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

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Graphical abstract
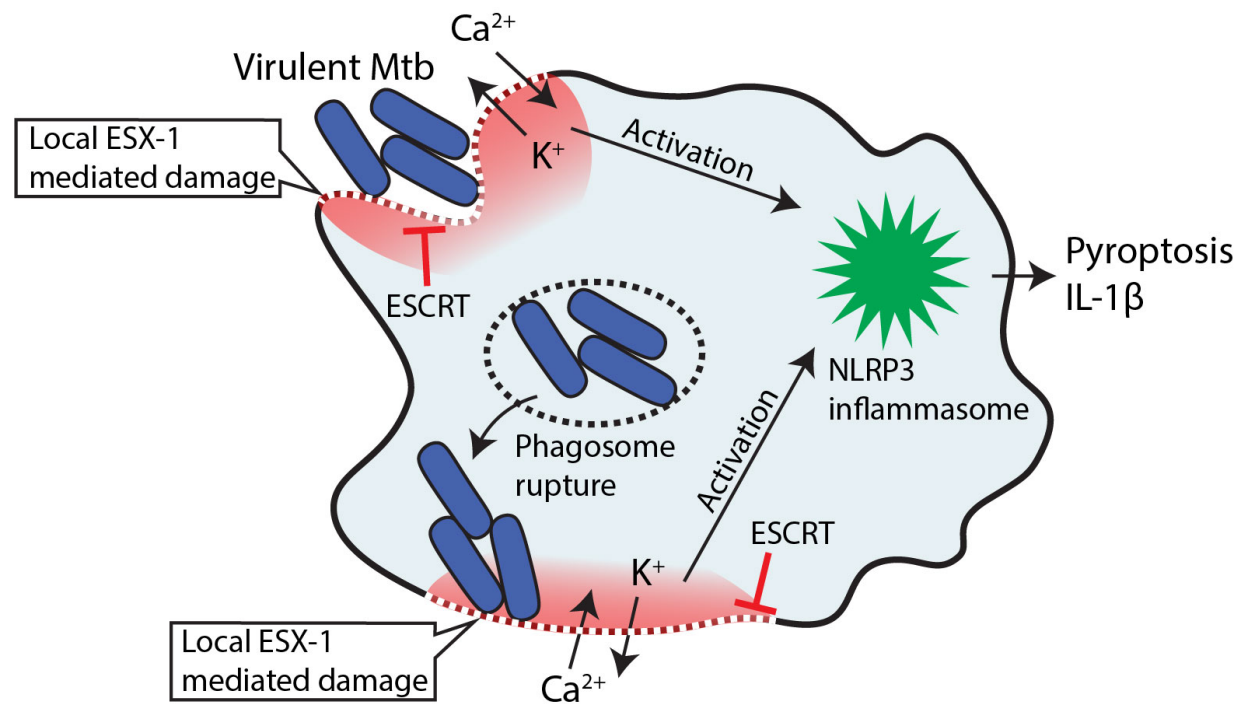
Abstract

Mycobacterium tuberculosis (Mtb) is a major global health problem and causes extensive cytotoxicity in patient cells and tissues. Here we define an NLRP3, caspase-1 and gasdermin D-mediated pathway to pyroptosis in human monocytes following exposure to Mtb. We demonstrate an ESX-1 mediated, contact-induced plasma membrane (PM) damage response that occurs during phagocytosis or from the cytosolic side of the PM after phagosomal rupture in Mtb infected cells. This PM injury in turn causes K+ efflux and activation of NLRP3 dependent IL-1β release and pyroptosis, facilitating the spread of Mtb to neighbouring cells. Further we reveal a dynamic interplay of pyroptosis with ESCRT-mediated PM repair. Collectively, these findings reveal a novel mechanism for pyroptosis and spread of infection acting through dual PM disturbances both during and after phagocytosis. We also highlight dual PM damage as a common mechanism utilized by other NLRP3 activators that have previously been shown to act through lysosomal damage.

Keywords

Mycobacterium tuberculosis, NLRP3 inflammasome, pyroptosis, gasdermin D, ESCRT membrane repair, human macrophages, CLEM, host-pathogen, infection biology

1 **Introduction**

2 *Mycobacterium tuberculosis* (Mtb) is a deadly human pathogen, causing about 1,6 million deaths

3 per year [1]. A pathological hallmark of Mtb infection is extensive necrosis in infected tissues [2].

4 Necrosis has long been regarded as an unregulated type of cell death, but recently several

5 programmed necrotic pathways have been identified [3,4]. A highly inflammatory form of

6 programmed necrosis is pyroptosis, occurring mainly in myeloid cells after pattern-recognition

7 receptor (PRR) activation. In the classical pathway, activation of nucleotide-binding

8 oligomerization domain-like receptors (NLRs) or absent in myeloma 2 (AIM2)-like receptors

9 (ALRs) by pathogen- or self-ligands drives the macromolecular assembly of an inflammasome

10 consisting of oligomerized NLRs or ALRs, the adaptor apoptosis-associated speck-like protein

11 containing a CARD (ASC), and caspase-1 [5–7]. Autocatalytic activation and cleavage of caspase-1

12 enables cleavage of pro-inflammatory cytokines interleukin (IL)-1β and IL-18, as well as the

13 pore-forming molecule gasdermin D (GSDMD) [8,9]. IL-1β is released through GSDMD pores and

14 in larger amounts during pyroptosis, the lytic cell death that often follows GSDMD pore

15 formation [10–13].

16

17 Canonical NLRP3 and AIM2 inflammasome activation has been implicated in IL-1β release

18 during Mtb infection in mouse and human macrophages [14–19]. The agonist of AIM2 is double-

19 stranded DNA [20–22] while the direct agonists of NLRP3 are not known. With few exceptions, two

20 steps are required for NLRP3 activation: The priming signal involves increased expression of

21 pro-IL-1β as well as inflammasome components such as NLRP3 itself, while the second signal is

22 characterized by a range of cell damage events such as potassium (K+) and chloride (Cl-) efflux,

23 mitochondrial dysfunction, metabolic changes, calcium fluxes, *trans*-Golgi disassembly and

4

24   lysosomal damage [7,23,32,24–31]. K+ efflux in particular is considered a key determinant of NLRP3

25   activation for a range of triggers [28] with some exceptions involving mitochondrial disruption and

26   mtROS production, e.g. by the small-molecule compound Imiquimod [29]. Mitochondrial

27   dysfunction seems closely tied to Mtb-induced necrosis as well [33,34], but whether this is related to

28   inflammasome activation is not clear. Inflammasome activation and pyroptosis by Mtb is

29   dependent on the type VII secretion system 6-kDa early secretory antigenic target (ESAT-6)

30   secretion system 1, ESX-1. ESX-1 secretes a range of protein substrates, and is postulated to

31   mediate phagosome permeabilization with full or partial translocation of Mtb into the cytosol [35–

32   38]. Phagosomal permeabilization presumably allows the release of Mtb DNA and activation of

33   AIM2 [19], and release of active cathepsin B from damaged phagolysosomes has been proposed as

34   a trigger for NLRP3 [24,39,40]. However, off target inhibitor effects and negative results in knockout

35   experiments have left the jury open on the role of cathepsin release in NLRP3 inflammasome

36   activation [28,41–43].

37

38   Here, we use time-lapse- and correlative microscopy to spatiotemporally resolve

39   Mtb-induced inflammasome activation and cell death at a single-cell level. We show that contact-

40   mediated PM damage caused by ESX-1 activity occurring either during phagocytosis, or from the

41   cytosol after phagosomal escape, drives K+ efflux and subsequent NLRP3 activation and

42   GSDMD-dependent pyroptosis of human macrophages. This mechanism is not unique to Mtb:

43   Our findings identify a common mechanism where PM damage caused by Mtb or crystals

44   triggers inflammasome activation, IL-1β release and pyroptosis unless the membrane damage is

45   balanced by repair mediated by the endosomal sorting complexes required for transport (ESCRT)

46   machinery [44].

47   **Results**

48   **Mtb H37Rv infection induces canonical NLRP3 activation followed by pyroptosis**

49   Based on previously published findings [15–19], we hypothesized that Mtb induces the assembly of

50   one of the ASC-dependent inflammasomes, NLRP3 or AIM2, upon infection of macrophages.

51   THP-1 macrophages expressing ASC-GFP were infected with Mtb H37Rv constitutively

52   expressing BFP (Mtb::BFP) and imaged after 24 hours or by time-lapse microscopy. The cell-

53   impermeable DNA dye DRAQ7 was present in the medium to assess cell death. ASC-GFP is

54   evenly distributed in the cytosol and appears as diffuse green until inflammasome activation

55   induces ASC aggregation, visible as bright specks [45]. Mtb induced ASC speck formation in

56   infected macrophages (Figure 1a and b) and specks were exclusively localized to dead cells as

57   indicated by DRAQ7-positive nuclei. The number of dead cells, ASC specks and IL-1β secretion

58   increased with increasing multiplicity of infection (MOI, Figure S1a). Treatment with the specific

59   NLRP3 inhibitor MCC950 [46] or increased extracellular concentrations of KCl inhibited the

60   formation of ASC specks, demonstrating that Mtb infection results in potassium efflux-driven

61   activation of the NLRP3 inflammasome (Figure 1b). Correspondingly, IL-1β secretion was

62   inhibited by MCC950 and KCl as well as the caspase inhibitors Z-VAD-FMK (pan-caspase) or

63   VX765 (caspase-1). Treatment with Z-VAD, VX765, MCC950 or KCl all reduced cell death

64   measured after 24 hours. Knocking down NLRP3 using CRISPR-Cas9 similarly reduced Mtb-

65   induced cell death and completely inhibited ASC speck formation (Figure 1c and Figure S1b).

66   Similar inhibitor- and NLRP3 KD results were obtained with the canonical NLRP3

67   inflammasome-inducing combination LPS and nigericin (Figure S1c-d). NLRP3-dependent IL-1β

68   release and cell death was verified in Mtb-infected primary human monocytes (Figure 1d),

69   indicating the physiological relevance of these findings. Live-cell time-lapse microscopy of

70   single Mtb-infected cells revealed a nuclear DRAQ7 signal within few minutes after ASC speck

6

71   formation, while the DRAQ7 signal remained low after ASC speck formation in THP-1 cells

72   deficient in GSDMD (Figure 1e, Figure S1b). Together our data demonstrate that Mtb induces

73   potassium efflux-driven activation of the canonical NLRP3 inflammasome upon infection of

74   human monocytes and macrophages, followed by GSDMD-dependent pyroptotic cell death with

75   release of IL-1β. The results also confirm ASC speck formation as a representative readout for

76   inflammasome activation by Mtb.

77

78   To gain a better understanding of the kinetics of pyroptosis caused by Mtb, we imaged THP1-

79   ASC-GFP cells infected with Mtb-BFP for 40 hours by time-lapse microscopy. We noted the

80   cumulative number of cell deaths by pyroptosis, apoptosis and necrosis, scored as illustrated in

81   Figure 1f and corresponding Movies S1, S2 and S3. Pyroptotic cells were characterized as those

82   forming a visible ASC speck followed by rapid influx of DRAQ7, while necrotic cells did not

83   form ASC specks and generally followed a slower progression with e.g. cessation of cell

84   migration prior to a slower influx of DRAQ7. Apoptotic cells were identified by a characteristic

85   blebbing morphology and no DRAQ7 influx. Pyroptosis was the dominating form of cell death

86   during the first 24 hours of infection. In line with this result, the necroptosis inhibitors Nec1s

87   (RIPK1 inhibitor) and GSK'872 (RIPK3 inhibitor) did not affect cell death or ASC speck

88   formation after 24 hours of infection (Figure S1e). Pyroptosis and necrosis primarily occurred in

89   infected cells, while the proportion of apoptotic cell death was larger in uninfected bystander

90   cells (Figure 1f). Additionally, the bacterial burden was higher in cells that formed ASC specks

91   than in infected cells that did not (Figure 1g). These results indicate that pyroptosis from Mtb

92   infection is a cell-intrinsic effect, i.e. a direct result of infection within single cells.

93

94   We reasoned the relative reduction in pyroptosis and increase in necrosis from 24-48 could be

95   due to prolonged stimulation of Toll-like receptor (TLR) signalling, which has been reported to

96   inhibit NLRP3 inflammasome activation [47]. Indeed, we observed that prolonged treatment (24h)

97   of THP-1 cells with TLR2 or 4 ligands prior to infection with Mtb decreased cell death and ASC

98   speck formation to a similar extent as treatment with MCC950 (Figure S1f).

99

100 **Pyroptosis causes severe cellular damage and allows for spread of Mtb**

101 Cell death is accompanied with morphological changes that can inform about which processes are

102 involved in execution of the particular cell death pathway. We investigated the ultrastructural

103 features of pyroptosis by correlative 3D light and electron microscopy (CLEM). Focused Ion

104 Beam -Scanning EM (FIB-SEM) was used to gain ultrastructural information with near-isotropic

105 3D resolution, using a correlation system we developed previously [48]. Uninfected THP1-ASC-

106 GFP cells displayed a normal, healthy morphology (Figure 2a). In comparison, infected,

107 pyroptotic cells (i.e. with visible ASC specks) had severe ultrastructural disruptions, including

108 fragmented and occasionally split nuclear membranes, large PM disruptions and leakage of

109 content to the extracellular space. Most organelles were no longer recognizable, with the

110 exception of mitochondria that could be discerned by their internal cisterna. The mitochondria

111 displayed a more rounded and less interconnected morphology than in the healthy control cell.

112 Mtb displayed a largely intact morphology, and some bacteria were observed in contact with the

113 extracellular space, suggesting a means of escape from pyroptotic cells. Cells that were treated

114 with LPS and nigericin and formed ASC specks displayed similar ultrastructural features as Mtb-

115 infected pyroptotic cells, with nuclear and PM disruptions, cytosol leakage and organelle damage

116 (Figure S2a).

117

118   To distinguish morphological changes that occurred as a consequence of pyroptosis from those

119   caused by Mtb infection prior to or during inflammasome activation, we infected cells for 5h with

120   Mtb-BFP in the presence of the caspase inhibitors Z-VAD and VX765. Cells were imaged live

121   during infection with DRAQ7 in the medium, and chemically fixed within two minutes of the last

122   captured frame. Cells with an ASC speck but without influx of DRAQ7 and no visible changes to

123   cell morphology were chosen for further imaging by FIB-SEM. The morphology was similar to

124   uninfected cells, with intact organelles (Golgi, ER, interconnected and elongated mitochondria), a

125   continuous PM and a double nuclear membrane (Figure 2c). Similar results were observed in

126   THP-1 GSDMD KD cells (Figure S2b). Hence, the dramatic morphological changes in pyroptotic

127   cells are a consequence of the pyroptotic cell death process itself.

128

129   Next, we investigated the structure of the ASC speck using the correlative imaging approach.

130   ASC Specks were visible in SEM images as aggregates with a branched structure and a size of

131   about 1 - 2,5 μm in THP1-ASC-GFP cells, both when infected with Mtb and when treated with

132   LPS and nigericin (Figure 2d-e). The structure corresponds well to that previously observed in a

133   zebrafish model [49]. The speck in the living (Z-VAD + VX765-treated) cell was located in an open

134   space in the cytosol of the cell, seemingly devoid of other organelles. As a control that the

135   observed ASC speck structure was not a consequence of ASC overexpression or tagging, we

136   treated primary human monocytes with LPS and nigericin, and visualised active caspase-1 by

137   FAM-YVAD-FMK (FLICA). Caspase-1 is stabilized in its active form on the inflammasome [50],

138   and we therefore hypothesized that the point with highest fluorescence intensity from FLICA

139   treatment would be the point where the speck was assembled. Indeed, by CLEM, we could

140   identify a similar branched structure in LPS and nigericin-treated primary human monocytes,

141   albeit with a smaller size than that observed in THP-1 cells (about 0,8 μm). To our knowledge,

142   this is the first time an ASC speck has been visualized in a primary human cell, without tagging

143   or overexpression of selected proteins.

