation. THP-1 ASC-GFP macrophages were infected by Mtb-BFP and imaged by time-lapse microscopy or at 24 h post infection (p.i.). a Cells were treated with DMSO control, caspase inhibitors (Z-VAD-FMK (pan-caspase) or VX765 (caspase-1)) or NLRP3 inhibitors (MCC950, KCl). DRAQ7+ cells and ASC specks were quantified at 24 h p.i. IL-1β release was determined by ELISA. b THP-1 ASC-mNeonGreen (WT) or NLRP3 KD cells were infected by Mtb and imaged 24 h p.i. c To assess the role of GSDMD in cell death in cells with ASC speck formation, THP-1 ASC-mNeonGreen (WT) or GSDMD KD cells were infected by Mtb and imaged 24 h p.i. c Primary human monocytes were treated with inhibitors and infected with Mtb, and LDH and IL-1β release were determined 24 h p.i. d Primary human macrophages were treated with inhibitors and infected with Mtb. LDH and IL-1β release were determined 24 h p.i. n = 3 and n = 5 biologically independent samples examined over two independent experiments. e Primary human macrophages were treated with inhibitors and infected with Mtb. LDH and IL-1β release were determined 24 h p.i. n = 3 and n = 5 biologically independent samples examined over two independent experiments. The prevalence of pyroptosis was assessed by quantifying the cumulative number of pyroptotic, necrotic and apoptotic cell death events in THP-1 ASC-GFP cells from a 45-h time-lapse experiment during infection with Mtb. Representative cell death events are shown. Scale bars 10 μm. d Data representative of two independent experiments. The bacterial burden (intracellular Mtb-BFP fluorescence) was determined immediately before ASC-speck formation in pyroptotic cells (n = 26), and at the average time of ASC-speck formation in cells surviving the 24-h experiment (n = 61). Lines indicate median values. Bars show mean ± s.e.m. in (a, b) and mean ± s.d. in (d, e). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, by one-way ANOVA with two-sided Dunnett’s test in (a, b), repeated measures ANOVA with two-sided Dunnett’s test in (d, e) and two-sided Mann-Whitney test in (g). Data in (a-c) representative of three independent experiments with n > 2000 cells in technical triplicates per condition. Source data are provided as a Source Data file.
bacterial burden was higher in cells that formed ASC specks than in infected cells that did not (Fig. 1g). These results indicate that pyroptosis from Mtb infection is a cell-intrinsic effect, i.e. a direct result of infection within single cells.

We reasoned the relative reduction in pyroptosis, and the increase in necrosis 24–48 h of infection could be due to prolonged stimulation of Toll-like receptor (TLR) signalling, which has been reported to inhibit NLRP3 inflammasome activation. Indeed, we observed that prolonged treatment (24 h) of THP-1 cells with TLR2 or TLR4 ligands prior to infection with Mtb decreased cell death and ASC speck formation to a similar extent as treatment with MCC950 (Supplementary Fig. 1j).

Pyroptosis causes severe damage and allows for spread of Mtb. Cell death is accompanied with morphological changes that can inform about the processes involved in execution of a particular cell death pathway. We investigated the ultrastructural features of pyroptotic cells using focused ion beam–scanning electron microscopy (FIB-SEM), allowing near-isotropic 3D resolution. Cells of interest were located by light microscopy using a correlation approach. Cells of interest were located by light microscopy using a correlation system we developed previously. Uninfected THP1-ASC-GFP cells displayed a normal, healthy morphology (Fig. 2a). In comparison, infected, pyroptotic cells (i.e. with visible ASC specks) had severe ultrastructural disruptions, including fragmented and occasionally split nuclear membranes, large PM disruptions, organelle damage and leakage of content to the extracellular space (Fig. 2b).

To distinguish morphological changes that occurred as a consequence of pyroptosis from those that occurred prior to or during inflammasome activation, we infected cells for 5 h with Mtb-BFP in the presence of the caspase inhibitors Z-VAD and VX765. Cells were imaged live during infection with DRAQ7 in the medium, and chemically fixed within 2 min of the last captured frame. Cells with an ASC speck but without influx of DRAQ7 and no visible changes to cell morphology were chosen for further imaging by FIB-SEM. The morphology was similar to uninfectected cells, with intact organelles, a continuous PM and a double nuclear membrane (Fig. 2c). Similar results were observed in THP-1 GSDMD KD cells (Supplementary Fig. 2b). Hence, the dramatic morphological changes in pyroptotic cells are a consequence of the pyroptotic cell death process itself.

Next, we investigated the structure of the ASC speck using the correlative imaging approach. ASC specks were visible in SEM images as aggregates with a branched structure and a size of about 1–2.5 μm in THP1-ASC-GFP cells, both when infected with Mtb and when treated with LPS and nigericin (Fig. 2d, e). The structure corresponds well to that previously observed in a zebrafish model. The speck in the living (Z-VAD + VX765-treated) cell was located in an open space in the cytosol of the cell, seemingly devoid of other organelles. To ensure that the ASC-speck structure was not a consequence of ASC overexpression or tagging, we treated primary human monocytes with LPS and nigericin, and visualised active caspase-1 by FAM-YVAD-FMK (FLICA). Caspase-1 is stabilised in its active form on the inflammasome, and we therefore hypothesised that the point with the highest fluorescence intensity from FLICA treatment would be the point where the speck was assembled. Indeed, by correlative light and EM (CLEM), we identified a similar branched structure in LPS and nigericin-treated primary human monocytes, albeit with a smaller size than that observed in THP-1 cells (~0.8 μm). Our data demonstrate that ASC specks also form in primary cells, without tagging or overexpression of selected proteins.

To elaborate on the possible consequences of pyroptosis on Mtb spreading, we investigated pyroptosis during time-lapse imaging. On average, ~25% of Mtb bacilli were lost into the medium immediately after pyroptosis (Fig. 2f), while the rest were trapped in the pyroptotic cell. However, Mtb that was trapped in pyroptotic cells was phagocytosed by neighbouring cells. Further, this induced ASC-speck formation with pyroptosis in the new host cell, thus propagating inflammasome activation and cell death (Fig. 2g; Supplementary Movie 4). This indicates that pyroptosis enables some bacteria to spread immediately after cell death, and that bacteria that are trapped in the ghost cell can also contribute to further local spread of infection by efferocytosis.

Mitochondrial depolarisation accompanies pyroptosis. It has been reported that Mtb lacking the region of difference 1 (MtbΔARD1), where most ESX-1-related genes are located, is deficient in inflammasome activation. Indeed, we observed that Mtb grown in the absence of the detergent tween-80 is more potent in inflammasome activation than Mtb grown in the presence of tween (our regular growth condition). Detergent is found by others to perturb the mycobacterial capsule, in particular decreasing the abundance of ESX-1-related proteins on the Mtb surface/capsule. Our data thus show that inflammasome activation correlates with ESX-1 activity and the integrity of the Mtb capsule, suggesting that deposition of ESX-1-related factors on the Mtb surface/capsule is important for Mtb inflammasome activation.