144

145   To elaborate on the possible consequences of pyroptosis on Mtb spreading as indicated by EM,

146   we investigated pyroptosis during time-lapse imaging. On average, about 25% of Mtb bacilli

147   were lost into the medium immediately after pyroptosis (Figure 2f), while the rest were trapped in

148   the pyroptotic cell. However, Mtb that were trapped in pyroptotic cells could be phagocytosed by

149   neighbouring cells. Further, this could induce ASC speck formation with pyroptosis in the new

150   host cell, thus propagating inflammasome activation and cell death (Figure 2g, Movie S4). This

151   indicates that pyroptosis allows some bacteria to spread immediately after cell death, and that

152   bacteria that are trapped in the ghost cell can also contribute to further local spread of infection

153   by efferocytosis.

154

155   **Mtb-induced inflammasome activation is dependent on ESX-1 but not on the tuberculosis-**

156   **necrotizing toxin (TNT) or destabilization of host mitochondria**

157   It has been reported that Mtb deleted in the Region of Difference 1 (MtbΔRD1), where most

158   ESX-1 related genes are located, are deficient in inflammasome activation [15–17]. Our results are in

159   line with these reports, as MtbΔRD1 caused significantly less ASC speck formation and cell

160   death of THP-1 cells than Mtb wild-type (Figure 3a). We also observed that Mtb grown in the

161   absence of the detergent tween-80 are more potent in inflammasome activation than Mtb grown

162   in the presence of tween (our regular growth condition). Detergent has been published to perturb

163   the mycobacterial capsule, in particular decreasing the abundance of ESX-1 related proteins on

164   the Mtb surface/capsule [51,52]. Our data thus show that inflammasome activation correlates with

165   ESX-1 activity and the integrity of the Mtb capsule, suggesting that deposition of ESX-1 related

166   factors on the Mtb surface/capsule is important for Mtb inflammasome activation.

167

168   Tuberculosis necrotizing toxin (TNT, the C-terminal end of the protein CpnT) is secreted from

169   Mtb and released into the host cell cytosol in an ESX-1 dependent manner, leading to

170   macrophage necroptosis [34,53,54]. We therefore wanted to investigate if TNT is contributing to the

171   ESX-1 dependent inflammasome activation. To this end, we infected THP1-ASC-GFP

172   macrophages with wildtype Mtb, MtbΔCpNT or MtbΔCpNT complemented either with the

173   catalytically active or inactive form of TNT (MtbΔCpNT::TNT and MtbΔCpNT::TNT*

174   respectively). We found that all Mtb mutants and complemented strains were similarly competent

175   in inflammasome activation, and that treatment with MCC950 had a similar effect across

176   bacterial strains in inhibiting ASC speck formation (Figure 3b and Figure S3a). This indicates

177   that TNT does not play a role in Mtb-induced inflammasome activation in our system.

178

179   Finally, we assessed the possible involvement of mitochondrial membrane perturbation prior to

180   inflammasome activation and pyroptosis, an event that could also be linked to the activity of

181   ESX-1 and related proteins. Mitochondrial damage has been strongly implicated in

182   inflammasome activation in general, and in Mtb-induced necrosis [25,29,33,34,55]. In particular, Mtb

183   H37Rv is reported to cause a loss in mitochondrial membrane potential ($\Delta\Psi_m$), which could be

184   inhibited by cyclosporine A (CsA), a cyclophilin D inhibitor that prevents formation of the

185   mitochondrial permeability transition pore [34,56]. However, we did not see an effect of CsA on

186   Mtb-induced inflammasome activation and pyroptosis after 24 hours of infection (Figure 3c). In

187   addition, we monitored the $\Delta\Psi_m$ by live-cell imaging of cells infected with Mtb-BFP and labelled

188   with tetramethylrhodamine ethyl ester (TMRE), a dye that is accumulated in mitochondria in

189   proportion to $\Delta\Psi_m$ [57]. We observed that $\Delta\Psi_m$ was stable in infected cells until ASC speck

190   formation, and that the potential quickly dropped after ASC speck formation (measured by a drop

191   in TMRE intensity, Figure 3d and Movie S5). This immediate drop in TMRE intensity after ASC

192   speck formation was abolished in THP-1 GSDMD KD macrophages, without any adverse effects

193   on the ability of the cells to form ASC specks, suggesting that GSDMD activity and possibly PM

194   disruption during cell death is required for mitochondrial destabilization (Figure 3e, Movie S6).

195   Similar results were obtained with LPS and nigericin treatment, where $\Delta\Psi_m$ dropped only after

196   ASC speck formation, and the drop was inhibited in GSDMD KD cells (Figure S3b and Movies

197   S7 and S8). We conclude that gross mitochondrial membrane disruption is not a prerequisite for

198   inflammasome activation caused by Mtb or LPS+nigericin but rather accompanies pyroptosis,

199   which is also in line with the intact mitochondrial morphology observed by EM in living cells

200   with an assembled inflammasome (Figure 2c).

201

202   **ESX-1-mediated phagosomal damage is a prerequisite for NLRP3 activation by**

203   **phagosomal Mtb**

204   One of the main effects of the ESX-1 secretion system is destabilization of phagosomal

205   membranes [36–38]. We therefore asked how phagosomal rupture is related to inflammasome

206   activation and pyroptosis. Galectin-3 (Gal3) binds to exposed glycosylated proteins usually

207   confined to the inner phagosomal membrane and has been used as a marker for ruptured

208   phagosomes [16,58]. We infected THP1-Gal3-mScarlet macrophages and observed that Gal3 was

209   indeed recruited to the vicinity of wild-type Mtb, but not to MtbΔRD1 (Figure 4a), and Gal3

210   recruitment was enhanced upon infection with Mtb grown in the absence of tween (Figure 4b).

211   Gal3 recruitment to Mtb phagosomes occurred prior to inflammasome activation in about 80% of

212   pyroptotic cells, and a typical sequence of events is depicted in Figure 4c and Movie S9.

213

214   Using CLEM, we confirmed that Gal3 accumulation corresponds to cytosolic contact of Mtb

215   (Figure 4d). Mtb in phagosomes devoid of Gal3 were surrounded by a tightly apposed and

216   continuous phagosomal membrane, in contrast to bacteria associated with Gal3 which had no

217   visible phagosomal membrane and were in direct contact with the host cell cytosol. Furthermore,

218   the CLEM approach revealed in great detail the presence of clusters of vesicles and membranous

219   structures adjacent to Gal3+ bacteria, but not the Gal3- and intact Mtb phagosomes. The

220   ultrastructure is similar to that previously observed for Mtb residing in LC3+ compartments in

221   lymphatic endothelial cells [59]. We therefore investigated the recruitment of LC3B in relation to

222   Gal3 by live-cell imaging of Mtb-BFP-infected THP-1 macrophages expressing mNeonGreen-

223   LC3B and Gal3-mScarlet. Indeed, we consistently observed recruitment of LC3B to intracellular

224   Mtb shortly after recruitment of Gal3 (Figure 4f, g and Movie S10), suggesting that ruptured Mtb

225   phagosomes are targeted by autophagy [60–62]. To investigate if autophagic targeting of Mtb lead to

226   formation of mature, acidified autophagosomes, THP-1 macrophages expressing Gal3-mScarlet

227   were labelled with LysoView633 (Figure S4a). Only ~20% of Mtb in ruptured phagosomes were

228   later found in acidified compartments indicating that autophagy of Mtb after phagosomal rupture

229   is inefficient (Figure S4b).

230

231   **Inflammasome activation by Mtb is independent of lysosomal damage and release of active**

232   **cathepsins**

233 The ability to disrupt phagosomes seems important for inflammasome activation by Mtb, and it

234 has been reported that phagosomal acidification is a prerequisite for the membrane damaging

235 activity of ESX-1 [63–65]. Additionally, active cathepsin release from ruptured phagolysosomes is a

236 suggested trigger for the NLRP3 inflammasome [24,39,40]. We therefore went on to investigate the

237   possible involvement of phagosomal acidification and release of active cathepsins in

238   inflammasome activation and pyroptosis by Mtb. Live-cell imaging of THP1-Gal3-mScarlet or

239   THP1-ASC-GFP cells in the presence of the pH sensitive dye LysoView633 revealed that Mtb

240   was equally efficient at escaping from acidified and neutral compartments (Figure 5a-c and

241   Movies S11 and S12), suggesting that phagosomal acidification is not a prerequisite for Mtb

242   escape into the cytosol. The LysoView signal in single cells was stable prior to ASC speck

243   formation, indicating that there is no general disruption or degradation of acidified lysosomes

244   prior to inflammasome activation (Figure 5d-e and Movie S13). In addition, we did not see any

245   effect on cell death or ASC speck formation when cells were treated with Bafilomycin A1

246   (BafA1) which inhibits lysosomal acidification by V-ATPase, or the pan cathepsin inhibitor

247   K777, during infection (Figure 5f), despite efficient cathepsin B inhibition (Figure S5).

248   Compared to K777, cathepsin B inhibitor Ca-074-Me was less potent and had clear off-target

249   effects on inflammasome activation during LPS+nigericin treatment (Figure S5). These results

250   demonstrate that Mtb causes inflammasome activation and pyroptosis independently of

251   phagolysosomal acidification and release of active cathepsins.

252

253   **Mtb carrying ESX-1 can directly damage the host cell plasma membrane**

254   K+ efflux precedes NLRP3 activation for a range of known triggers [28]. Common for many of

255   those triggers is that they make the PM permeable to K+, e.g. by opening a membrane resident

256   pore (P2X7, pannexin-1) or by inserting and making new pores (MLKL, complement, bacterial

257   pore forming toxins) [66]. We and others have shown that Mtb-induced NLRP3 inflammasome

258   activation is dependent on K+ efflux, and it has also been shown that Mtb ESX-1 can be

259   haemolytic [67]. Moreover, PM damage has been observed during Mtb infection and PM repair

260   protects macrophages from necrotic cell death [68]. However, whether Mtb could directly cause PM

261   damage or if PM damage is a consequence of Mtb induced intrinsic cell death pathways, was not

262   addressed. We therefore wanted to investigate if Mtb could damage the macrophage PM, and if

263   this could be involved in inflammasome activation by Mtb. To monitor PM integrity, we made a

264   THP-1 reporter cell line with an mNeonGreen fluorescent protein tag on the Ca2+ binding

265   protein Apoptosis-Linked Gene 2 (ALG-2), which is recruited to sites of PM damage by calcium

266   influx [69–71]. We imaged Mtb-BFP infected THP-1 macrophages by time-lapse confocal

267   microscopy and observed ALG-2 recruitment in infected cells, almost 90% of which occurred in

268   close vicinity to Mtb bacteria (Figure 6a, b and Movies S14 and S15). We observed ALG-2

269   recruitment to Mtb both without prior Gal3 recruitment and subsequent to Gal3 recruitment, with

270   a distribution close to 50/50 between the two categories. This suggests that Mtb can damage the

271   host cell PM either during phagocytosis or following phagocytosis and phagosome rupture.

272

273   As damage to the PM from the cytosolic side by already internalized bacteria has (to our

274   knowledge) not been described before, we wished to verify that ALG-2 recruitment to Gal3-

275   positive Mtb indeed occurred at the PM. TIRF microscopy is only sensitive to the region within

276   ~100 nm from the substrate, meaning it specifically detects events occurring at the PM [72]. We

277   imaged infected THP-1 macrophages with widefield and total internal reflection (TIRF)

278   microscopy simultaneously (Figure 6c and Movie S16). From widefield imaging we could see

279   Gal3 recruitment to intracellular Mtb that were out of range for TIRF, while ALG-2 recruitment

280   was observed in TIRF and WF modes when Mtb came into the range of the TIRF excitation,

281   demonstrating that ALG-2 recruitment following Gal3 also occurs at the PM.

282

283   To better understand the extent of PM damage marked by ALG-2 events, we imaged cytosolic

284   calcium levels using the fluorescent indicator Calbryte-590 during time-lapse microscopy of

15

285   Mtb-infected THP-1 cells. We observed a consistent influx of calcium at timepoints of ALG-2

286   recruitment (Figure 6d, e and Movie S17), which is expected considering that ALG-2 is known to

287   be recruited by calcium influx. Calcium influx was not observed at timepoints of Gal3

288   recruitment to Mtb phagosomes (Figure 6e, Figure S6a and Movie S18). We also imaged infected

289   THP-1 macrophages with the membrane impermeable RNA/DNA dye propidium iodide (PI)

290   present in the medium. We noted a consistent local influx of PI in regions of Mtb-localised ALG-

291   2 recruitment events, in contrast to control cells with no apparent ALG-2 events (Figure 6f, g,

292   Movie S19). Taken together, these data demonstrate that Mtb can damage the macrophage PM in

293   a manner depending on close proximity between the two. The damage caused by Mtb makes the

294   PM permeable to both ions (Ca^{2+}, K^+) and larger molecules such as PI.

295

296   To investigate the ultrastructure of Mtb-associated PM damage we performed live-cell CLEM.

297   After live-cell imaging and rapid fixation, recent ALG-2 recruitment events were imaged by

298   confocal or Airyscan microscopy, and further by FIB-SEM tomography (Figure 6h, Figure S6b).

299   At the location of fluorescent ALG-2 signal and directly adjacent to Mtb, multiple 50-100 nm

300   sized vesicles were visible, indicative of ESCRT-mediated PM repair [70,73].

301

302   **Plasma membrane damage caused by Mtb or Silica activates the NLRP3 inflammasome**

303   Knowing the dependency on K+ efflux for NLRP3 activation, we hypothesized that the ability of

304   Mtb to cause PM damage (hence making it permeable to ions) would be linked to inflammasome

305   activation. We therefore imaged THP1-ALG2-mNeonGreen/Gal3-mScarlet/ASC-mIRFP670

306   macrophages by time-lapse microscopy during infection with Mtb-BFP. An example time course

307   is shown in Figure 7a and Movie S20. We recorded the time points of all Gal3 (phagosomal

308   rupture) and ALG-2 (PM damage) recruitment events in cells forming ASC specks, along with

16

309   the time point of ASC speck formation. PM damage preceded ASC speck formation in 87% of

310   cells infected with Mtb and occurred closer in time to ASC speck formation than Gal3

311   recruitment to Mtb phagosomes (Figure 7b). While the median time from phagosomal rupture to

312   ASC speck formation was 2 h, the median time from PM damage to ASC speck formation was 1

313   h. Moreover, within 20 minutes before ASC speck formation, 48% of cells had ALG-2 events

314   and 23% of cells had Gal3 events, compared to 1% and 4%, respectively, in infected control cells

315   over an average 20-minute period (Figure 7c). Thus, especially PM damage events are strongly

316   enriched in the time before ASC specks, connecting these events at the single-cell level. When

317   cells were infected with MtbΔRD1, which does not damage host cell membranes, we did not

318   observe any ALG-2 recruitment and ASC speck formation was significantly reduced, as noted

319   earlier (Figure 3a). Treatment with the phagocytosis inhibitor cytochalasin D during infection

320   with Mtb only partially inhibited ASC speck formation after 24 hours, supporting that Mtb can

321   induce inflammasome activation through PM damage from outside of the cell (Figure 7d).

322   Finally, when inflammasome activation was inhibited by MCC950 or extracellular KCl during

323   infection, the number of ALG-2 events per cell remained constant while ASC speck formation

324   was inhibited as earlier, confirming that PM damage happens prior to NLRP3 inflammasome

325   activation (and is not caused by e.g. active GSDMD) (Figure 7e and Figure S7a).

326

327   ALG-2 and the ALG-2 interacting protein X (ALIX) are early components of ESCRT-mediated

328   PM repair [69,70]. We went on to inhibit PM repair by knocking down ALG-2 or ALIX by CRISPR-

329   Cas9 in THP-1 macrophages. Mtb caused significantly more inflammasome activation and

330   pyroptosis both in ALG-2 and ALIX KD macrophages after 24 hours of infection, compared to

331   control cells (Figure 7f and Figure S7b). Together, these results demonstrate that Mtb-induced

17

332   PM damage is a trigger for NLRP3 inflammasome activation in macrophages, and that PM repair

333   mechanisms negatively regulate inflammasome activation and pyroptosis caused by Mtb.