Tuberculosis-necrotising toxin (TNT, the C-terminal end of the channel protein with necrosis-inducing toxin (CpnT)) is secreted from Mtb and released into the host cell cytosol in an ESX-1-dependent manner, leading to macrophage necroptosis. We therefore wanted to investigate if TNT contributes to the ESX-1-dependent inflammasome activation. To this end, we infected THP-1 ASC-GFP macrophages with wild-type Mtb, MtbΔcpnT or MtbΔcpnT complemented with cpnT containing either the catalytically active or inactive form of TNT (MtbΔcpnT::cpnT and MtbΔcpnT::cpnT, respectively). We found that all Mtb mutants and complemented strains were similarly competent in inflammasome activation, and that treatment with MCC950 had a similar effect across bacterial strains in inhibiting ASC speck formation (Fig. 3b; Supplementary Fig. 3a). This indicates that TNT does not play a role in Mtb-induced inflammasome activation in our system.

Finally, we assessed the possible involvement of mitochondrial membrane perturbation prior to inflammasome activation and pyroptosis, an event that could also be linked to the activity of ESX-1 and related proteins. Mitochondrial damage has been strongly implicated in inflammasome activation in general, and in Mtb-induced necrosis. In particular, infection with Mtb H37Rv was reported to cause a loss in mitochondrial membrane potential (ΔΨm), which was inhibited by cyclosporine A (CsA), a cyclophilin D inhibitor that prevents formation of the mitochondrial permeability transition pore. However, we did not see an effect of CsA on Mtb-induced inflammasome activation and pyroptosis after 24 h of infection (Fig. 3c). In addition, we monitored the ΔΨm by live-cell imaging of cells infected with Mtb-BFP and labelled with tetramethylrhodamine
ethyl ester (TMRE), a dye that is accumulated in the mitochondria in proportion to ΔΨm (ref. 61). We observed that ΔΨm was stable in infected cells until ASC-speck formation, and that the potential quickly dropped after ASC-speck formation as measured by a drop in TMRE intensity (Fig. 3d; Supplementary Movie 5). This immediate drop in TMRE intensity after ASC-speck formation was abolished in THP-1 GSDMD KD macrophages, without any adverse effects on the ability of the cells to form ASC specks, suggesting that GSDMD activity and possibly PM disruption during cell death are required for mitochondrial...
destabilisation (Fig. 3e; Supplementary Movie 6). Similar results were obtained with LPS and nigericin treatment, where ΔΨm dropped only after ASC-speck formation, and the drop was inhibited in GSDMD KD cells (Supplementary Fig. 3b and Supplementary Movies 7 and 8). We conclude that gross mitochondrial membrane disruption is not a prerequisite for inflammasome activation caused by Mtb or LPS and nigericin, but rather accompanies pyroptosis. These results are also consistent with the intact mitochondrial morphology observed by EM in living cells with an assembled inflammasome (Fig. 2c).

Phagosomal damage is a prerequisite for NLRP3 activation. One of the main effects of the ESX-1 secretion system is destabilisation of phagosomal membranes. We therefore asked how phagosomal rupture is related to inflammasome activation and pyroptosis. Galectin-3 (Gal-3) binds to exposed glycosylated proteins usually confined to the inner phagosomal membrane, and has been used as a marker for ruptured phagosomes. We infected THP1-Gal-3-mScarlet macrophages and observed that Gal-3 was indeed recruited to the vicinity of wild-type Mtb, but not to the vicinity of MtbΔRD1 (Fig. 4a), and Gal-3 recruitment was enhanced upon infection with Mtb grown in the absence of...
tween (Fig. 4b). Gal-3 recruitment to ruptured Mtb phagosomes occurred prior to inflammasome activation in about 80% of pyroptotic cells, and a typical sequence of events is depicted in Fig. 4c and Supplementary Movie 9.

Using CLEM, we confirmed that Gal-3 accumulation corresponds to cytosolic contact of Mtb (Fig. 4d). Mtb in phagosomes devoid of Gal-3 were surrounded by a tightly apposed and continuous phagosomal membrane, in contrast to bacteria associated with Gal-3 that had no visible phagosomal membrane, and were in direct contact with the host cell cytosol (Fig. 4d, e). Furthermore, the CLEM approach revealed in great detail the presence of clusters of vesicles and membranous structures only in regions where Gal-3 was recruited close to Mtb. The ultrastructure of Gal-3-positive compartments is similar to that...
previously observed for Mtb residing in LC3\(^+\) compartments in lymphatic endothelial cells.\(^{63}\) We therefore investigated the recruitment of LC3B in relation to Gal-3 by live-cell imaging of Mtb-BFP-infected THP-1 macrophages expressing mNeonGreen-LC3B and Gal-3-mScarlet. Indeed, we observed recruitment of LC3B around intracellular Mtb shortly after recruitment of Gal-3 in 98% of events (Supplementary Movie 10), suggesting that ruptured Mtb phagosomes are targeted by autophagy\(^{64-66}\). To investigate if autophagic targeting of Mtb leads to formation of mature, acidified autophagosomes, THP-1 macrophages expressing Gal-3-mScarlet were labelled with LysoView633 (Supplementary Fig. 4a). Only ~20% of Mtb in ruptured phagosomes was later found in acidified compartments. Together with the observation by FIB-SEM that Mtb associated with Gal-3 phagosomes retained cytosolic contact, these results suggest that autophagosomal sequestration of Mtb after phagosomal rupture is inefficient (Supplementary Fig. 4b).

**Mtb inflammasome activation is independent of pH and cathepsins.** The ability to disrupt phagosomes seems important for inflammasome activation by Mtb, and it has been reported that phagosomal acidification is a prerequisite for the membrane-damaging activity of ESX-1 (refs. \(^{67-69}\)). In addition, active cathepsin release from ruptured phagolysosomes has been suggested as a trigger of NLRP3 inflammasome activation\(^{23,40,41}\). We therefore went on to investigate the possible involvement of phagosomal acidification and release of active cathepsins in inflammasome activation and pyroptosis by Mtb. Live-cell imaging of THP1-Gal-3-mScarlet or THP1-ASC-GFP cells in the presence of the pH-sensitive dye LysoView633 revealed that Mtb was equally efficient at escaping from acidified and neutral compartments (Fig. 5a–c; Supplementary Movies 11 and 12), suggesting that phagosomal acidification is not a prerequisite for Mtb escape into the cytosol. The LysoView signal in single cells was stable prior to ASC-speck formation, indicating that there is no general disruption or degradation of acidified lysosomes prior to inflammasome activation (Fig. 5d, e; Supplementary Movie 13). In addition, we did not see any effect on cell death or ASC-speck formation when cells were treated with Bafilomycin A1 (BafA1) which inhibits lysosomal acidification by V-ATPase, or the pan-cathepsin inhibitor K777, during infection (Fig. 5f), despite efficient cathepsin B inhibition (Supplementary Fig. 5). Compared with K777, cathepsin B inhibitor Ca-074-Me was less potent and had clear off-target effects on inflammasome activation during LPS and nigericin treatment (Supplementary Fig. 5).

These results demonstrate that Mtb causes inflammasome activation and pyroptosis independently of phagolysosomal acidification and release of active cathepsins.

**Mtb ESX-1 directly damages the host cell plasma membrane.** K\(^+\) efflux precedes NLRP3 activation for a range of known triggers\(^{27}\). Common for many of those triggers is that they permeabilise the PM to K\(^+\), e.g. by opening a membrane-resident pore (P2X7, pannexin-1) or by inserting and making new pores (Mixed Lineage Kinase Domain Like protein (MLK), complement, bacterial pore-forming toxins)\(^{70}\). We and others have shown that Mtb-induced NLRP3 inflammasome activation is dependent on K\(^+\) efflux, and it has also been shown that Mtb ESX-1 can be haemolytic\(^{71}\). Moreover, PM damage has been observed during Mtb infection, and PM repair protects macrophages from necrotic cell death\(^{72}\). However, whether Mtb could directly cause PM damage or if PM damage is a consequence of Mtb-induced intrinsic cell death pathways, was not addressed. To monitor PM integrity, we made THP-1 reporter cell lines with an mNeonGreen fluorescent protein tag on the Ca\(^2+\)-binding protein Apoptosis-Linked Gene 2 (ALG-2), which is recruited to sites of PM damage by calcium influx\(^{73,75}\). We imaged Mtb-BFP-infected THP-1 macrophages by time-lapse confocal microscopy and observed ALG-2 recruitment in infected cells, almost 90% of which occurred in close vicinity to Mtb bacteria (Fig. 6a, b; Supplementary Movies 14 and 15). Mtb infection also caused ALG-2 recruitment close to Mtb in primary human macrophages (Supplementary Fig. 6a). We observed ALG-2 recruitment near Mtb both without prior Gal-3 recruitment and subsequent to Gal-3 recruitment around Mtb, with a distribution close to 50/50 between the two categories (Fig. 6b). This result suggests that Mtb damages the host cell PM either during phagocytosis or following phagocytosis and phagosome rupture.

As damage to the PM from the cytosolic side by already-internalised bacteria has not been described before, we examined whether ALG-2 recruitment next to Gal-3+ Mtb compartments indeed occurred at the PM. Total internal reflection fluorescence (TIRF) microscopy is only sensitive to the region within ~100 nm from the substrate, meaning it specifically detects events occurring at the PM\(^{76}\). We imaged infected THP-1 macrophages with wide-field and TIRF microscopy simultaneously (Fig. 6c; Supplementary Movie 16). Since the TIRF microscope is outside the BSL3 facility, we used an Mtb auxotroph strain with intact ESX-1 function (Mtb mc\(^{2+}\)). From wide-field imaging, we observed Gal-3 recruitment to intracellular Mtb phagosomes that...
were out of range for TIRF, while ALG-2 recruitment was observed in TIRF and wide-field modes when Mtb came into the range of the TIRF excitation, demonstrating that ALG-2 recruitment following Gal-3 also occurs at the PM.

To better understand the extent of PM damage marked by ALG-2 events, we imaged cytosolic calcium levels using the fluorescent indicator Calbryte-590 during time-lapse microscopy of Mtb-infected THP-1 cells. We observed a consistent influx of calcium when ALG-2 was recruited (Fig. 6d, e; Supplementary Movie 17), which is expected considering that ALG-2 recruitment following Gal-3 also occurs at the PM.

To investigate the ultrastructure of Mtb-associated PM damage, we performed live-cell CLEM. After live-cell imaging and rapid fixation, recent ALG-2 recruitment events were imaged by confocal or Airyscan microscopy, and further by FIB tomography (Fig. 6h; Supplementary Fig. 6c). At the location of fluorescent ALG-2 signal and directly adjacent to Mtb, multiple 50–100-nm-sized vesicles were visible, indicative of ESCRT-mediated PM repair.

**Plasma membrane damage activates the NLRP3 inflammasome.** Based on the dependency of NLRP3 activation on K⁺ efflux as shown by us and others, we hypothesised that the ability of Mtb to cause PM damage and its subsequent permeabilisation to ions would be linked to inflammasome activation. We therefore imaged THP-1-ASC-mIRFP670 macrophages by time-lapse microscopy during infection with Mtb-BFP. An example time course is shown in Fig. 7a and Supplementary Movie 20. We recorded the time points of all Gal-3 (phagosomal rupture) and ALG-2 (PM damage) recruitment events in cells later forming ASC specks.
along with the time point of ASC-speck formation. PM damage preceded ASC-speck formation in 87% of cells infected with Mtb and occurred closer in time to ASC-speck formation than Gal-3 recruitment to Mtb phagosomes (Fig. 7b). While the median time from phagosomal rupture to ASC speck formation was 2 h, the median time from PM damage to ASC-speck formation was 1 h. Moreover, within 20 min before ASC-speck formation, 48% of cells had ALG-2 events and 23% of cells had Gal-3 events, compared with 1% and 4%, respectively, in infected control cells over an average 20-min period (Fig. 7c). Thus, especially PM damage events occur much more frequently before ASC specks form, connecting these events at the single-cell level. When cells
**Fig. 6** Mtb-carrying ESX-1 can directly damage the host cell plasma membrane. THP-1 macrophages expressing Gal-3 (magenta) and/or ALG-2 (green) were infected with Mtb-BFP (blue) and imaged by time-lapse microscopy and FIB-SEM. 

- **a** Time-lapse images showing ALG-2 recruitment to PM-Mtb contact points either independent of or after Gal-3 recruitment. Arrows point to recent Gal-3 or ALG-2 events, and circular insets highlight a bacterium that first ruptures its phagosome (Gal-3 recruitment) and then damages the PM (ALG-2 recruitment). Data representative of three independent experiments.
- **b** Quantification of localisation of ALG-2 events with respect to Mtb, and of ALG-2 events occurring independent of or after Gal-3 recruitment to ruptured Mtb phagosomes. \( n = 213 \) and \( n = 321 \) events from three independent experiments. Mean ± s.e.m. shown.
- **c** Simultaneous wide-field (WF, cell interior) and TIRF (plasma membrane) time-lapse imaging of ALG-2 and Gal-3 during Mtb infection. Representative of \( n > 10 \) experiments. To verify that ALG-2 recruitment is indicative of PM damage, \( \text{Ca}^{2+} \) and propidium iodide (PI) fluxes were monitored during Mtb infection. 
- **d** Time lapse of \( \text{Ca}^{2+} \) influx (Calbryte-590, grey) upon ALG-2 recruitment close to Mtb. Data representative of five independent experiments. 
- **e** Calbryte-590 signal over time during ALG-2 or Gal-3 recruitment (\( n = 10 \) events per condition). Median ± IQR shown.
- **f** Time lapse of PI influx during Mtb-localised ALG-2 recruitment. Arrows point to ALG-2 recruitment and PI influx events occurring subsequently in the same region of the cell. Data representative of two independent experiments.
- **g** PI signal and the cumulative PI influx in single cells during ALG-2 events (\( n = 55 \)) compared with neighbouring cells without ALG-2 events (\( n = 37 \)). Median ± IQR and median values shown of all PI influx events from one experiment. Data representative of two independent experiments. ****\( p < 0.0001 \) by two-sided Mann–Whitney test.

**Fig. 7** Plasma membrane damage by Mtb activates the NLRP3 inflammasome. THP-1 cells with ALG-2 (green), Gal-3 (magenta) and ASC (grey) were infected by Mtb-BFP (blue) and imaged live for up to 24 h. 