334

335   Silica is another NLRP3 stimulus that supposedly triggers inflammasome activation by

336   destabilizing host cell phagolysosomes after uptake [24]. We therefore thought it interesting to

337   study NLRP3 activation by silica in the same system as we used for Mtb infection. TIRF and

338   widefield microscopy of Gal3 and ALG-2 tagged THP-1 macrophages revealed Gal3 recruitment

339   to intracellular silica crystals and ALG-2 recruitment to sites at the PM (Figure 7g and Movie

340   S21), indicating that silica crystals are also capable of disrupting phagosomal membranes and the

341   PM. A typical time course of events culminating in ASC speck formation is shown in Figure 7h

342   and Movie S22. PM damage preceded ASC speck formation after silica stimulation in most cells

343   (90%). Further, silica-induced ALG-2 events were closer in time to ASC specks than Gal3

344   events, similar to what we observed during Mtb infection (Figure 7i). 71% of cells that assembled

345   ASC specks had an ALG-2 recruitment event within the last 20 minutes before ASC speck

346   formation, while about 25% of the cells had a Gal3 recruitment event within the same period

347   (Figure 7i). Nigericin, which directly depletes the cell of K^+ as a K^+ ionophore, or imiquimod,

348   which is an NLRP3 trigger acting independently of K+ efflux [29], did not result in any visible Gal3

349   or ALG-2 recruitment events prior to inflammasome activation (FigS7c, d and Movies S23 and

350   S24).

351

352   Further, we investigated if lysosomal damage using the lysosome-targeted photosensitizer

353   $TPCS_{2a}$ [74] could activate NLRP3 (Figure S7e, f and Movie S25). However, despite loss of

354   lysosomal pH and Gal3 recruitment indicating lysosomal damage after blue light excitation in the

355   presence of $TPCS_{2a}$, we did not observe ALG-2 events at the PM or ASC speck formation. These

18

356   data show evidence that PM damage (with subsequent K+ efflux) is a central event upstream of

357   NLRP3 inflammasome activation by the "lysosomal triggers" Mtb and silica, and that lysosomal

358   damage itself is not sufficient to activate NLRP3.

359

360   **Discussion**

361   Studies of the molecular mechanisms involved in the interaction between *Mycobacterium*

362   *tuberculosis* and host cells are complicated by the variability of this interaction. Bacterial

363   containment, replication and host cell death through different programmed or unregulated death

364   pathways are all possible outcomes. The heterogeneity makes average measures hard to interpret,

365   and they are unsuited to distinguish between cause and effect. Long-term studies at the single cell

366   level give a better insight into these questions, allowing to study separate populations in a cell

367   culture and multiple events in the same cell and over time. We capitalize on this advantage to

368   gain several insights into Mtb-host interactions. We focus on the temporal regulatory events that

369   orchestrate NLRP3 activation and pyroptosis, two phenomena that are still not well understood,

370   especially in the context of a complex pathogen infection. Both AIM2 and NLRP3 have been

371   implicated in IL-1β release during Mtb infection [15,16,18,19], while the occurrence of pyroptosis as a

372   distinct route of cell death has been less clear [75,76]. Here we establish that NLRP3 is activated and

373   that caspase1- and GSDMD-dependent pyroptosis is an important cell death mechanism during

374   Mtb infection in human cells, together leading to release of IL-1β. Intriguingly, NLRP3

375   activation and pyroptosis is triggered by PM damage caused by Mtb during phagocytosis or after

376   phagosomal escape. Similar results were obtained with silica crystals, suggesting PM damage as

377   a common mechanism of NLRP3 activation and pyroptosis by sterile and pathogenic agents

378   holding membrane disruptive properties.

379

380   Necroptosis is another necrotic cell death pathway induced by Mtb, suggested to be caused by the

381   secreted enzyme TNT [33,34]. In some cases, there is cross-talk between necroptosis and NLRP3

382   activation and pyroptosis [77,78], but our results indicate that these are distinct processes during Mtb

383   infection. We do not see an effect of necroptosis inhibitors or TNT mutants, and by time-lapse

384   imaging we show that destabilization of mitochondria accompanies pyroptosis rather than causes

385   inflammasome activation in the NLRP3 pathway. The mitochondrial integrity is similar both in

386   response to Mtb and to LPS+nigericin. The discrepancy between our results and those of Pajuelo

387   et al. [34] with regards to cell death by TNT and necroptosis inhibitors is likely due to the longer

388   infection time used by Pajuelo and colleagues: our analyses are mostly performed after 24 hours

389   of infection where pyroptosis is most prominent, but we also see an increase in necrotic cell death

390   at later timepoints.

391

392   At the single cell level, highly infected cells at earlier stages of infection (first 24h) are the most

393   prone to pyroptosis, and there is a strong correlation between ESX-1 activity damaging host cell

394   membranes and NLRP3 activation. Other groups have also shown the correlation between

395   bacterial burden and cell death [76,79,80]. One could hypothesize that *in vivo* pyroptosis would occur

396   during a phase of infection with presence of larger amounts of Mtb, such as during active phases

397   of the disease where Mtb needs to spread [80]. Interestingly, although IL-1β is a central cytokine in

398   a successful immune response against tuberculosis [81], a gain of function mutation in NLRP3 has

399   been associated with poor clinical outcome in tuberculosis patients [82].

400

401   The precise mechanism of NLRP3 activation has evaded the community for over a decade.

402   Although there are alternative routes proposed, inhibition of NLRP3 activation by high

403   concentrations of extracellular KCl positions K+ efflux as the predominant upstream effector of

404   NLRP3 activation by a wide range of triggers. This is also the case for Mtb, as we and others

405   have shown [14,39]. The similarities in ASC speck structure and progression of pyroptosis during

406   Mtb infection and the canonical NLRP3 trigger nigericin, which directly depletes the cell of K+

407   as a K+/H+ ionophore, indicates that potential differences are mainly linked to how K+ efflux

408   occurs. Lysosomal damaging agents are one of the most clinically relevant classes of NLRP3

409   triggers [83]. Cathepsin release from damaged lysosomes, especially of Cathepsin B, is one

410   commonly proposed connection, although how this further causes K+ efflux has been unclear

411   [24,39,40]. One main source of this confusion is the widespread use of the Cathepsin B inhibitor Ca-

412   074-Me, which appears to inhibit NLRP3 activation independent of Cathepsin B inhibition (our

413   results and [28,41–43]). We also show that although Mtb can damage acidified phagolysosomes, this

414   is not required for NLRP3 activation, and there is no general loss of lysosomal content prior to

415   NLRP3 inflammasome activation. Instead, PM damage appears to be the key event to trigger

416   NLRP3 during Mtb infection. Also, in response to silica crystals, a canonical lysosome-damaging

417   NLRP3 trigger, we observe PM damage prior to NLRP3 activation. Permeabilization of PM by

418   e.g. bacterial pore-forming toxins is well known to cause ion fluxes down their electrochemical

419   gradients and thus activate NLRP3 through the K+ efflux route [66]. PM damage during

420   phagocytosis of Mtb, silica and likely other similar membrane damaging agents explains some of

421   the NLRP3 activation events, but there is also a second possible route. Our imaging data show

422   that PM damage can also occur from the cytosolic side after uptake, if Mtb or silica has damaged

423   the phagosome and subsequently come into contact with the PM. Thus, a straight forward

424   explanation for how something that primarily damages phagolysosomes can cause K+ efflux and

425   NLRP3 activation emerges. Strikingly, the opposite case with extensive, but localized and

426   controlled lysosomal damage caused by a lysosomal photosensitiser does not cause NLRP3

427   activation. Since PM damage is actively repaired through many routes, including through Ca2+ -

21

428   dependent ESCRT machinery [44,69–71], it is also interesting to note that this branch of ESCRT

429   emerges as a negative regulator of NLRP3 activation and cell death during Mtb infection. The

430   ESCRT machinery was also recently shown to be involved in repair of phagosomes damaged by

431   mycobacteria [84,85] and to regulate pyroptosis or necroptosis by repairing GSDMD pores [86] or

432   MLKL pores [87], respectively, further pointing to the importance of the ESCRT machinery in

433   regulation of inflammation and securing cell viability.

434

435   How ESX-1 damages membranes is not well understood. Several papers indicate a central role

436   for the secreted protein ESAT-6, acting through membrane lysis or membrane pore formation

437   [35,63,65,88,89]. However, this has recently been attributed to detergent contamination, and a contact-

438   induced form of gross membrane disruption caused by undefined ESX-1 activity has been

439   proposed [67]. ESX-1 secreted substrates are also retained in the mycobacterial capsule, and ESAT-

440   6 has been observed to quickly disappear upon contact between bacteria and host macrophages

441   [51,52]. Our results also indicate contact-induced damage being most likely, as damage events are

442   localized to contact points between Mtb and the PM. The damage upon contact could be caused

443   by ESX-1-dependent factors in the bacterial capsule or other factors that are diminished by the

444   presence of the detergent tween, as Mtb cultured without tween showed increased membrane

445   damaging activity and higher inflammasome activation and cell death. The damage also appears

446   to be severe, as shown by ultrastructural investigations, the simultaneous recruitment of Gal3 and

447   loss of lysosomal pH in phagolysosomes, and the simultaneous recruitment of ALG-2 and PI and

448   calcium influx during PM damage. This is different from previously described bacterial toxins

449   that cause membrane damage by pore formation. Furthermore, the membrane damaging activity

450   of ESX-1 is unique in that it can act from both sides of the PM. Our ultrastructural investigations

451   of sites of PM damage close to Mtb indicate that the PM repair response in the affected cell is

452   quickly initiated, complicating a direct observation of what the damage initially looks like.

453

454   In conclusion we show a K+ efflux dependent, NLRP3-caspase-1-GSDMD axis for pyroptotic

455   cell death and IL-1β release during Mtb infection of human macrophages and monocytes. We

456   emphasize the importance of membrane damage through activity of the ESX-1 secretion system,

457   upstream of NLRP3 activation at the single cell level. Rather than phagolysosomal damage itself

458   causing NLRP3 activation, we show that PM damage occurring during phagocytosis or from the

459   cytosol after phagosomal damage is the cause of K+ efflux and subsequent NLRP3 activation by

460   Mtb. In this scenario, NLRP3 acts as a sensor for cell integrity rather than as a sensor for Mtb in

461   the cytosol or release of lysosomal enzymes.

462

463   Mtb needs to escape from host macrophages to efficiently replicate and spread to new hosts.

464   Based on our data we propose a model where preservation of membrane integrity is key to

465   contain Mtb infections. The ESCRT machinery repairs damages caused by Mtb to the plasma

466   membrane and phagosomes whereas autophagy sequesters ruptured Mtb phagosomes. Inefficient

467   countermeasures by the host cell allows for potassium efflux and NLRP3 inflammasome

468   activation, in which case pyroptosis is inevitable and facilitates the spread of Mtb to

469   neighbouring cells and eventually to new hosts. Cell necrosis pathways are thus attractive as

470   possible targets for host-directed therapeutic strategies [90]. We also show that PM damage is a

471   shared mechanism with the crystalline NLRP3 trigger silica. Crystalline triggers cause sterile

472   inflammatory diseases in the lungs and the cardiovascular system, and the mechanism of NLRP3

473   activation by these so-called lysosomal triggers has so far been elusive. Our findings point to PM

474   damage as a shared mechanism for this group of NLRP3 activators, extending the relevance

475   beyond tuberculosis disease.

476

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487

488   **Author contributions**

489   Conceptualization: THF, KSB and MSB; Investigation: KSM, MSB, SU, RS, HK, AM, SEÅ,

490   TÅS; Writing – initial draft: THF, KSB, MSB; Writing – review and editing: THF, KSB, MSB;

491   Supervision and Funding acquisition: THF. All authors discussed the results and commented on

492   the manuscript.

493

494   **Declaration of Interests**

495   The authors declare no competing interests.

496

Materials and Methods

All reagents used are listed in supplementary table 1.

Experimental models

THP-1 cells were maintained in RPMI 1640 supplemented with L-glutamine, 10 mM HEPES and 10% FCS, and passaged regularly to keep the cell density between 0.2 and $1x10^6$/mL. Cell lines were routinely tested for mycoplasma. Human 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 Olavs Hospital (Trondheim, Norway). 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 $mc^2$6206 auxotroph strain 50μg/mL L-leucine and 24μg/mL D-pantethonate were added to the growth medium.

Lentiviral production was performed in HEK293T cells as described [91] using 3rd generation packaging system with pMDL.g/pRRE, pMD2.G and pRSV-Rev and JetPrime (PolyPlus) as transfection reagent. Virus-containing supernatant were harvested 2 and 3 days post transfection and THP-1 cells were transduced in the supernatant by 90 minutes spinoculation at 32°C, 1000g in the presence of 8μg/mL polybrene, and selected in 1μg/mL puromycin, 10μg/mL blasticidine or 100μg/mL Hygromycin for 1 week. Finally, the cells were selected for a consistent moderate

520 expression level of fluorescent constructs by 1-2 rounds of FACS (BD Aria II). CRISPR

521 modified THP-1 cells were used directly as a polyclonal population after puromycin selection.

522 Mtb H37Rv, Mtb H37RvΔRD1 and Mtb mc^{2}6206 were transformed with msp12::EBFP2 and

523 selected on hygromycin (65 µg/mL) 7H10 plates (Difco/Becton Dickinson).

524

525 **Method details**

526

527 **Generation of new vectors**

528 Gateway cloning was used to generate lentiviral expression vectors. Human Galectin-3, human

529 ALG-2 (THP-1 cDNA), human ASC, human LC3B (synthetic GeneArt Strings, ThermoFisher),

530 mScarlet (synthetic), mRuby3, mNeonGreen, mIRFP670 and SNAP$_f$ tag were PCR amplified and

531 cloned into Gateway pEntry vectors. All pEntry constructs were verified by Sanger sequencing

532 (GATC Biotech) before further use. PuroR in pLex307 was replaced with BlastR or HygR to

533 generate Gateway lentiviral destination vectors with corresponding antibiotic resistances. 2-

534 fragment Gateway recombination with pLex307, pLex307-Blast or pLex307-Hyg were

535 performed to generate ASC-mNeonGreen, ASC-mIRFP670, Galectin-3-mScarlet, Galectin-3-

536 SNAP, mNeonGreen-LC3B and mNeonGreen-ALG-2 constructs. Guide RNAs targeting

537 GSDMD, NLRP3, ALG-2 or ALIX were cloned into LentiCrispr v2 by BsmBI digestion and

538 ligation.

539

540 **Verification of CRISPR knock-down**

541 Knock-down in the cell pool was verified by cleavage assay (GeneArt genomic cleavage assay,

542 ThermoFisher), sequencing and TIDE analysis [92] and/or western blotting. Antibodies and

543   dilutions for western blot were NLRP3 (1:1000), GSDMD (1:1000), ALG-2 (1:500) and ALIX

544   (1:1000).

545

546   **Macrophage and monocyte infection**

547   Before use, THP-1 cells were differentiated at a concentration of 300.000 cells/mL in medium

548   containing 100 ng/mL PMA for 3 days, washed in cell medium and rested 1 day prior to

549   experiments. Primary monocytes were selected by plastic adherence of PBMCs for 1 hour,

550   followed by 3x washing in HBSS, and cultured in RPMI with 10% A+ serum (blood bank of St

551   Olavs Hospital, Trondheim, Norway). Mtb bacteria were grown until OD_{600} was 0.4-0.55 (log

552   phase) then pelleted at 2400g for 10 minutes, resuspended in RPMI with 10% A+ serum to

553   opsonise bacteria prior to infection, sonicated 2 times for 5 s at 70% power (Branson Digital

554   Sonifier, S-450D), and clumped bacteria were removed by centrifuging at 300g for 4 minutes.

555   The supernatant containing bacteria was diluted in RPMI with 10% A+ serum to the indicated

556   MOI assuming 1 $OD_{600}=3x10^8$ bacteria/mL, and applied to cells for 45 minutes with or without

557   inhibitors, followed by washing in HBSS and replacement of the media to normal cell medium

558   with 10% A+ with or without inhibitors, or Leibovitz L-15 CO_2-independent medium with 10%

559   A+ for live cell imaging. The typical condition of MOI 20 infection gave about 50% infection

560   rate with 1-20 bacteria per macrophage. Inhibitor concentrations were DMSO control (1:400),

561   zVAD-FMK (50 µM) VX765 (50µM), MCC950 (10 µM), KCl (40mM), Nec1s (10 µM), GSK-

562   872 (5µM), Cyclosporin A (5 µM), K777 (15µM) or Bafilomycin A1 (50 nM). Supernatants

563   were harvested for ELISA or LDH assays after 24 hours, and analysed by human IL-1β kit or

564   LDH cytotoxicity kit according to the manufacturer's instructions.

565

566   **Live/fixed cell imaging**

567   THP-1 cells were seeded in 35mm glass bottom dishes (Ibidi) or 96-well glass bottom plates

568   (Cellvis) as above. For some experiments bacteria were added at a lower MOI and not washed to

569   be able to follow the entire course of infection. Cells were imaged on a Leica SP8 confocal

570   microscope with a 37°C incubator using a 40x1.3 or 20x0.75 oil immersion objective or 10x0.4

571   air objective at the timepoints indicated in the text. TIRF/Widefield imaging was done on a Zeiss

572   Laser TIRF 3, with a 63x1.46 oil objective and Hamamatsu EMX2 EMCCD camera. For time-

573   lapse imaging by confocal, typically 6 fields of view comprising >500 cells in total were imaged

574   at 30-45 s intervals, while TIRF/WF was done on one to four fields of view comprising 10-40

575   cells at 10-30s intervals. For some experiments, DRAQ7 (0.15µM), 10 nM TMRE (10 nM),

576   LysoView 633 (1:10 000) or Calbryte-590 (5µM) was preincubated for 30-60 minutes, and added

577   to the cell medium during Mtb infection. Calbryte-590 was only preincubated and not re-added.