- **a** Time lapse of ALG-2, Gal-3 and ASC dynamics during Mtb infection, with the respective events indicated by arrows. Scale bar 10 µm. Data representative of three independent experiments.
- **b** Timing of Mtb-localised Gal-3 and ALG-2 events compared with ASC-speck formation. \( n = 200 \) ALG-2 and \( n = 262 \) Gal-3 events analysed from three independent experiments. Lines indicate median values. ***\( p < 0.0001 \) by two-sided Mann–Whitney test.
- **c** Percentage of pyroptotic cells with Gal-3 and ALG-2 events within 20 min before ASC-speck formation, compared with the percentage of cells in the surviving, infected population with events in an average 20-min period. \( n = 104 \) pyroptotic and \( n = 137 \) surviving cells from three independent experiments.
- **d** Percentage of ASC specks 24 h.p.i. in cells treated with the indicated concentration (in µM) of cytochalasin D. **\( p < 0.01 \) by one-way ANOVA with two-sided Dunnett’s test. \( n > 2000 \) cells per condition in triplicate, representative of two independent experiments. Mean ± s.e.m. shown.
- **e** Average number of Mtb-localised ALG-2 events per cell during 24 h of infection in the absence or presence of NLRP3 inhibitors. \( n = 732 \) events from three independent experiments, mean ± s.e.m. of five fields of view shown.
- **f** Quantification of cell death and ASC specks 24 h.p.i. in THP1-ASC-mNeonGreen (WT) cells and in cells depleted of the ESCRT-associated proteins ALG-2 or ALIX. Mean ± s.e.m. shown. \( n > 2000 \) cells per condition in triplicate, *\( p < 0.05 \), **\( p < 0.01 \) by one-way ANOVA with two-sided Dunnett’s test. Data representative of three independent experiments. Source data are provided as a Source Data file.
Both AIM2 and NLRP3 have been implicated in IL-1β release during Mtb infection of macrophages, while the occurrence of pyroptosis as a distinct route of cell death has been less clear. Here we establish that ESX-1-mediated PM damage causes K+ efflux, NLRP3 activation and subsequent caspase-1-mediated IL-1β release in THP-1 cells and human primary monocytes and macrophages. We also demonstrate at the single-cell level that NLRP3 activation causes rapid pyroptosis through caspase-1 and GSDMD in THP-1 cells. We emphasise that pyroptosis is an all-or-nothing response after ASC-speck formation, consistent with previous reports. In primary human monocytes and macrophages, NLRP3 inhibitors reduced cell death, although the dependence on caspase-1 was less clear for macrophages. It has previously been reported that monocytes require less stimulation than macrophages to activate an NLRP3-mediated inflammatory response, and PMA differentiation of THP-1 cells upregulates pro-inflammatory cytokines and NLRP3 pathway components, thus potentially making them more prone to activation of this pathway. In a recent report, even in response to the canonical NLRP3 trigger LPS and nigericin, a low proportion (<5%) of human macrophages formed ASC specks, ascribed to a regulatory role of alternative NLRP3 isoforms. Accordingly, the poorer inhibition of pyroptosis in macrophages than in monocytes/THP-1 is likely due to a lower frequency of macrophages responding with inflammasome activation and IL-1β release, which are the cells prone to undergo pyroptosis.

Furthermore, caspase inhibitors have differing efficacies in preventing pyroptosis, despite potent inhibition of IL-1β release and there is also crosstalk between cell death pathways and caspase redundancy during inflammasome activation. Together with long time courses of infection, these factors could contribute to why caspase-1 inhibitors are less efficient than NLRP3 inhibition in preventing primary cell death, which is consistent with previous reports of caspase-independent cell death during Mtb infection.

We show at the single-cell level that a high bacterial burden is linked to NLRP3 activation, and other groups have also shown the correlation between bacterial burden and cell death. One could hypothesise that pyroptosis would occur in vivo in tissue regions where Mtb replication results in high bacterial loads, such as in necrotising granulomas during active phases of the disease when Mtb needs to spread. Further, although IL-1β is a central cytokine in a successful immune response against tuberculosis, there is also crosstalk between cell death pathways and the inflammasome. Thus, further studies in relevant in vivo systems will be necessary to clarify the prevalence and importance of NLRP3 activation and the pyroptotic pathway during different stages of tuberculosis disease.

Necroptosis is another necrotic cell death pathway induced by Mtb, mainly caused by the secreted NAD+ glycohydrolase TNT. In some cases, there is crosstalk between necroptosis and NLRP3 activation and pyroptosis, but our results indicate that these are distinct processes during Mtb infection. We do not see an effect of necroptosis inhibitors or TNT-deficient mutants in THP-1 cells on NLRP3 activation, while in primary human monocytes and macrophages, cell death was reduced by RIPK3 inhibition, suggesting that both pathways are indeed activated in more physiological systems. The discrepancy between our results and those of Pajuelo et al. with regard to cell death by TNT and necroptosis inhibitors in THP-1 cells is likely due to the longer infection time used by Pajuelo and colleagues: our analyses are mostly performed after 24 h of infection where pyroptosis is most prominent, but we also see an increase in necrotic cell death at later time points. Mitochondrial damage and depolarisation have been linked to necrosis during Mtb infection, both necroptosis and other forms of necrosis. By time-lapse imaging, we show that destabilisation of mitochondria...
accompanied by pyroptosis rather than causing inflammasome activation in the NLRP3 pathway. In summary, our results highlight that Mtb-induced pyroptosis is an independent pathway distinct from other programmed necrotic pathways such as necroptosis, although both pathways and others can and likely do occur during Mtb infection in vivo. Which pathway is triggered first is likely determined both by the state of the host cell (e.g. activation state, immune environment or previous stimulation) and the nature of the damage by Mtb, where more severe damage causes K\(^+\) efflux and pyroptosis, while lesser damage could allow, e.g. TNT secretion and subsequent activation of necroptosis.

The precise mechanism of NLRP3 activation has evaded the community for over a decade. Although alternative routes were proposed, inhibition of NLRP3 activation by high concentrations of extracellular KCl positions K\(^+\) efflux as the predominant upstream effector of NLRP3 activation. This is also the case for Mtb, as we and others have shown\(^{40,96}\). The similarities in ASC-speck structure and progression of pyroptosis after NLRP3 activation by Mtb infection or nigericin, which is a K\(^+\)/H\(^+\) ionophore, suggest that differences are linked to the mechanism of K\(^+\) efflux. Lysosomal damaging agents are one of the most clinically relevant classes of NLRP3 triggers\(^{97}\). Cathepsin release from damaged lysosomes, especially of Cathepsin B, is one commonly proposed connection, although how this further causes K\(^+\) efflux has been unclear\(^{43,40,41}\). A main source of this confusion is the widespread use of the Cathepsin B inhibitor Ca-074-Me, which appears to inhibit NLRP3 activation independent of Cathepsin B inhibition\(^{37,42–44}\) (Supplementary Fig. 5). We show that although Mtb can damage acidified phagolysosomes, this is not required for NLRP3 activation, and there is no general loss of lysosomal content prior to NLRP3 inflammasome activation. Instead, PM damage appears to be the key event to trigger NLRP3 during Mtb infection. Also, in response to silica crystals, a canonical lysosome-damaging NLRP3 trigger, we observe PM damage prior to NLRP3 activation. PM permeabilisation can cause ion fluxes down their electrochemical gradients and thus activate NLRP3 by K\(^+\) efflux\(^{70}\).

PM damage during phagocytosis of Mtb and silica explains some of the NLRP3 activation events, but our imaging data show that PM damage can also occur from the cytosolic side after uptake, if Mtb or silica damage the phagosome and subsequently come into contact with the PM. This mechanism explains how something that primarily disrupts phagolysosomes can cause K\(^+\) efflux and NLRP3 activation. Strikingly, localised and controlled lysosomal damage caused by a lysosomal photosensitiser will not subsequently damage the PM and did not cause NLRP3 activation. Since PM damage is actively repaired through many routes, including the Ca\(^{2+}\)-dependent ESCRT machinery\(^{45,73–75}\), it is also interesting to note that this branch of ESCRT emerges as a suppressor of NLRP3 activation and cell death during Mtb infection. The ESCRT machinery was also recently shown to be involved in repair of phagosomes damaged by mycobacteria\(^{98,99}\).
and to regulate pyroptosis or necroptosis by repairing GSDMD with or without inhibitors, followed by washing in HBSS and replacement of the medium and rested 1 day prior to experiments. Primary human monocytes were differentiated at a concentration of 300,000 cells/mL in medium containing 10% fetal bovine serum, rhGM-CSF (50 ng/ml), and IL-4 (5 ng/ml) for 3 days, washed in cell medium and rested 1 day prior to experiments. Primary human monocytes were selected by plastic adherence of PBMCs for 3 h, followed by 3x washing in HBSS, and cultured in RPMI with 10% A+ serum (blood bank of St. Olav’s Hospital, Trondheim, Norway). For primary macrophage experiments, primary monocytes were selected by a CD14 bead isolation (Miltenyi Biotech) and seeded out in RPMI with 5% A+ serum and 50 ng/ml rhGM-CSF (Peprotech, Gibco-ThermoFisher) for 3 days. At the third day, the media was replaced with normal cell medium with 5% A+ serum.

**Methods**

**Reagents.** All reagents used in this paper are listed in Supplementary Table 1.

**Experimental models.** THP-1 cells (ATCC Cat#TIB-202, ASC-GFP from Prof. Emad Alnemri) were maintained in RPMI 1640 supplemented with 1-glutamine, 10 mM HEPES and 10% FCS, and passaged regularly to keep the cell density between 0.2 and 1 x 10^6/mL. Cell lines were routinely tested for mycoplasma.

Peripheral blood mononuclear cells (PBMCs) were isolated with lymphoprep from buffy coats obtained from healthy volunteers (both male and female) at the blood bank of St. Olav’s Hospital (Trondheim, Norway), or directly from the blood of healthy volunteers (both male and female) after informed consent. Collection of human blood was approved by the Regional Committee for Medical and Health Research Ethics in Central Norway.

Mtb strains were grown at 37 °C in Middlebrook 7H9 medium supplemented with 0.2% glycerol, 0.05% Tween-80 and oleic acid, albumin, dextrose and catalase (OADC). For Mtb mc2606 auxotroph strain, 50 µg/mL leucine and 24 µg/mL D-pantothenate were added to the growth medium. Mtb bacteria were grown in the absence of Tween-80 for 24 h prior to infection in experiments where no-tween growth conditions are indicated.

Lentiviral production was performed in HEK293T cells using 3rd generation packaging system pMDLg-pRRE, pMD2.G and pRSV-Rev and JetPrime (PolyPlus) as transfection reagents according to the manufacturer’s instructions. Virus-containing supernatant was harvested 2–3 days post transfection, and filtered through 0.45-µm PES membrane filters. THP-1 cells were transduced in the supernatant by 90 min of spinoculation at 32 °C, 1000 g in the presence of 8 µg/mL polybrene, and selected in 1 µg/mL puromycin or 100 µg/mL blasticidin or 100 µg/mL hygromycin for 1 week. Finally, the cells were selected for a consistent moderate expression level of fluorescent constructs by 1–2 rounds of FACS (BD Aria II).

CRISPR-modified THP-1 cells were used directly as a polyclonal population after puromycin selection. Mtb H37Rv, Mtb H37RvΔK1 and Mtb mc2606 were transformed with mps12:EBFP2 and selected on hygromycin (65 µg/mL) T710 plates (Difico/Becton Dickinson).

**Generation of new vectors.** Gateway cloning was used to generate lentiviral expression vectors. Human Galectin-3, human ALG-2 (THP-1 cDNA), human ASC, human LC3B (synthetic GeneArt Strings, Thermofisher), mScarlet (synthetic), mRuby3, mNeonGreen, mRFP670 and SNAP, tag were PCR-amplified and cloned into Gateway pEntry vectors. All pEntry constructs were verified by Sanger sequencing (GATC Biotech) before further use. PurOR in pLEX37 was replaced with BlastR or HygR to generate Gateway lentiviral destination vectors with the corresponding antibiotic resistance. Two-fragment Gateway recombination with pLEX37, pLEX37-Blast or pLEX37-Hyg was performed to generate ASC-mNeonGreen, ASC-mRFP670, Galectin-3-mScarlet, Galectin-3-SNAP, mNeonGreen-LC3B and mNeonGreen-ALG-2 constructs. Guide RNAs targeting GSDMD, NLRP3, ALG-2 or ALIX were cloned into LentiCrispr v2 by BsmBI digestion and ligation.

**Verification of CRISPR knockdown.** Knockdown in the cell pool was verified by cleavage assay (GeneArt genomic cleavage assay, Thermofisher), sequencing and TIDE analysis and/or by western blotting. Uncropped and unprocessed western blot scans are available in the Source Data File. Antibodies and dilutions for western blot were NLRP3 (1:1000), GSDMD (1:1000), ALG-2 (1:500) and ALIX (1:1000).

**Macrophage and monocyte infection and stimulation.** Before use, THP-1 cells were differentiated at a concentration of 300,000 cells/mL in medium containing 10% fetal bovine serum to MOI 20, unless otherwise indicated, assuming 1 OD600 = 10^8 bacteria/mL. For primary human monocyte experiments, the second centrifugation was done at 200 g for 1 min, and bacteria were resuspended in 5% A+ serum (blood bank of St. Olav’s Hospital, Trondheim, Norway). For primary macrophage experiments, primary monocytes were selected by a CD14 bead isolation (Miltenyi Biotech) and seeded out in RPMI with 5% A+ serum and 50 ng/mL rhGM-CSF (Peprotech, Gibco-ThermoFisher) for 3 days. At the third day, the media was replaced with normal cell medium with 5% A+ serum.