578   In cells with SNAP tag, cells were labelled with 1:200 SNAP-Cell 647-SiR for 15 minutes,

579   washed 3x in cell medium and rested for 30 minutes before use. For lysosomal damage by

580   photosensitizer, $TPSC_{2a}$ was added to medium (0.4µg/mL) for 16 h and cells were washed and

581   incubated for 4h without $TPSC_{2a}$. THP1 cells were stained or not stained with Cresyl Violet

582   (1µM, 5 minutes) exposed to 405 nm light (10%, 1 second), and imaged. Galectin-3

583   accumulation on phagosomes during infection with different Mtb strains was determined by

584   fixing cells in 4% PFA for 20 minutes at 1, 4 and 24 h post infection, and imaging 16 fields of

585   view with 40x objective per well.

586

587   **FIB/SEM sample preparation and correlative imaging**

588   For correlative imaging experiments two growth substrates were used, with different optical

589   microscopy and fixation approaches. In the first approach THP1 ASC-GFP Gal3-RFP cells were

590   seeded and infected as above on moulded aclar slides. Aclar substrates were placed in 24-well

591   plates and imaged live using 10x objective as above. After 24 hours, cells were fixed in 2.5%

592   glutaraldehyde in 100mM PIPES for 1h, placed upside-down in 35mm glass bottom dishes and

593   cells of interest were re-imaged using 63X1.2W objective on a Leica SP8 confocal microscope.

594   In the second approach THP-1 cells were seeded and infected as above on moulded polymer

595   coverslips (ibidi) mounted in 8 well sticky-Slides (ibidi). Samples were imaged using 40X1.3oil

596   objective on a Leica SP8 confocal microscope and fixated by adding 8% paraformaldehyde

597   (PFA) and 0.2% GA in 200mM PIPES directly into imaging media at a 1:1 ratio. After 20

598   minutes fixation media was changed to fresh 4% PFA and 0.1% GA in 100mM PIPES. Cells with

599   interesting events during live microscopy were then re-imaged with a 40X1.2Imm AutoCorr

600   objective on a Zeiss LSM880 microscope using the Airyscan mode. After re-imaging fixation

601   medium was changed to 2% PFA and 2.5% GA in 100mM PIPES and fixated at 4°C over night.

602   Cells were then prepared for FIB/SEM microscopy and relocated as previously described

603   (Beckwith et al. 2015). Briefly, cells were post-fixed and contrasted in potassium ferrocyanide

604   and osmium tetroxide, and further *en block* stained in uranyl acetate. Cells were gradually

605   dehydrated and embedded in Durcupan (Sigma-Aldrich) and cured at 60 degrees for a total of 3

606   days. Samples were attached to SEM sample stubs with a drop of Durcupan before the last day of

607   curing. Before FIB/SEM imaging, a layer of 40nm platinum/palladium was deposited on the

608   samples. FIB/SEM imaging and tomography experiments were performed on a Helios G4 dual-

609   beam FIB/SEM from FEI, using an acceleration voltage of 2kV or 3kV for the electron beam,

610   immersion mode and the in-column mirror detector. Slice thickness was typically set to 20nm or

611   25nm. Initial alignments, scaling and simple adjustments (brightness/contrast) were done in Fiji,

612   while 3D reconstructions were performed semi-manually in Avizo or Dragonfly.

613

614   **Cathepsin B activity assay**

615   THP1 cells were differentiated in 24-well plates as above. 1 h prior to lysis, inhibitors (K777 and

616   Ca074-Me) were added at concentrations indicated in the text. Cathepsin B activity assay was

617   performed as described [93] with some modifications. Briefly, cells were lysed in 200µL of lysis

618   buffer (0.1% Triton X-100, 250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1

619   mM EDTA, 1 mM EGTA, 0.5 mM Pefabloc SC, pH=7.5) on ice for 20 minutes. 50µL of lysate

620   was mixed with 50µL of Cathepsin B reaction buffer (50 mM sodium acetate, 4 mM EDTA, 0.5

621   mM Pefabloc SC, 8 mM Dithiothreitol (DTT) and 50 µM Z-RR-AMC, pH=6.0), incubated at

622   30°C for 5 minutes, and fluorescence intensity was monitored every 30 s for 20 minutes (ex

623   355/em 460 nm, POLARstar Omega microplate reader).

624

625   **Quantification and statistical analysis**

626

627   **Image analysis**

628   For quantification of cell death and ASC specks at defined time points with and without

629   inhibitors or knock-down, 16 fields of view containing n>2000 cells in total were imaged per

630   condition. CellProfiler was used to automatically segment and count live cells and ASC specks

631   based on the ASC-GFP signal, and dead cells based on the DRAQ7 signal. Bacterial burden per

632   cell was measured from live cell time-lapse movies by mean EBFP2 intensity in infected cells

633   immediately prior to ASC speck formation, and compared to cells surviving the duration of the

634   experiment. The time point for the control cells was chosen to be the average time point for ASC-

635   speck induction. Traces of DRAQ-7, TMRE or LysoView signal were generated by manually

636   selecting ROIs around cells with or without ASC specks forming.

637   Galectin-3 association with Mtb was scored by a trained model in Ilastik and further analysed by

638   CellProfiler. During live-cell imaging the time point for galectin-3 or ALG-2 recruitment events

639　was determined by the first visible accumulation at a bacterial phagosome in a cell later forming

640　an ASC speck. Data were plotted and statistically analysed using Python or GraphPad Prism,

641　while images and figures were prepared using FIJI and Adobe Illustrator. TIRF/WF images of

642　mNeonGreen-ALG-2 and Galectin-3 mScarlet were unmixed by linear unmixing due to

643　substantial cross-excitation of mScarlet with 488 nm laser.

644

645　**Statistical analysis**

646　Detailed statistical analysis for individual experiments are listed in each figure legend. This

647　includes the statistical test performed, the parameters shown, and number of cells, replicates and

648　independent experiments as appropriate. Data comparing mean values of technical replicates of

649　representative experiments or independent experiments were analysed by two-tailed unpaired

650　Student's t test (two groups) or one-way ANOVA (more groups). For one-way ANOVA

651　corrections for multiple comparisons were applied for comparisons with one control group

652　(Dunnett's) or all groups (Tukey's). For comparisons of non-parametric data (fluorescent

653　intensity or event timing) Mann-Whitney U-test was used instead. GraphPad Prism 8.0 was used

654　to perform all statistical analysis and determine p-values, with p-value<0.05 considered

655　significant.

656

657　**Data and Software Availability**

658　The data that support the findings of this study and the custom scripts and pipelines for image

659　analysis are available from the Lead Contact upon reasonable request.

660

661　**Supplemental Information**

662   Supplemental Table 1 and Figures 1-7 with legends are attached following the main Figures in

663   the merged manuscript and uploaded as a separate file.

664

665   Supplemental Movies 1-25 show time-lapse imaging results underlying main and supplemental

666   Figures and are uploaded to the Immunity server:

667   **Movie S1.** Confocal time-lapse imaging of THP1 ASC-GFP (green) infected by Mtb::BFP (blue)

668   undergoing pyroptosis in the presence of Draq7 (magenta).

669

670   **Movie S2.** Confocal time-lapse imaging of THP1 ASC-GFP (green) infected by Mtb::BFP (blue)

671   undergoing non-pyroptotic necrosis in the presence of Draq7 (magenta).

672

673   **Movie S3.** Confocal time-lapse imaging of THP1 ASC-GFP (green) not infected by Mtb::BFP

674   (blue) undergoing apoptosis in the presence of Draq7 (magenta).

675

676   **Movie S4.** Confocal time-lapse imaging of THP1 ASC-GFP (green) infected by Mtb::BFP (blue)

677   undergoing ASC speck formation, pyroptosis and bacterial dissemination, in the presence of

678   Draq7 (magenta).

679

680   **Movie S5.** Confocal time-lapse imaging of THP1 ASC-mNeonGreen stained with the

681   mitochondrial membrane potential indicator TMRE (red, 10nM) and infected by Mtb::BFP

682   (blue).

683

684 **Movie S6.** Confocal time-lapse imaging of THP1 ASC-mNeonGreen (green) depleted for

685 GSDMD by CRISPR, stained with the mitochondrial membrane potential indicator TMRE (red,

686 10nM) and infected by Mtb::BFP (blue).

687

688 **Movie S7.** Confocal time-lapse imaging of THP1 ASC-mNeonGreen (green) stained with the

689 mitochondrial membrane potential indicator TMRE (red, 10nM) and treated with LPS+nigericin.

690

691 **Movie S8.** Confocal time-lapse imaging of THP1 ASC-mNeonGreen (green) depleted for

692 GSDMD by CRISPR, stained with the mitochondrial membrane potential indicator TMRE (red,

693 10nM) and treated with LPS+nigericin.

694

695 **Movie S9.** Confocal time-lapse imaging of THP1 ASC-GFP (green)/Gal3-mRuby3 (magenta)

696 infected by Mtb::BFP (blue).

697

698 **Movie S10.** Confocal time-lapse imaging of THP1 mNeonGreen-LC3B (green)/Gal3-mScarlet

699 (magenta) infected by Mtb::BFP (blue).

700

701 **Movie S11.** Confocal time-lapse imaging of THP1 Gal3-mScarlet (magenta) stained by

702 LysoView633 (green) and infected by Mtb::BFP (blue).

703

704 **Movie S12.** Confocal time-lapse imaging of THP1 Gal3-mScarlet (magenta) stained by

705 LysoView633 (green) and infected by Mtb::BFP (blue).

706

707 **Movie S13.** Confocal time-lapse imaging of THP1 ASC-GFP (red) stained by LysoView633

708 (green) and infected by Mtb::BFP (blue).

709

710 **Movie S14.** Confocal time-lapse imaging of THP1 mNeonGreen-ALG-2 (green)/Gal3-mScarlet

711 (magenta) infected by Mtb::BFP (blue).

712

713 **Movie S15.** Confocal time-lapse imaging of THP1 mNeonGreen-ALG-2 (green)/Gal3-mScarlet

714 (magenta) infected by Mtb::BFP (blue).

715

716 **Movie S16.** Simultaneous widefield and TIRF time-lapse imaging of THP1 mNeonGreen-ALG-2

717 (green)/Gal3-mScarlet (magenta) infected by Mtb::BFP (blue).

718

719 **Movie S17.** Widefield time-lapse imaging of THP1 mNeonGreen-ALG-2 (green) stained with

720 Calbryte-590 calcium indicator (grey) and infected by Mtb::BFP (blue).

721

722 **Movie S18.** Widefield time-lapse imaging of THP1 Gal3-SNAP(SiR) (magenta) stained with

723 Calbryte-590 calcium indicator (grey) and infected by Mtb::BFP (blue).

724

725 **Movie S19.** Confocal time-lapse imaging of THP1 mNeonGreen-ALG-2 (green) in the presence

726 of extracellular propidium iodide (20μg/mL, magenta) and infected by Mtb::BFP (blue).

727

728 **Movie S20.** Confocal time-lapse imaging of THP1 mNeonGreen-ALG-2 (green)/Gal3-mScarlet

729 (magenta)/ASC-mIRFP670 (grey) infected by Mtb::BFP (blue).

730

731 **Movie S21.** Simultaneous widefield and TIRF time-lapse imaging of THP1 mNeonGreen-ALG-2

732 (green)/Gal3-mScarlet (magenta) stimulated by silica (100μg/mL).

733

734 **Movie S22.** Confocal time-lapse imaging of THP1 mNeonGreen-ALG-2 (green)/Gal3-mScarlet

735 (magenta)/ASC-mIRFP670 (grey) stimulated by silica (100μg/mL).

736

737 **Movie S23.** Confocal time-lapse imaging of THP1 mNeonGreen-ALG-2 (green)/Gal3-mScarlet

738 (magenta)/ASC-mIRFP670 (grey) stimulated by LPS (10ng/mL) + nigericin (10μM).

739

740 **Movie S24.** Confocal time-lapse imaging of THP1 mNeonGreen-ALG-2 (green)/Gal3-mScarlet

741 (magenta)/ASC-mIRFP670 (grey) stimulated by LPS (10ng/mL) + imiquimod (20μg/mL).

742

743 **Movie S25.** Simultaneous widefield and TIRF time-lapse imaging of THP1 mNeonGreen-ALG-2

744 (green, TIRF and WF)/Gal3-mScarlet (magenta, TIRF and WF)/ASC-mIRFP670 (grey, WF),

745 loaded with $TPCS_{2a}$ (0.4 µg/mL, 16h pulse, 4h chase) and exposed to 405 nm blue light prior to

746 imaging.

747

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962

REAGENTS TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit mAb to human NLRP3	CST	Cat #13158S; RRID:AB_2798134
Rabbit pAb to human GSDMD	Novus Bio	NBP2-33422; RRID:AB_2687913
Rabbit pAb to human ALG-2	ThermoFisher	PA5-27639: RRID:AB_2545115
Rabbit pAb to human ALIX	Novus Bio	NBP1-90201; RRID:AB_11023702
Bacterial and Virus Strains		
Mtb H37Rv	ATCC	Cat #27294
Mtb H37RvΔRD1	Prof. R.Brosch	
Mtb H37Rv mc^{2}6206	Prof. W. Jacobs	(Jain *et al.*, 2014)
Mtb H37Rv::EBFP2	This work	
Mtb H37RvΔRD1::EBFP2	This work	
Mtb H37Rv mc^{2}6206::EBFP2	This work	
Mtb H37RvΔCpNT	Prof. M. Niederweiss	(Danilchanka *et al.*, 2014)
Mtb H37RvΔCpNT::TNT	Prof. M. Niederweiss	(Danilchanka *et al.*, 2014)
MtbH37Rv ΔCpNT::TNT* (catalytically inactive)	Prof. M. Niederweiss	(Danilchanka *et al.*, 2014)
Chemicals, Peptides, and Recombinant Proteins		
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich	Cat # P1585
Lymphoprep		
zVAD-FMK	Invivogen	Cat # tlrl-vad
VX-765	Invivogen	Cat # inh-vx765i
MCC950	Sigma-Aldrich	Cat #PZ0280
Potassium chloride	Sigma-Aldrich	Cat #P4504
Nec-1s	BioVision	Cat # 2263
GSK'872	BioVision	Cat # 2673
Cyclosporin A	Sigma-Aldrich	Cat # 30024
K777	Adipogen	Cat #AG-CR1-0158-M001
Ca-074-Me	Merck	Cat # 205531
Bafilomycin A1	Sigma-Aldrich	Cat # B1793
Cytochalasin D	Sigma-Aldrich	Cat #C8273
DRAQ7	Biolegend	Cat #424001
Tetramethylrhodamine ethyl ester perchlorate (TMRE)	Sigma-Aldrich	Cat # 87917
LysoView 633	Biotium	Cat # 70058
Calbryte-590	AAT Bioquest	Cat # 20700
SNAP-Cell 647-SiR	New England Biolabs	Cat # S9102S
Cresyl Violet	Sigma-Aldrich	Cat # C5042
TPSC$_{2a}$	PCI Biotech	

Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride	Sigma-Aldrich	Cat # C5429
FAM-YVAD-FMK	ImmunoChemistry Technologies	Cat #97
Critical Commercial Assays		
Human IL-1β DuoSet ELISA	R&D systems	Cat # DY201
LDH Cytotoxicity Detection Kit	Takara Bio	Cat # MK401
Experimental Models: Cell Lines		
THP-1	ATCC	Cat#TIB-202 RRID:CVCL_0006
THP-1 ASC-GFP	Prof. Emad Alnemri	(Fernandes-Alnemri *et al.*, 2007)
Human PBMC	St. Olavs blood bank	
Oligonucleotides		
GSDMD sgRNA: GGACAGGCAAAGATCGCAGG	This work	
NLRP3 sgRNA: GCTGCAAGCTGGCCAGGTAC	This work	
ALG-2 sgRNA: TGCCGGACCAGAGCTTCCTG	This work	
ALIX sgRNA: CTTAAGTCGAGAGCCGACCG	This work	
Recombinant DNA		
pEGFP-hGal3	Addgene	#73080
pCI-ASC-HA	Addgene	#41553
pKanCMV-mRuby3-10aa-H2B	Addgene	#74258
mNeonGreen-C1	Allele Biotech	
pBAD/His-miRFP670	Addgene	#79984
pSNAPf	New England Biosciences	#E9100S
pEntry-Galectin-3-L1-R5	This work	
pEntry-ASC-L1-R5	This work	
pEntry-ALG-2-L5-L2	This work	
pEntry-LC3B-L5-L2	This work	
pEntry-mNeonGreen-L1-R5	This work	
pEntry-mNeonGreen-L5-L2	This work	
pEntry-mScarlet-L5-L2	This work	
pEntry-mRuby3-L5-L2	This work	
pEntry-SNAPf-L5-L2	This work	
pEntry-mIRFP670-L5-L2	This work	
pLex307-Puro	Addgene	#413922
pLex307-Blast	This work	
pLex307-Hyg	This work	
LentiCrispr v2	Addgene	#52961
pMDL.g/pRRE	Addgene	#12251
pMD2.G	Addgene	#12259
pRSV-Rev	Addgene	#12253
pLex-Hyg-ASC-mNeonGreen	This work	
pLex-Hyg-ASC-mIRFP670	This work	
pLex-Blast-Galectin3-mScarlet	This work	

pLex-Blast-Galectin3-mRuby3	This work	
pLex-Blast-Galectin3-SNAPf	This work	
pLex-Hyg-mNeonGreen-LC3B	This work	
pLex-Hyg-mNeonGreen-ALG-2	This work	
msp12::EBFP2	Addgene	#30177
Software and Algorithms		
Graphpad Prism 8.0		graphpad.com/scientific-software/prism/
ImageJ-Fiji – Version: 2.0.0-rc-68/1.52h		imagej.net/Fiji/Downloads
CellProfiler 3.1.5		www.cellprofiler.org
Ilastik 1.3.0		www.ilasik.org
Adobe Illustrator 2019		www.adobe.com
Anaconda Python 3.7		https://www.anaconda.com/distribution/
Custom code for image analysis (Python)	This work	
Avizo	FEI	https://www.fei.com/software/avizo/
Dragonfly 4.0	Object Research Systems	https://www.theobjects.com/dragonfly/
Other		
#1.5 96 well glass bottom plate	Cellvis	Cat #P96-1.5H-N
35mm glass bottom dish	Ibidi	Cat #81218-200
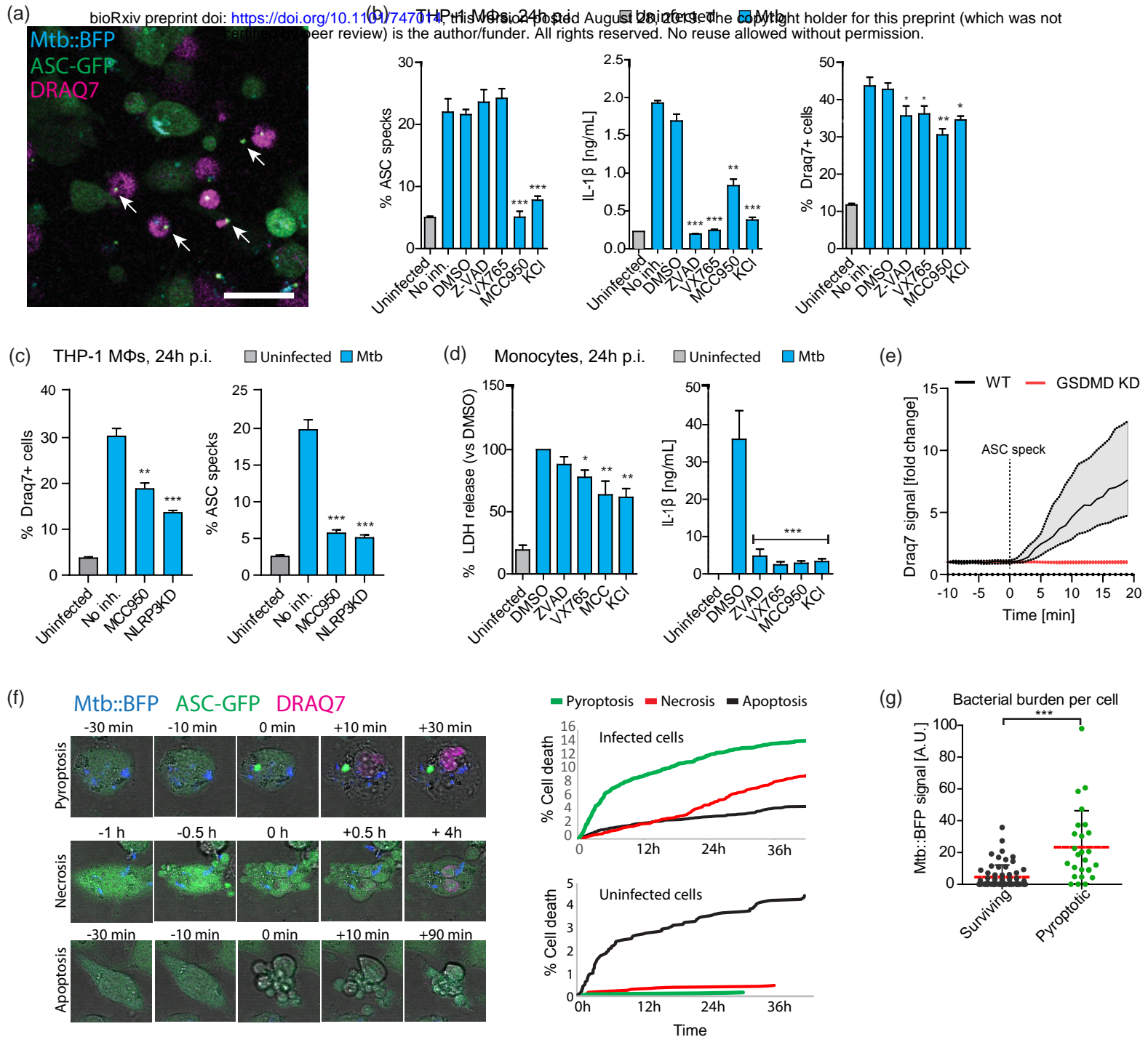
Figure 1. Mtb H37Rv infection induces canonical NLRP3 activation followed by pyroptosis

THP1 ASC-GFP macrophages were infected by MtbH37Rv::BFP at MOI 20, and imaged by time-lapse microscopy or at 24 hours post infection (p.i.).

(**a**) ASC-GFP specks observed as bright green specks (white arrows), while diffuse cytosolic green indicates live ASC-GFP expressing THP1 cells. DRAQ7 binds DNA in the nuclei of dead cells (magenta).

(**b**) THP1 ASC-GFP cells were treated with DMSO, Z-VAD-FMK (50µM), VX765 (50µM), MCC950 (10µM) or KCl (40mM) and infected with Mtb at MOI 20. DRAQ7+ cells and ASC specks were quantified at 24h p.i. (n>2000 cells in triplicates per condition). IL-1β release was determined by ELISA. Bars show mean±SEM.

(**c**) THP1 ASC-mNeonGreen wild-type or NLRP3 knockdown cells were infected by Mtb and imaged 24h p.i.

(**d**) Primary human monocytes were treated with inhibitors and infected with Mtb as before, and LDH and IL-1β release were determined 24h p.i. Bars show mean±SD for 4 donors.

(**e**) THP1 ASC-mNeonGreen wild-type or GSDMD knockdown cells were infected with Mtb and imaged by time-lapse microscopy. DRAQ7 intensity during ASC speck formation was measured in single cells in WT (n=57) and GSDMD KD (n=81) cells. Median±IQR shown.

(**f**) The cumulative number of pyroptotic, necrotic and apoptotic cell death events in THP1 ASC-GFP cells was quantified from a 40h time-lapse experiment during infection with Mtb. Representative cell death events in each class are shown.

(**g**) The bacterial burden (intracellular Mtb::BFP fluorescence) was determined immediately prior to ASC speck formation (n=26), and at the average time of ASC speck formation, in cells surviving a 24h time-lapse experiment (n=61). Lines indicates median values.

*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, by one-way ANOVA with Dunnett's test in (a)-(d), and Mann-Whitney test in (g). Data in (a)-(e) representative of 3-5 independent experiments. See also Figure S1.

Figure 2. Pyroptosis causes severe cellular damage and allows for spread of Mtb

Ultrastructural comparison by FIB-SEM tomography between (**a**) uninfected (**b**) infected, pyroptotic and (**c**) infected, living cell with an activated inflammasome. THP1 ASC-GFP cells were infected with Mtb H37Rv::BFP (MOI 20) and fixed after (**b**) 24 hours or (**c**) 5h in the absence or presence of Z-VAD FMK (50µM) and VX765 (50µM), respectively. The uninfected cell (**a**) is from a 24h infection experiment but was found to be uninfected from confocal microscopy and FIB-SEM. Insets (i), (ii) and (iii) highlight mitochondria/ER/Golgi, plasma membrane morphology and nuclear membrane morphology, respectively. Yellow stars indicate mitochondria, blue arrowheads ER, orange arrowheads Golgi, red arrowheads indicate plasma membrane ruptures and black arrowheads indicate nuclear membranes. Black inset scalebars 1µm.

(**d**) Structure of ASC specks induced by Mtb infection in THP1 ASC-GFP cells. Cell number 1) and 2) are the same as in b) and c), respectively. Cells were imaged by correlative confocal microscopy and FIB-SEM, and ASC specks were reconstructed in 3D from the respective FIB-SEM image stacks.

(**e**) Confocal and FIB-SEM correlative microscopy of ASC specks in THP1 ASC-GFP cells and primary human monocytes primed for 3h by LPS (10ng/ml) and treated with 6.7µM nigericin for 1-2h. Monocytes were additionally treated with FLICA caspase-1 reagent (FAM-YVAD-FMK, 20µM) for visualization of active caspase-1 at the inflammasome.

(**f**) Quantification of intracellular Mtb::BFP fluorescence in single cells before and after ASC speck formation and pyroptosis for n=10 representative cells.

(**g**) THP1 ASC-GFP (green) cells in DRAQ7 containing medium (magenta) and infected with Mtb::BFP (MOI 20, blue) imaged by time-lapse confocal microscopy for 24h. Arrowheads indicate Mtb that is phagocytosed twice during the time course, and arrows indicate ASC specks. See also Figure S2.

(a) THP-1 MΦs, 24h p.i.

(b) THP-1 MΦs, 24h p.i.

(c) THP-1 MΦs, 24h p.i.

(d)

(e)

Figure 3. Mtb-induced inflammasome activation is dependent on ESX-1 but not on TNT or destabilization of host mitochondria
(**a**) THP1 ASC-GFP macrophages were infected with Mtb H37Rv ΔRD-1 or Mtb H37Rv cultured in the presence or absence of tween-80 at MOI 20.
(**b**) THP1 ASC-GFP cells were not treated or treated with MCC950 (10µM) and infected with Mtb H37Rv or Mtb H37RvΔCpNT (MOI 20).
(**c**) THP1 ASC-GFP cells were treated with DMSO or cyclosporine A (CsA, 5µM) and infected by Mtb H37Rv MOI 20.
In (**a-c**) ASC specks and DRAQ7+ cells were quantified for n>2000 cells per condition, *p<0.05, **p<0.01, ***p<0.001, by one-way ANOVA with Dunnett's test. Bars show mean±SEM. Data representative of 3 independent experiments.
(**d**) Representative time-lapse images of THP1 ASC-mNeonGreen (green) cells or THP1 ASC-mNeonGreen cells depleted of GSDMD by CRISPR-Cas9, with mitochondria stained by TMRE (red, 10nM) and infected with Mtb::BFP (blue) during ASC speck formation.
(**e**) Quantification of TMRE intensity, indicative of mitochondrial membrane potential before and after ASC speck formation in WT and GSDMD depleted cells (n=57 and n=81 cells). Median±IQR shown. Representative of 2 independent experiments. See also Figure S3.
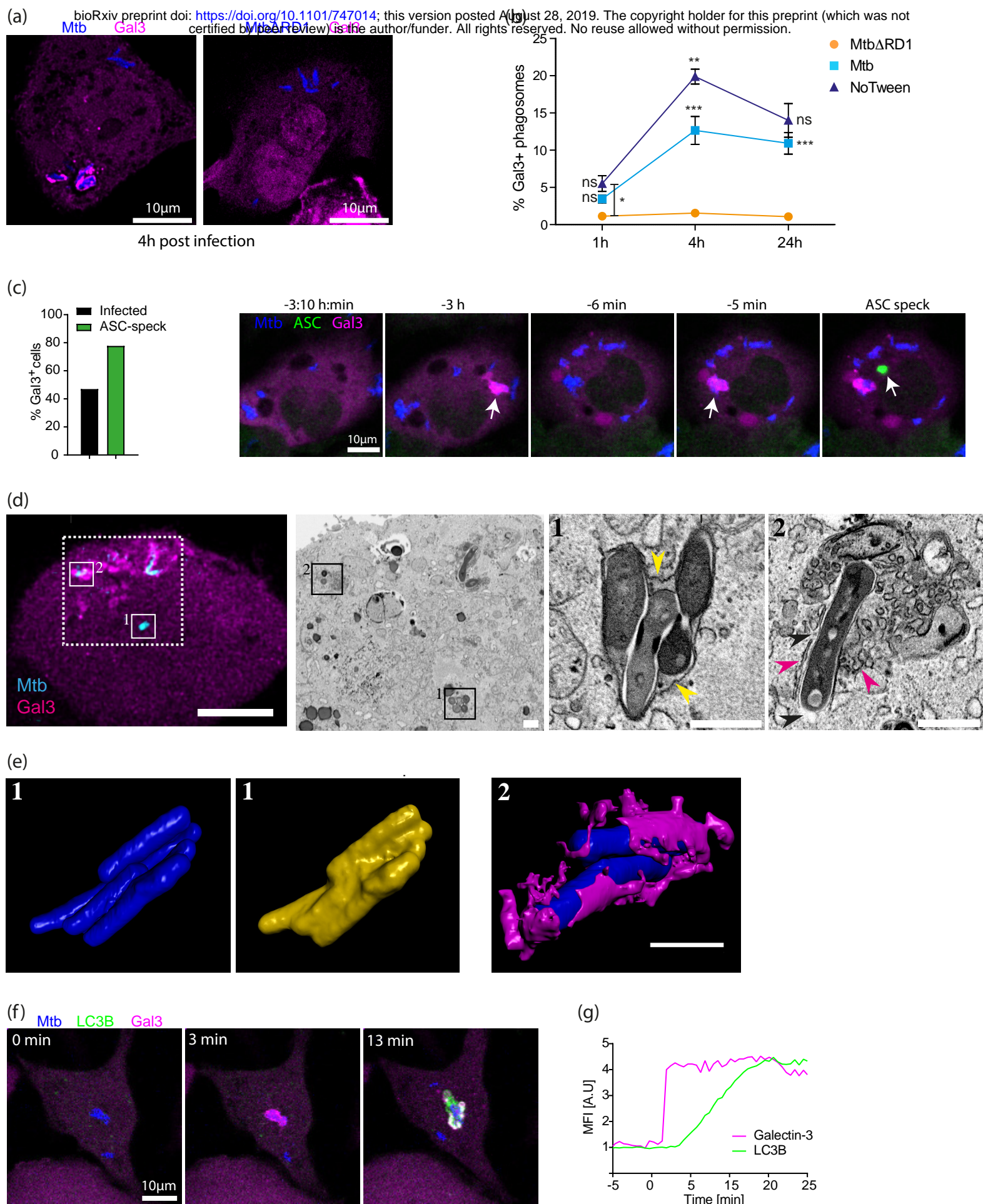
Figure 4. ESX-1-mediated phagosomal damage is a prerequisite for NLRP3 activation by phagosomal Mtb

Figure 4. ESX-1-mediated phagosomal damage is a prerequisite for NLRP3 activation by phagosomal Mtb

(**a**) Representative images of THP1 Galectin3-mScarlet (magenta) cells infected with MtbH37Rv::BFP or Mtb H37RvΔRD1::BFP (blue), 4h p.i.,

(**b**) THP1 Gal3-mScarlet cells were fixed at indicated time points post infection with Mtb H37Rv::BFP, Mtb H37Rv ΔRD1::BFP or Mtb H37Rv::BFP cultured with or without tween-80 (all MOI 20) and imaged by confocal microscopy. The percentage of Gal3$^+$ phagosomes was quantified (n>4000 phagosomes per condition, 3 independent experiments) by automated image analysis. Phagosomes with more than one bacterium were considered single phagosomes. *p<0.05, **p<0.01, ***p<0.001, by one-way ANOVA with Tukey's test. Statistics are calculated compared to the closest lower condition, except for the star between ΔRD1 and noTween at 1h as indicated. Mean±SEM shown.