Mtb bacteria were grown until OD_600=0.4–0.5 (log phase), then pelleted at 2000 g for 10 min, resuspended in RPMI with 10% A+ serum to opsonise bacteria prior to infection and sonicated 2–3 times for 5 s at 70% power (Branson Digital Sonifier, S-450D). Clumped bacteria were removed by centrifuging at 300 g for 4 min. The supernatant containing bacteria was diluted in RPMI with 10% human A+ serum to MOI 20, unless otherwise indicated, assumed 1 OD_600=3 x 10^8 bacteria/mL. For primary human macrophage experiments, the first centrifugation was done at 200 g for 1 min, and bacteria were resuspended in 5% A+ serum (to reduce the background of the LDH readout). Bacteria were applied to cells for 45 min (THP-1 and monocytes) or 4 h (primary human macrophages) with or without inhibitors, followed by washing in HBSS and replacement of the medium to normal cell medium with 10% or 5% A+ serum with or without inhibitors, or Lebovitz L-15 CO2-independent medium with 10% A+ serum for live-cell imaging. The typical condition of MOI 20 infection gave ~50% infection rate with 1–20 bacteria per macrophage. Inhibitor concentrations were DMSO control (1:400), z-VAD-FMK (50 µM) for THP-1 cells, 20 µM for primary human cells, Z-VAD-FMK (50 µM), MCC950 (40 µM), VX765 (50 µM), GSK872 (5 µM), Cyclosporin A (5 µM), K777 (15 µM) or Bafilomycin A1 (50 nM). Supernatants were harvested for ELISA or LDH assays after 24 h, and analysed by
human IL-1β kit or LDH cytotoxicity kit according to the manufacturer’s instructions.

In all incubation experiments, the following conditions were used: LPS (10 ng/ml), LPS, 3 h), FSL-1 (10 ng/ml), Pam3Cys4K (10 ng/ml), nigericin (10 µM, 1 h, unless otherwise stated), imiquimod (20 µg/ml, 1 h) and silica (100–200 µg/ml). When used in combination, inhibitors were added 30 min before and were present during stimulation.

**Live-/fixed-cell imaging.** THP-1 cells were seeded in 35-mm glass-bottom dishes (Ibidi) or 96-well glass-bottom plates (Cellvis) as above. For some experiments, bacteria were incubated for 30 min prior to staining to ensure cellular association. For some experiments, cell medium was only preincubated and not re-added. In cells with SNAP tag, cells were labelled with SNAP-Cell 647-SiR for 15 min, washed 3× in cell medium and rested for 30 min before fixation. For quantification of cell death, CellTiter-Blue (10 nM), LysoView633 (1:10 000) or Calbryte-590 (5 µM) was preincubated for 15 min. After fixation, samples were imaged on a Zeiss Laser TIRF 3, with a 63 × 1.46 oil objective and Hamamatsu EMX2 EMCCD camera. For quantification of cell death, (by DRAQ7 signal) and ASC specks at defined time points with and without inhibitors or knockdown, 16 fields of view containing n ≥ 2000 cells in total were imaged per condition in triplicate. For time-lapse imaging by confocal microscopy, typically six fields of view comprising >500 cells in total were imaged at 30–45 s intervals, while TIRF/FI was done on one to four fields of view comprising 10–40 cells at 30–50 s intervals. Fields of view were defined based on plate coordinates, without bias from observation of the sample, and all cells or events within every captured field of view were included in the analysis. For some experiments, DRAQ7 (0.15 µM), TMRE (10 nM), LysoView633 (1:10 000) or Calbryte-590 (5 µM) was preincubated for 30–60 min in cell medium, 1× and rested for 15 min before fixation. Cells with cell death were only preincubated and not re-added. In cells with SNAP tag, cells were labelled with 1:200 SNAP-Cell 647-SiR for 15 min, washed 3× in cell medium and rested for 30 min before fixation. For lysosomal damage by photosensitizer, TPC_2 was added to the medium (10 µM) for 16 h, and cells were washed and incubated in fresh medium without TPC_2. THP-1 cells were stained or not stained with Cresyl Violet (1 µM, 5 min) exposed to 405-nm light (10%, 1 s), and imaged. Galectin-3 accumulation on phagosomes during infection with different Mtb strains was determined by fixing cells in 4% PFA for 20 min at 1, 4 and 24 h post infection, and imaging 16 fields of view with 40× objective per well. Caspase-1 activity was detected in monocytes using FAM-FLICA Caspase-1 Assay kit according to the manufacturer’s instructions.

**Immunofluorescence analysis of ALG-2 in primary macrophages.** Primary human macrophages were isolated, differentiated and infected with Mtb auxotroph (Mtb H37Rv mc26206::EBFP2) as previously described. Cells were fixed on ice 2 h 15 min post infection in methanol-acetone solution (50% methanol and 50% acetone). Cells were immediately stored at −20 °C. The following day, the fixative was exchanged and cells were added to the cells for 1 h at room temperature. Blocking was done in PBS with 20% human serum for 20 min. Cells were incubated with ALG-2 antibody (1:50 in PBS with 20% human serum) overnight at 4 °C, with the second antibody Alexa Fluor 647 (1:500 in PBS with 20% human serum) for 1 h and finally washed in PBS × 3. Images were acquired using a Zeiss LSM880 microscope and fluorescent images were deconvolved using Huygens Professional v18.10 (Scientific Volume Imaging, The Netherlands, http://svi.nl).

**FIB-SEM sample preparation and correlative imaging.** For correlative imaging experiments, two growth substrates were used, with different optical microscopy and fixation approaches. In the first approach, THP1-ASC-GFP Gal-3-RFP cells were seeded and infected as above on aclar slides micropatterned by thermocuring aclar substrates were placed in 24-well plates and imaged live using 10× objective as above. After 24 h, cells were fixed in 2.5% glutaraldehyde in 100 mM PIPES for 1 h, placed upside-down in 35-mm glass-bottom dishes and cells of interest were re-imaged using 63 × 1.2 W objective on a Zeiss LSM880 microscope and fluorescent images were deconvolved by linear unmixing due to substantial cross-excitation of mScarlet with ALG-2 after 2 h 15 min post infection in methanol. After fixation, cells were immersed in 1 mM EDTA, 1 mM EGTA and 0.5 mM Pefabloc SC, pH 7.5) on ice for 20 min. In total, 50 µl of lysate was mixed with 50 µl of Cathespin B reaction buffer (50 mM sodium acetate, 4 mM EDTA, 0.5 mM Pefabloc SC, 8 mM dithiothreitol (DTT) and 50 µM Z-RR-AMC, pH = 6.0), incubated at 30 °C for 5 min and fluorescence intensity was monitored every 30 s for 20 min (exc 355/em 460 nm, POLARStar Omega microplate reader).

**Image analysis.** Cell death pathways (Fig. 1) were identified according to the following guidelines: pyroptotic cells were characterised as those forming a visible ASC speck followed by rapid influx of DRAQ7, while necrotic cells did not form ASC specks and generally followed a slower progression with, e.g., cessation of cell migration prior to a slower influx of DRAQ7. Apoptotic cells were identified by a characteristic membrane blebbing and no DRAQ7 influx. CellProfiler was used to unmix images and count live cells and ASC specks based on the ASC-GFP signal, dead cells based on the DRAQ7 signal and Mtb uptake based on Mtb-BFP signal overlapping with ASC-GFP signal in live cells. Bacterial burden per cell was also measured from live-cell time-lapse movies by mean Mtb-BFP intensity in infected cells immediately prior to ASC speck formation, and compared with cells surviving the duration of the experiment. The time point for the control cells was chosen to be the average time point for ASC-speck induction. Traces of DRAQ7, TMRE, Calbryte-590, PI or LysoView signal were generated by manually selecting ROIs around cells with or without ASC specks forming and measuring the mean intensity in the ROI over time. The data were aligned according to the time point of the first visible sign of the event under consideration and plotted as mean ± IQR.