(**c**) THP1 ASC-GFP/Galectin3-mRuby3 cells were imaged by confocal time-lapse for 24h, and the occurrence of Mtb-associated Gal3+-events in infected, surviving cells (n=85) compared to cells undergoing pyroptosis (n=56 cells) was quantified. A representative time-lapse showing Gal3-mRuby3 (magenta) accumulation at Mtb::BFP phagosomes (blue) prior to ASC speck (green) formation.

(**d**) Correlative confocal and FIB-SEM imaging of a non-pyroptotic THP1 ASC-GFP/Galectin3-mRuby3 (magenta) macrophage infected with Mtb H37Rv::BFP (cyan) for 24 hours. Dashed square indicates FIB-SEM area, and higher magnification insets are numbered (1) and (2) for Gal3$^-$ and Gal3$^+$ Mtb. Yellow arrowheads in (1) indicate the intact phagosomal membrane; magenta arrowheads in (2) indicate patches of host cell membrane that are recruited around a bacterium, while absence of membrane is indicated by black arrowheads. Scale bars are 10 µm, 5 µm, 1 µm and 1 µm respectively.

(**e**) 3D reconstructions of FIB-SEM tomography data of the bacteria from insets 1 and 2 (blue), along with the corresponding intact phagosomal membrane (yellow) or patches of host cell membrane (magenta).

(**f**) THP1 mNeonGreen-LC3B (green)/Galectin3-mScarlet (magenta) macrophages were infected with Mtb H37Rv::BFP (blue) and imaged by time lapse microscopy.

(**g**) Normalised fluorescent intensities of one representative Gal3 and LC3B event of n>280 events in 2 independent experiments is shown. *See also Figure S4.*
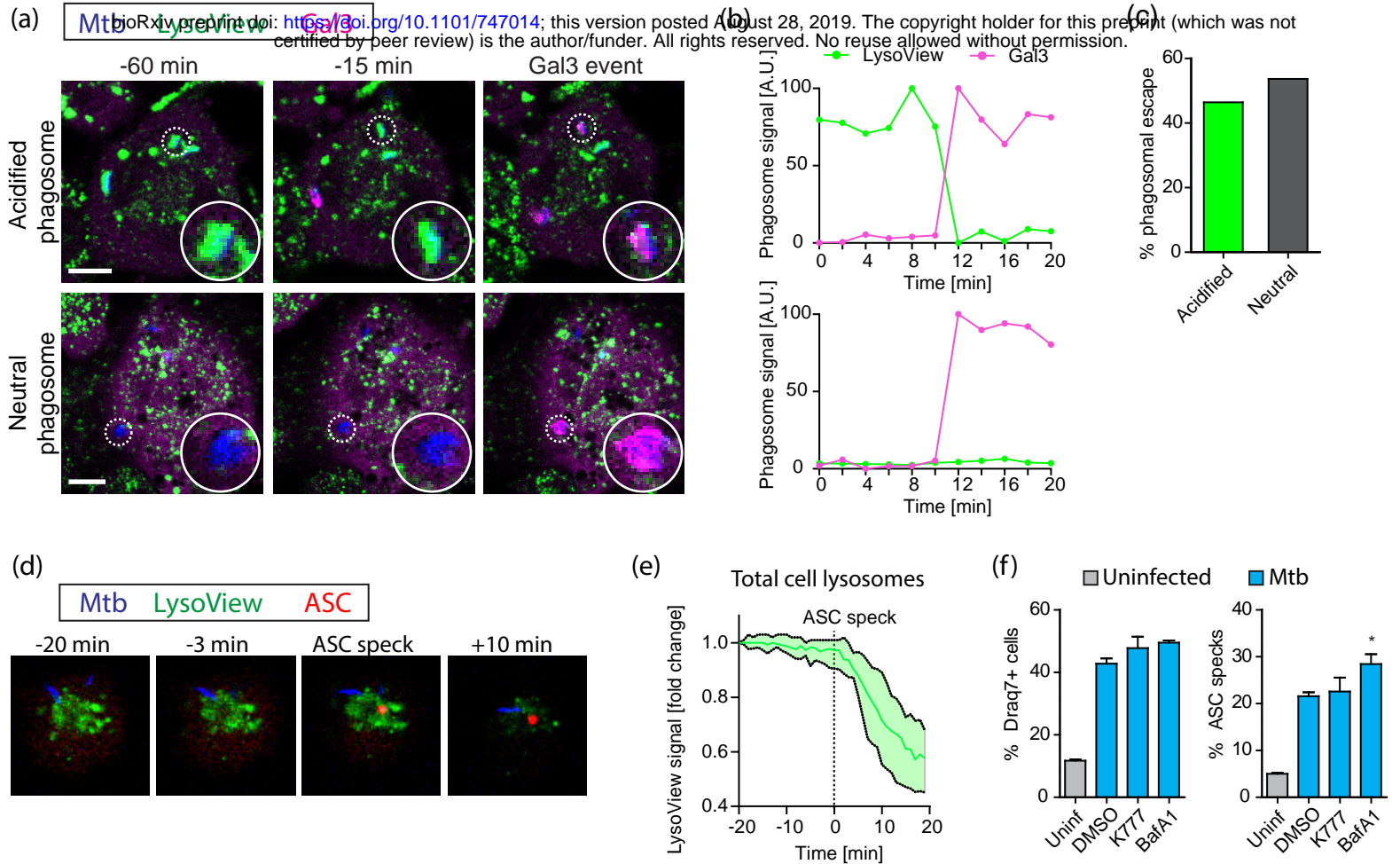
Figure 5. Inflammasome activation by Mtb is independent of lysosomal damage and release of active cathepsins

(**a**) Representative images from 24 hours of time-lapse confocal imaging of THP1 Galectin-3-mScarlet (magenta) macrophages labelled with LysoView633 (green) and infected with Mtb H37Rv::BFP (blue).

(**b**) Representative traces of Galectin-3 and LysoView signals for the Mtb phagosomes highlighted in (a).

(**c**) Quantification of the occurrence of Mtb-associated Gal3+ events occurring on acidified (LysoView+) and neutral (LysoView-) phagosomes. n=69 Mtb phagosomes were analysed.

(**d**) Representative images from confocal time-lapse microscopy of ASC speck formation in THP1 ASC-GFP (red) cells labelled with LysoView633 (green) infected by Mtb H37Rv::BFP (blue).

(**e**) Quantification of the average LysoView signal in single cells before and after ASC speck formation. n=27 cells analysed, median±IQR shown.

(**f**) THP1 ASC-GFP macrophages were treated with DMSO, K777 (15 µM) or BafA1 (50 nM) and infected with Mtb H37Rv. After 24h, ASC specks and DRAQ7+ cells were quantified for n>2000 cells per condition, and the data were analysed by one-way ANOVA with Dunnett's test. Mean±SEM shown. Data representative of 3 independent experiments. See also Figure S5.
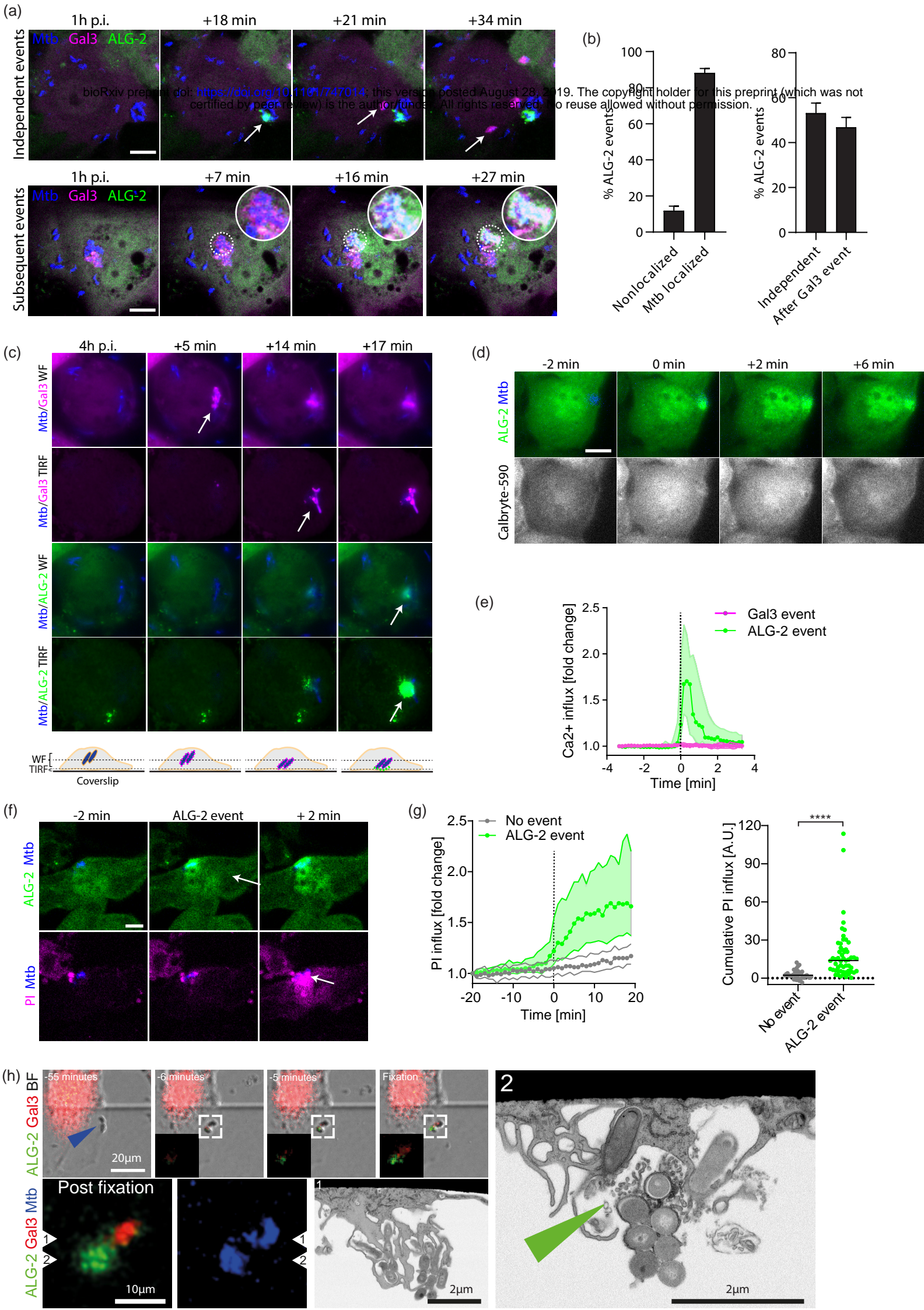
Figure 6. Mtb carrying ESX-1 can directly damage the host cell plasma membrane

THP1 Galectin3-mScarlet/mNeonGreen-ALG-2, THP1 mNeonGreen-ALG-2 or THP1 mNeonGreen-ALG-2/Galectin-3-SNAP(SiR) macrophages were infected with Mtb H37Rv::BFP or Mtb H37Rv mc^{2}6206 stained by eFluor450 and imaged live by confocal (Rv::BFP) or widefield and TIRF microscopy (Rv mc^{2}6206).

(**a**) Representative confocal time-lapse data showing ALG-2 (green) recruitment to plasma membrane-Mtb (blue) contact points either independent of or after Galectin-3 (magenta) recruitment.

(**b**) Quantification of ALG-2 events independent of or after Galectin-3 recruitment to Mtb phagosomes and the localization of ALG-2 events. n=213 and n=321 events from 3 independent experiments analysed.

(**c**) Simultaneous widefield (WF) and TIRF time-lapse imaging of ALG-2 (green) and Galectin-3 (magenta) during Mtb infection (blue). Representative of n>10 experiments with similar events.

(**d**) Time-lapse of Ca^{2+} influx (Calbryte-590, grey) upon ALG-2 (green) recruitment to Mtb (blue).

(**e**) Quantification of Calbryte-590 signal during ALG-2 recruitment and Gal3 recruitment events (n=10 events per condition). Median±IQR shown.

(**f**) Representative time-lapse images of local extracellular propidium iodide (PI, 20µg/mL) influx during ALG-2 (green) recruitment to Mtb H37Rv::BFP (blue).

(**g**) Quantification of 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, representative of 2 independent experiments. ****p<0.0001 by Mann-Whitney test.

(**h**) Time-lapse confocal imaging of Mtb-localized ALG-2 event indicated by arrow in THP-1 mNeonGreen-ALG-2 cells infected by Mtb H37Rv::BFP. After time-lapse imaging, cells were fixed and imaged by confocal and FIB-SEM tomography. The FIB-SEM imaging plane is perpendicular to the confocal image and indicated by triangles. Location of ALG-2 on confocal image is indicated by green triangle in FIB-SEM. Confocal scale bars: 10 µm. *See also Figure S6.*
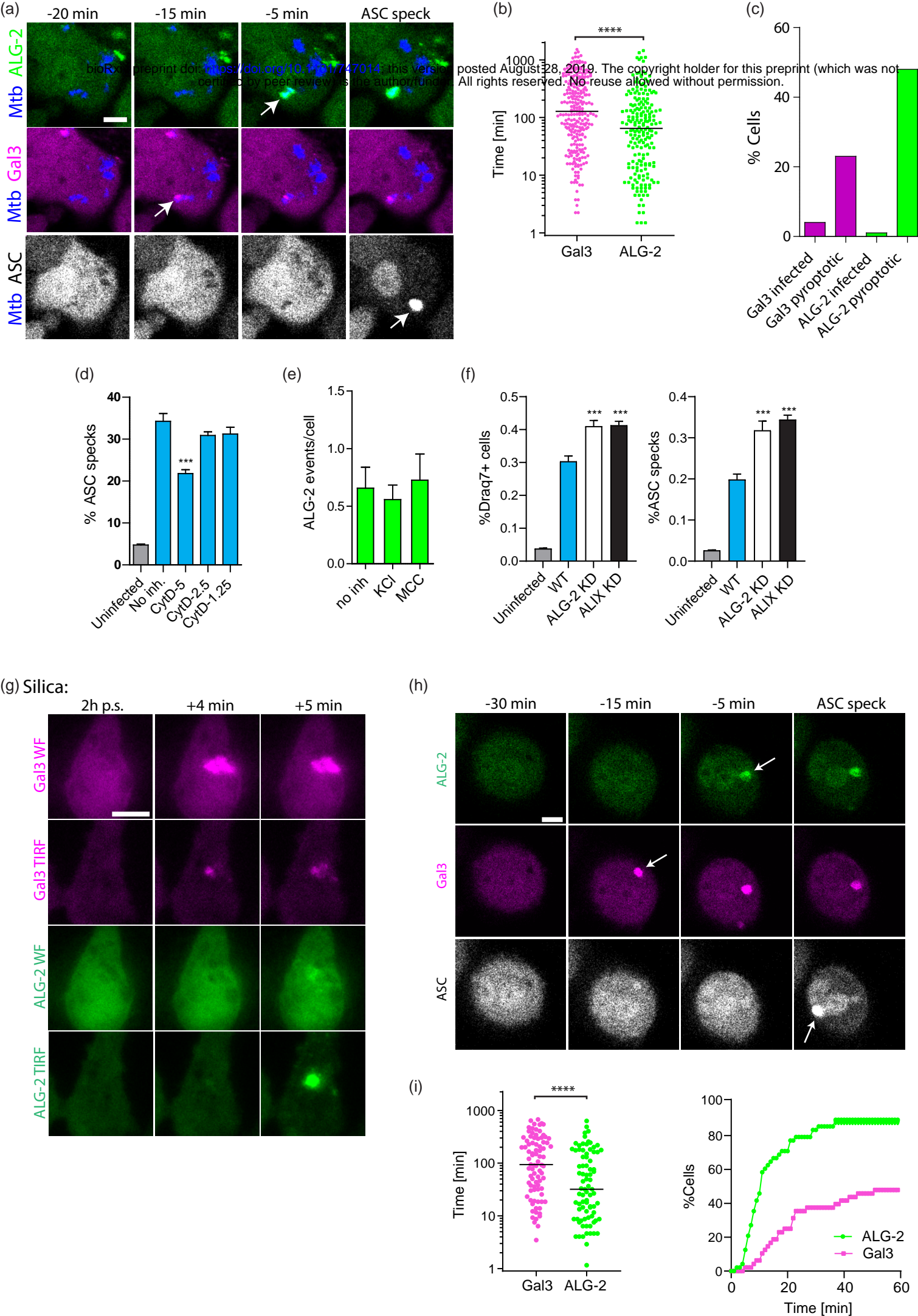
Figure 7. Plasma membrane damage caused by Mtb or Silica activates the NLRP3 inflammasome

Figure 7. Plasma membrane damage caused by Mtb or Silica activates the NLRP3 inflammasome

THP1 mNeonGreen-ALG-2/Galectin-3-mScarlet/ASC-mIRFP670 cells were infected by Mtb H37Rv::BFP (MOI 10) and imaged by time-lapse microscopy for 24h.

(**a**) Representative time-lapse of ALG-2 (green), Gal3 (magenta) and ASC (grey) dynamics during Mtb infection (blue).

(**b**) Timing of Mtb-localized Gal-3 and ALG-2 events compared to ASC speck formation in all cells forming ASC specks. n=200 ALG-2 and n=262 Gal-3 events analysed from 3 independent 24h time-lapse experiments. Lines indicate median values. ****p<0.0001 by Mann-Whitney test.

(**c**) Percentage of cells with ALG-2 and Gal3 events in the population of cells forming ASC specks (pyroptotic) within 20 minutes before ASC speck formation, compared to the percentage of cells in the surviving infected population with events in an average 20 min period.

(**d**) % ASC specks in cells infected with Mtb H37Rv::BFP MOI5 for 24h in the presence or absence of the indicated concentration (in µM) of the inhibitor cytochalasin D

(**e**) Average number of Mtb-localized ALG-2 events per cell during 24h infection in the absence or presence of KCl (40mM) or MCC950 (10µM).

(**f**) Quantification of DRAQ7+ cells and ASC specks 24h p.i. (MOI 20) in THP1 ASC-mNeonGreen (WT) cells and WT cells depleted of ALG-2 or ALIX by CRISPR-Cas9. n>2000 cells per condition, and the data was analysed by one-way ANOVA with Dunnett's test. Mean±SEM shown, *p<0.05, **p<0.01. Data representative of 2 independent experiments.

(**g**) Simultaneous widefield (WF) and TIRF time-lapse imaging of ALG-2 (green) and Gal-3 (magenta) during 4h treatment with silica (100µg/mL).

(**h**) Representative confocal time-lapse images of a cell forming an ASC speck (grey) during 24h treatment with 100µg/mL silica.

(**i**) Cells were treated with 100µg/mL silica. Timing of Gal-3 and ALG-2 events compared to ASC speck formation in all cells forming ASC specks. n=82 ALG-2 and n=85 Galectin-3 events analysed from 3 independent 24h time-lapse experiments. Lines indicate median values. Quantification of the percentage of cells with ALG-2 or Gal-3 events in the given time interval before ASC speck formation in single cells. n=48 cells from 3 independent 24h time-lapse experiments. ****p<0.0001 by Mann-Whitney test. Confocal scale bars: 10 µm. *See also Figure S7.*
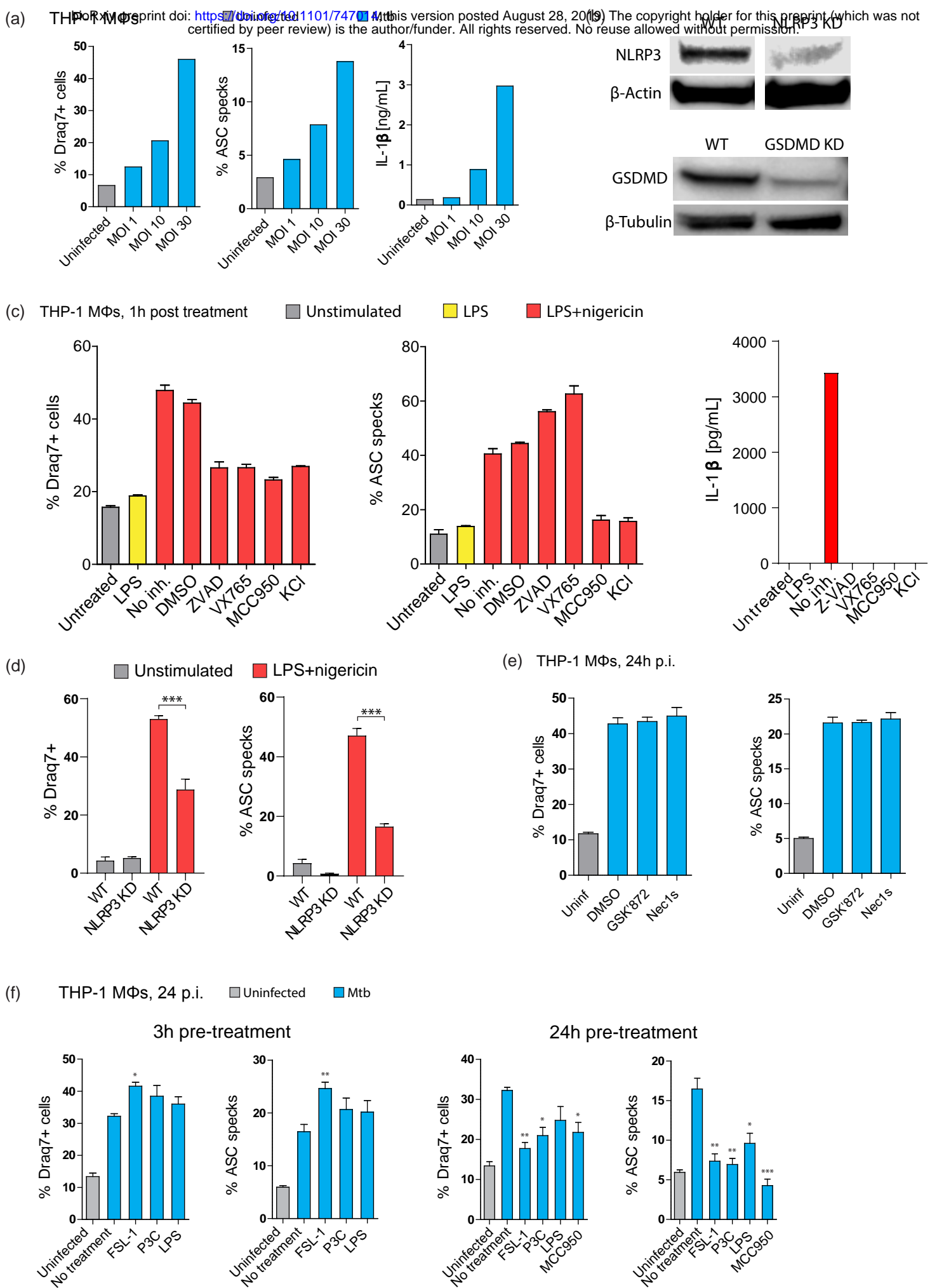
Figure S1, related to main Figure 1

Figure S1, related to main Figure 1.

(**a**) THP1 ASC-GFP cells were infected with different MOI of MtbH37Rv. Draq7+ cells, ASC specks and IL-1β release was measured after 24h. Data representative of 2-3 independent experiments.
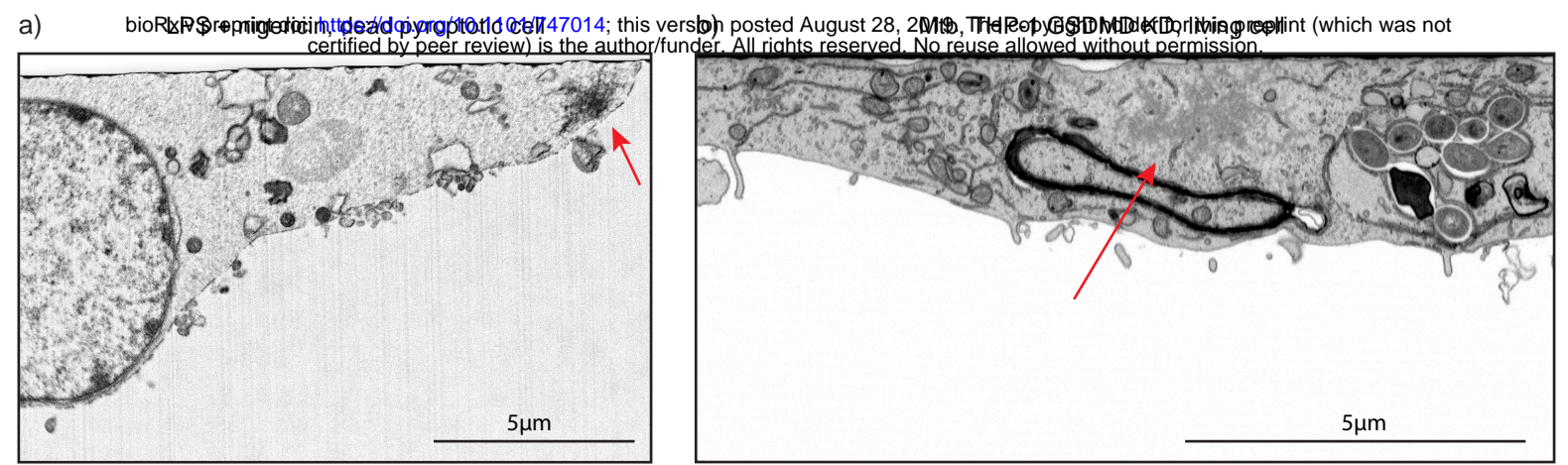
(**b**) THP1 ASC-mNeonGreen cells and THP1 ASC-mNeonGreen depleted of NLRP3 or GSDMD by CRISPR-Cas9 were lysed and protein levels measured by western blotting.

(**c**) THP1 ASC-GFP cells were stimulated by LPS (10ng/mL, 3h), not treated or treated with DMSO, Z-VAD-FMK (50µM), VX765 (50µM), MCC950 (10µM) or KCl (40mM) and nigericin (10µM, 1h). Draq7+ cells and ASC specks were quantified from n>2000 cells per condition in triplicate, and IL-1β in the supernatant was quantified by ELISA.

(**d**) THP1 ASC-mNeonGreen cells and THP1 ASC-mNeonGreen depleted of NLRP3 by CRISPR-Cas9 were stimulated by LPS and nigericin as above, and Draq7+ cells and ASC specks were quantified from n>2000 cells in triplicates.

(**e**) THP1 ASC-GFP cells were treated by DMSO, GSK'872 (5µM) or Nec1s (10µM) and infected by Mtb H37Rv (MOI 20). Draq7+ cells and ASC specks were quantified 24h p.i. for n>2000 cells per condition in triplicate.

(**f**) THP1 ASC-GFP cells were untreated or stimulated with FSL-1, Pam3Cys4K or LPS (all 10ng/mL) for 3h or 24h prior to infection with MtbH37Rv and analysed 24h p.i. for Draq7+ cells and ASC specks. n>2000 cells per condition in triplicate were quantified.

a)

Figure S2, related to main Figure 2. Single slices from FIB-SEM tomography of (**a**) THP1 ASC-GFP cell treated with LPS (10ng/mL, 3h) and nigericin (10μM, 1h) (**b**) THP1 ASC-mNeonGreen depleted of GSDMD by CRISPR-Cas9, infected with Mtb H37Rv and fixed after ASC speck formation while the cell was still alive (DRAQ7 negative and normal morphology). ASC specks indicated by arrows.

(a) THP1 ASC-GFP 24h p.i.

(b) LPS+nigericin, 1h | ASC-GFP TMRE
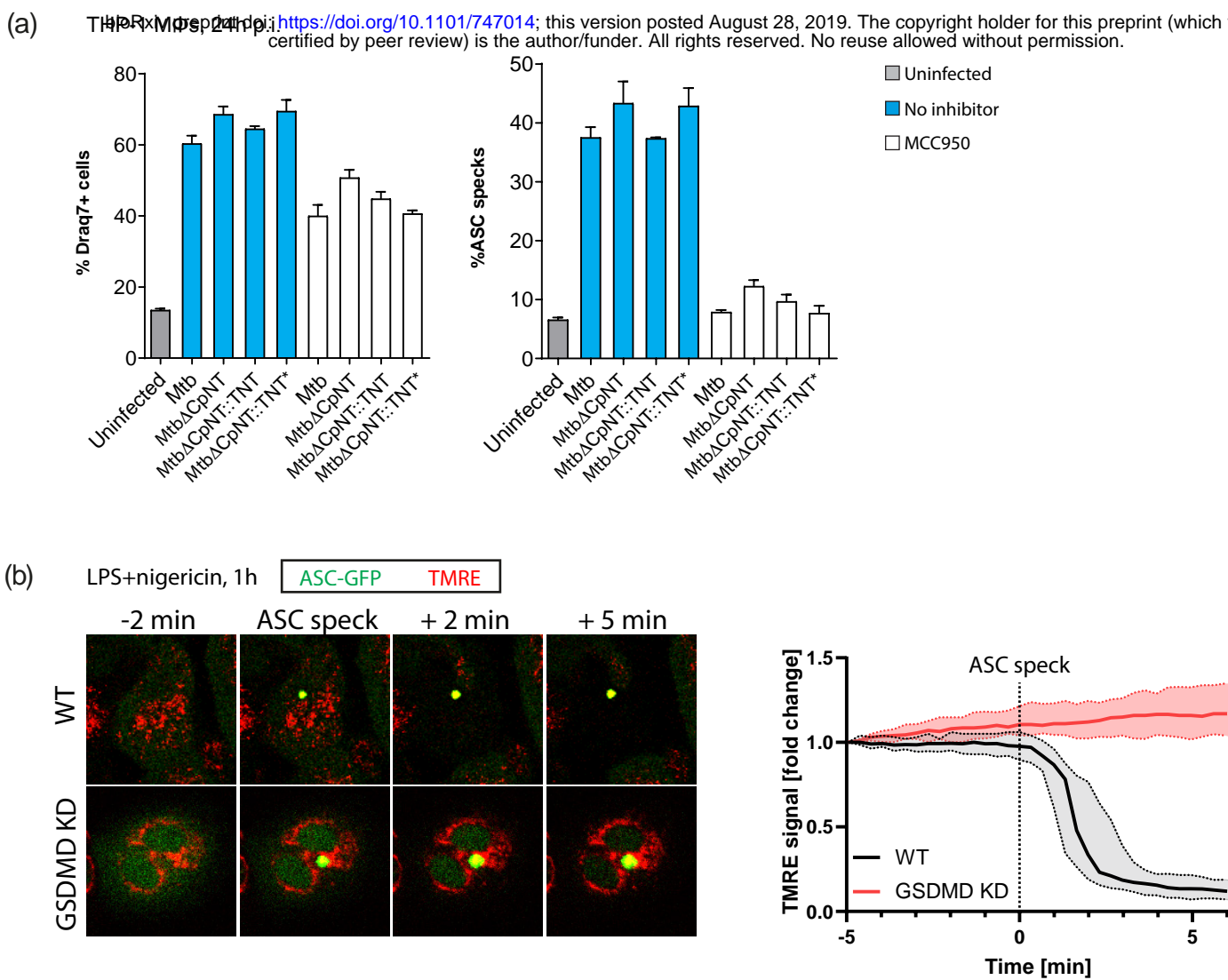
Figure S3, related to main Figure 3.