In fixed cells images of Galectin-3 colocalisation with Mtb, the colocalisation was scored by a trained model in Imaris and further analysed by CellProfiler. For analysis of ALG-2 recruitment events compared with Gal-3 recruitment events, only events where a complete overlap between increased Gal-3 signal and Mtb was followed by an overlap with an increased ALG-2 signal were scored as “ALG-2 after Gal-3” events. Data were plotted and statistically analysed using Python or GraphPad Prism, while images and figures were prepared using Fiji and Adobe Illustrator. TIRF/WF images of mNeonGreen-ALG-2 and Galectin-3 mScarlet were unmixed by linear unmixing due to substantial cross-excitation of mScarlet with 488-nm laser.

**Statistical analysis.** Detailed statistical analyses for individual experiments are listed in each figure legend. This includes the statistical test performed, the parameters shown and number of cells, replicates and independent experiments as appropriate. Data comparing the mean values of technical replicates of representative or independent experiments were analysed by two-sided unpaired Student’s t test (two groups) or one-way ANOVA (more groups). For comparisons of non-parametric data (fluorescent intensity or event timing), Mann-Whitney U test was used. GraphPad Prism 8.0 was used to perform all statistical analyses and determine p values, with p value <0.05 considered significant.

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

**Data availability**. All data are available from the corresponding author upon reasonable request. The source data underlying Figs. 1, 2f, 3a, c, e, 4b, c, 5a, 6b, e, 7a, b are provided as a Source Data file.

**Code availability.** The Python scripts for aligning intensity time-trace data and the CellProfiler and Ilastik pipelines used in this study are available from the corresponding author upon reasonable request.
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References
1. World Health Organization. Global tuberculosis report 2019 (WHO, 2019).
2. Dorhoi, A. & Kaufmann, S. H. E. Pathology and immune reactivity: understanding multidimensionality in pulmonary tuberculosis. Semin. Immunopathol. 38, 153–166 (2016).
3. Berge, T., Vanden, Linkernann, A., Jouan-Lhanouet, S., Walczak, H. & Vandenabeele, P. Regulated necrosis: the expanding network of non-apoptotic cell death pathways. Nat. Rev. Mol. Cell Biol. 15, 135–147 (2014).
4. Galluzzi, L. et al. Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. Cell Death Differ. 25, 486–541 (2018).
5. Rathinam, V. A. K. & Fitzgerald, K. A. Inflammation: the molecular activation and regulation to therapeutics. Nature 486, 477–489 (2019).
6. Ding, J. et al. Pore-forming activity and structural autoinhibition of the gasdermin D peptidoglycan receptor. Nature 580, 678–682 (2020).
7. Zhou, R., Yazdi, A. S., Menu, P. & Tschopp, J. A role for mitochondria in Gout–Pyroptosis: the in vivo role of ASC for NLRP3 inflammasome activation. J. Immunol. 187, 4744–4753 (2011).
8. Saiga, H. et al. Critical role of AIM2 in Mycobacterium tuberculosis infection. Infect. Immunol. 79, 1371–1384 (2011).
9. Lee, J. P. et al. Cutting edge: caspase-1 independent IL-1 production is critical for host resistance to Mycobacterium tuberculosis and does not require TLR signaling in vivo. J. Immunol. 184, 3326–3330 (2010).
10. Horio, T. et al. Activation of the NLRP3 inflammasome by Mycobacterium tuberculosis is uncoupled from susceptibility to active tuberculosis. Eur. J. Immunol. 42, 374–384 (2012).
11. Hornung, V. et al. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature 458, 514–518 (2009).
12. Bürckstümmer, T. et al. An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. Nat. Immunol. 10, 266–272 (2009).
13. Fernandes-Alnemri, T., Yu, J.-W., Datta, P., Wu, J. & Alnemri, E. S. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. Nature 458, 509–513 (2009).
14. Martino, F., Petrelli, V., Mayor, A., Tardivel, A. & Tschopp, J. Gout–Pyroptosis: the in vivo role of ASC for NLRP3 inflammasome activation. Nature Comm. 10, 2270 (2019).
15. Zhou, R., Yazdi, A. S., Menu, P. & Tschopp, J. A role for mitochondria in Gout–Pyroptosis: the in vivo role of ASC for NLRP3 inflammasome activation. J. Immunol. 187, 4744–4753 (2011).
16. Saiga, H. et al. Critical role of AIM2 in Mycobacterium tuberculosis infection. Infect. Immunol. 79, 1371–1384 (2011).
17. Mayer-Barber, K. D. et al. Cutting edge: caspase-1 independent IL-1 production is critical for host resistance to Mycobacterium tuberculosis and does not require TLR signaling in vivo. J. Immunol. 184, 3326–3330 (2010).
18. Horio, T. et al. Activation of the NLRP3 inflammasome by Mycobacterium tuberculosis is uncoupled from susceptibility to active tuberculosis. Eur. J. Immunol. 42, 374–384 (2012).
19. Hornung, V. et al. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature 458, 514–518 (2009).
20. Bürckstümmer, T. et al. An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. Nat. Immunol. 10, 266–272 (2009).
21. Fernandes-Alnemri, T., Yu, J.-W., Datta, P., Wu, J. & Alnemri, E. S. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. Nature 458, 509–513 (2009).
22. Martino, F., Petrelli, V., Mayor, A., Tardivel, A. & Tschopp, J. Gout–Pyroptosis: the in vivo role of ASC for NLRP3 inflammasome activation. Nature Comm. 10, 2270 (2019).
23. Zhou, R., Yazdi, A. S., Menu, P. & Tschopp, J. A role for mitochondria in Gout–Pyroptosis: the in vivo role of ASC for NLRP3 inflammasome activation. J. Immunol. 187, 4744–4753 (2011).
24. Saiga, H. et al. Critical role of AIM2 in Mycobacterium tuberculosis infection. Infect. Immunol. 79, 1371–1384 (2011).
25. Mayer-Barber, K. D. et al. Cutting edge: caspase-1 independent IL-1 production is critical for host resistance to Mycobacterium tuberculosis and does not require TLR signaling in vivo. J. Immunol. 184, 3326–3330 (2010).
26. Horio, T. et al. Activation of the NLRP3 inflammasome by Mycobacterium tuberculosis is uncoupled from susceptibility to active tuberculosis. Eur. J. Immunol. 42, 374–384 (2012).
27. Hornung, V. et al. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. Nature 458, 514–518 (2009).
28. Bürckstümmer, T. et al. An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. Nat. Immunol. 10, 266–272 (2009).
29. Fernandes-Alnemri, T., Yu, J.-W., Datta, P., Wu, J. & Alnemri, E. S. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. Nature 458, 509–513 (2009).
30. Martino, F., Petrelli, V., Mayor, A., Tardivel, A. & Tschopp, J. Gout–Pyroptosis: the in vivo role of ASC for NLRP3 inflammasome activation. Nature Comm. 10, 2270 (2019).
31. Zhou, R., Yazdi, A. S., Menu, P. & Tschopp, J. A role for mitochondria in Gout–Pyroptosis: the in vivo role of ASC for NLRP3 inflammasome activation. J. Immunol. 187, 4744–4753 (2011).
32. Zhao, X. et al. Bcl-xL mediates RIPK3-dependent necrosis in nutrient-deprivation-induced necrotic host cell death by abrogating mitochondrial membrane permeability transition. Nat. Commun. 10, 688 (2019).
60. Chen, M., Gan, H. & Remold, H. G. A mechanism of virulence: virulent Mycobacterium tuberculosis strain H37Rv, but not attenuated H37Ra, causes significant mitochondrial inner membrane disruption in macrophages leading to necrosis. J. Immunol. 176, 3707–3716 (2006).
61. Scaduto, R. C. & Grottyehann, L. W. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. Biophys. J. 76, 469–477 (1999).
62. Paz, I. et al. Galectin-3, a marker for vacuole lysis by invasive pathogens. Cell. Microbiol. 12, 530–544 (2010).
63. Lerner, T. R. et al. Lymphoid endothelial cells are a replicative niche for Mycobacterium tuberculosis. J. Clin. Invest. 126, 1093–1108 (2016).
64. Gutierrez, M. G. et al. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell 119, 753–766 (2004).
65. Watson, R. O., Mananullo, P. S. & Cox, J. X. Extracellular M. tuberculosis DNA targets bacteria for autophagy by activating the host DNA-sensing pathway. Cell 150, 803–815 (2012).
66. Watson, R. O. et al. The cytosolic sensor cGAS detects Mycobacterium tuberculosis DNA to induce type I interferons and activate autophagy. Cell Host Microbe 17, 811–820 (2015).
67. De Jonge, M. I. et al. ESAT-6 from Mycobacterium tuberculosis dissociates from its putative chaperone CFP-10 under acidic conditions and exhibits membrane-lysing activity. J. Bacteriol. 189, 6028–6034 (2007).
68. Simeone, R. et al. Cytosolic access of Mycobacterium tuberculosis: critical impact of phagocytosis acidification control and demonstration of occurrence in vivo. PLoS Pathog. 11, e1004650 (2015).
69. De Leon, J. et al. Mycobacterium tuberculosis ESAT-6 exhibits a unique membrane-interacting activity that is not found in its ortholog from non-pathogenic Mycobacterium smegmatis. J. Biol. Chem. 287, 44184–44191 (2012).
70. Gong, T., Yang, Y., Jin, T., Jiang, W. & Zhou, R. Orchestration of NLRP3 inflammasome activation by ion fluxes. Trends Immunol. 39, 393–406 (2018).
71. Conrad, W. H. et al. Mycobacterial ESX-1 secretion system mediates host cell lysis through bacterium contact-dependent gross membrane disruptions. Proc. Natl Acad. Sci. USA 114, 1371–1376 (2017).
72. Divangahi, M. et al. Mycobacterial tuberculosis evades macrophage defenses by inhibiting plasma membrane repair. Nat. Immunol. 10, 899–906 (2009).
73. Scheffer, L. L. et al. Mechanism of Ca2⁺-triggered ESCRT assembly and regulation of cell membrane repair. Nat. Commun. 5, 5646 (2014).
74. Jimenez, A. J. et al. ESCRT machinery is required for plasma membrane repair. Science 343, 1247136 (2014).
75. Sønder, S. L. et al. Annexin A7 is required for ESCRT III-mediated plasma membrane repair. Cell 169, 2156–2164 (2017).
76. Mattheyses, A. L., Simon, S. M. & Rappoport, J. Z. Imaging with total internal reflection fluorescence microscopy for the cell biologist. J. Cell Sci. 123, 3621 (2010).
77. Hanson, P. L., Roth, R., Lin, Y. & Heuser, J. E. Plasma membrane deformation by circular arrays of ESCRT-III protein filaments. J. Cell Biol. 180, 389–402 (2008).
78. Konečný, P., Ehrlich, R., Gulumian, M. & Jacobs, M. Immunity to the dual threat of silica exposure and Mycobacterium tuberculosis. Front. Immunol. 9, 3069 (2019).
79. Berg, K. et al. Disulfonated tetraphenyl chlorin (TPCS2a), a novel photosensitizer developed for clinical utilization of photochemical internalization. Photochem. Photobiol. Sci. 10, 1637 (2011).
80. Zeng, X. et al. Autophagy regulates cell death and its consequences. Cell 169, 2156–2164 (2017).
81. Tu, D. & Vincen, J. E. Pyroptosis versus necroptosis: similarities, differences, and crosstalk. Cell Deathiffer. 26, 99–114 (2019).
82. Taabazuing, C. Y., Okondu, M. C. & Bachovchin, D. A. Pyroptosis and apoptosis pathways engage in bidirectional crosstalk in monocytes and macrophages. Cell Chem. Biol. 24, 507–514 (2017).
83. Orning, P. et al. Pathogen blockade of TAK1 triggers caspase-8-dependent cleavage of gasdermin D and cell death. Science 362, 1064–1069 (2018).
84. Antoneopoulos, C. et al. Caspase-8 as an effector and regulator of NLRP3 inflammasome signaling. J. Biol. Chem. 290, 20167–20184 (2015).
85. Mahamed, D. et al. Intracellular growth of Mycobacterium tuberculosis after macrophage cell death leads to serial killing of host cells. eLife 6, e22028 (2017).
86. Repasy, T. et al. Intracellular bacillary burden reflects a burst size for Mycobacterium tuberculosis in vivo. PLoS Pathog. 9, e1003190 (2013).
87. Mayer-Barber, K. D. & Yan, B. Clash of the cytokine titans: counter-regulation of interleukin-1 and type I interferon-mediated inflammatory responses. Cell. Mol. Immunol. 14, 22–35 (2017).
88. Abate, E. et al. Polymorphisms in CARD8 and NLRP3 are associated with extrapulmonary TB and poor clinical outcome in active TB in Ethiopia. Sci. Rep. 9, 3126 (2019).
89. Gutierrez, K. D. et al. MLK1 activation triggers NLRP3-processed and release of IL-1β independently of gasdermin-D. J. Immunol. 198, 2156–2164 (2017).
90. Gao, L.-Y. et al. A mycobacterial virulence gene cluster extending RD1 is required for cytolsis, bacterial spreading and ESAT-6 secretion. Mol. Microbiol. 53, 1677–1693 (2004).
91. Smith, J. et al. Evidence for pore formation in host cell membranes by ESX-1-secreted ESAT-6 and its role in Mycobacterium marinum escape from the vacuole. Infect. Immun. 76, 5478–5487 (2008).
92. Kaufmann, S. H. E., Dorhoi, A., Hotchkiss, R. S. & Bartenschlager, R. Host-directed therapies for bacterial and viral infections. Nat. Rev. Drug Discov. 17, 35–56 (2018).
93. Tiscornia, G., Singer, O. & Verma, I. M. Production and purification of lentiviral vectors. Nat. Protoc. 1, 241–245 (2006).
94. Brinkman, E. K., Chen, T., Amendola, M. & van Steenel, B. Easy quantitative assessment of genome editing by sequence trace decomposition. Nucleic Acids Res. 42, e168–e168 (2014).
95. Jäättelä, M. & Nylandsted, J. Quantification of lysosomal membrane permeabilization by cytosolic cathepsin and β-N-Acetyl-glucosaminidase activity measurements. Cold Spring Harb. Protoc. 11, 1017–1023 (2015).

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Competing interests
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Additional information

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