(**a**) THP1 ASC-GFP cells were infected with Mtb H37Rv, Mtb H37RvΔCpNT, Mtb H37RvΔCpNT::TNT and Mtb H37RvΔCpNT::TNT* (catalytically inactive TNT) in the absence or presence of MCC950 (10μM). Draq7+ cells and ASC specks were quantified 24h p.i. for n>2000 cells per condition in triplicate.

(**b**) THP1 ASC-GFP cells or THP1 ASC-mNeonGreen cells depleted of GSDMD by CRISPR-Cas9 were labelled with TMRE (10nM) and treated by LPS (10ng/mL) for 3h and nigericin (10μM) and imaged by time-lapse microscopy. Representative images of TMRE and ASC during ASC speck formation are shown. Quantification of TMRE intensity in single cells during ASC speck formation in ASC-mNeonGreen (n=67 cells) and ASC-mNeonGreen GSDMD KD cells (n=44 cells). Median±IQR shown.

(a) Mtb LysoView633 Gal3
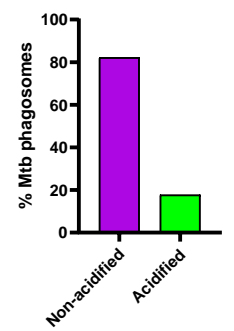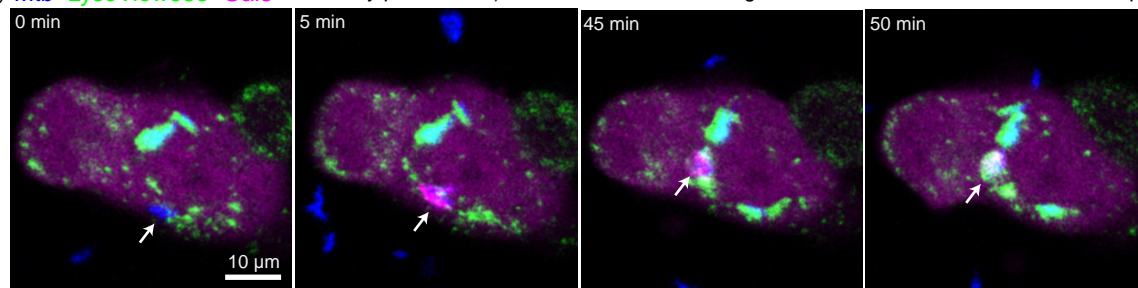
Figure S4, related to main Figure 4.
(**a**) Images from confocal time-lapse microscopy of THP1-Gal3-mScarlet cells labelled with LysoView633 and infected by Mtb H37Rv::BFP.
(**b**) Proportion of Mtb phagosomes gaining a LysoView signal indicating acidification after a Gal3 event, within 24h or until cell death occurred. n=63 events from one representative experiment.

(a)
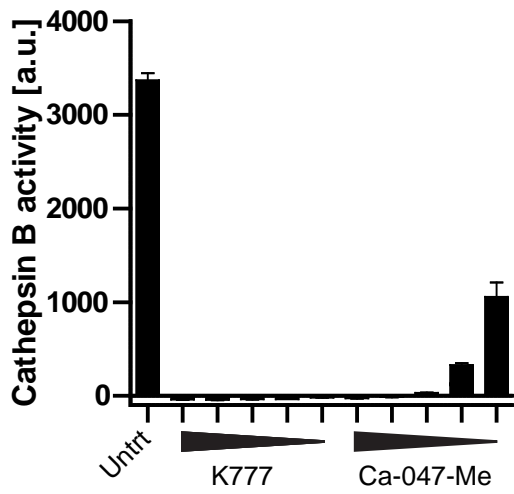
(b) LPS + nigericin
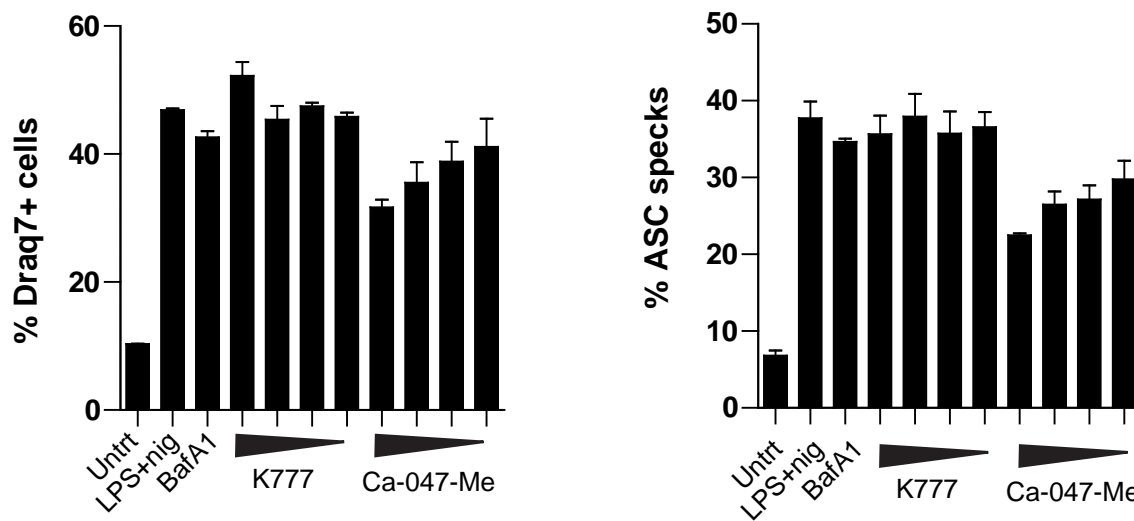
Figure S5, related to main Figure 5.
(**a**) Cathepsin B activity measured by Vmax of Z-RR-AMC conversion in total cell lysates without or with 1h pre-treatment by cathepsin inhibitors K777 (30, 15, 7.5, 3.75, 1.6 µM) or Ca-074-Me (30, 15, 7.5, 3.75, 1.6 µM) for 1h. Mean±SEM of technical triplicates.
(**b**) Dose response of LPS+nigericin-treated THP-1 macrophages to Bafilomycin A1 (50 nM), K777 (30, 15, 7.5, 3.75 µM) and Ca-074-Me (30, 15, 7.5, 3.75 µM). Cells were primed with 10ng LPS for 3h, pre-treated with inhibitors for 30min than treated with 5µM nigericin for 2h.

(a)

(b)
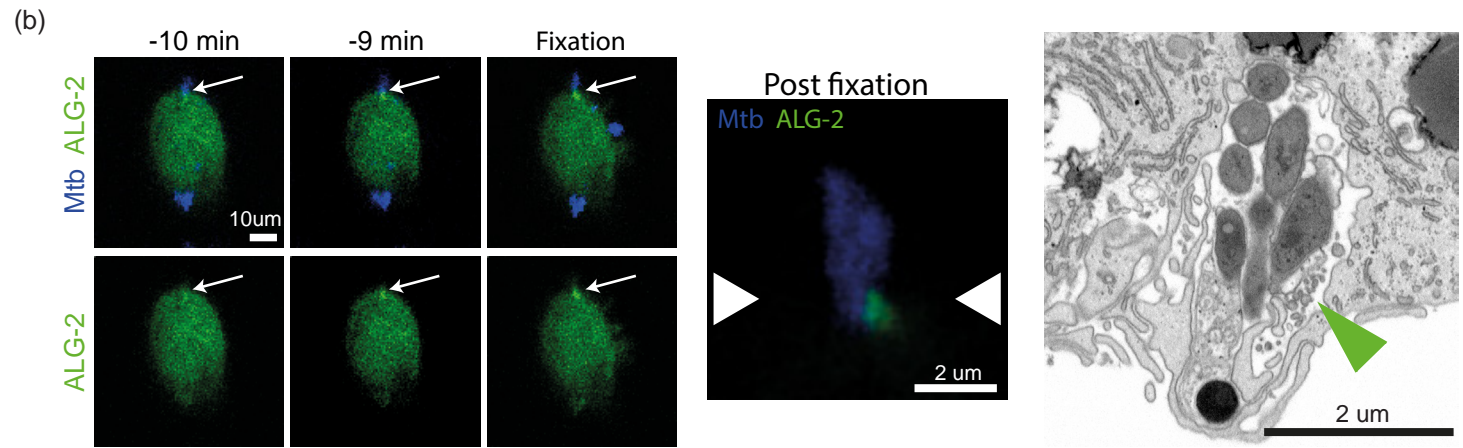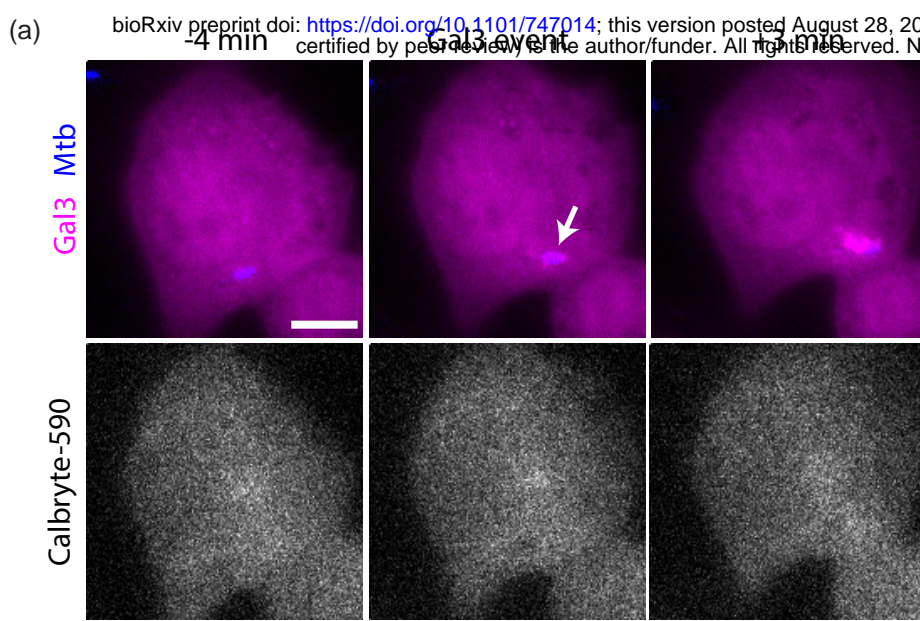
Figure S6, related to main Figure 6.

(**a**) Representative images from time-lapse microscopy of Ca2+ sensitive Calbryte-590 signal during an Mtb localized Gal3 event in THP1 Galectin-3-SNAP (SiR) cells.

(**b**) Time-lapse confocal imaging of Mtb-localized ALG-2 event indicated by arrow in THP-1 mNeonGreen-ALG-2 cells infected by Mtb H37Rv::BFP. After time-lapse imaging, cells were fixed and imaged by Airyscan and FIB-SEM tomogra-
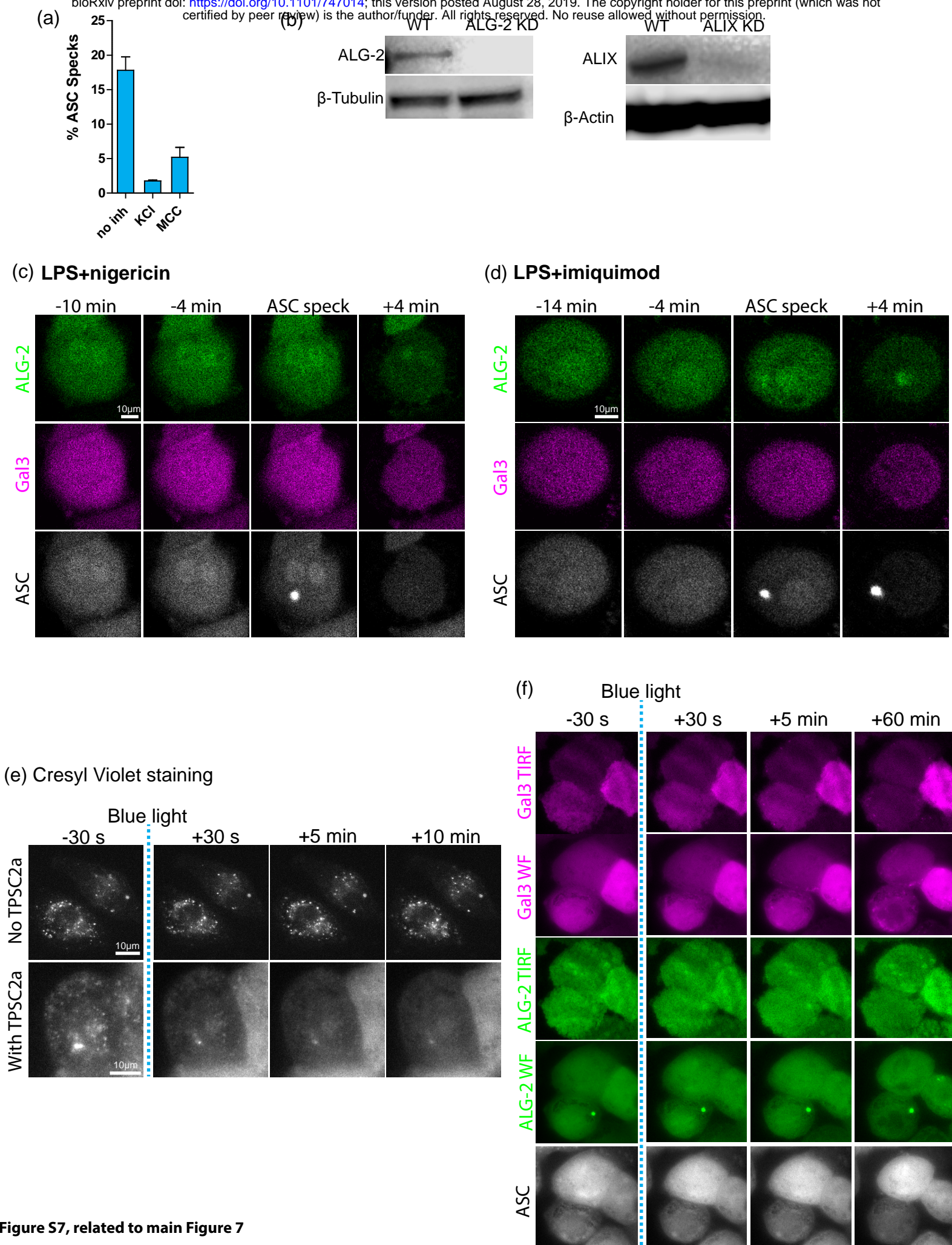
(a)

(b) WT · ALG-2 KD   WT · ALIX KD

(c) **LPS+nigericin**

(d) **LPS+imiquimod**

(e) Cresyl Violet staining

(f)

Figure S7, related to main Figure 7

Figure S7, related to main Figure 7.

(**a**) Quantification of ASC speck formation from 24h time-lapse experiments of THP1 mNeonGreen-ALG-2/Galectin-3-mScarlet/ASC-mIRFP670 cells not treated or treated by KCl (40mM) or MCC950 (10μM) and infected by Mtb H37Rv::BFP, corresponding to experiment in Figure 7. n>200 cells analysed per condition, mean±SD of 5 fields of view shown.

(**b**) ALG-2 or ALIX protein levels in lysates of THP1 ASC-mNeonGreen or ASC-mNeonGreen cells depleted of ALG-2 or ALIX by CRISPR-Cas9.

(**c**) THP1 mNeonGreen-ALG-2 (green)/Galectin-3-mScarlet (magenta)/ASC-mIRFP670 (grey) cells treated with LPS (10ng/mL, 3h) and imaged by time-lapse confocal microscopy during treatment with nigericin (10μM). Representative of n=100 similar events.

(**d**) THP1 mNeonGreen-ALG-2 (green)/Galectin-3-mScarlet (magenta)/ASC-mIRFP670 (grey) cells treated with LPS (10ng/mL, 3h) and imaged by time-lapse confocal microscopy during treatment with imiquimod (20μg/mL). Representative of n=132 similar events.

(**e**) THP1 cells with lysosomes labelled with Cresyl Violet (grey) and pulsed (16h) and chased (4h) with TCSP2a (0.4μg/mL), then exposed to 1s of 10% power 405 laser in a widefield microscope and imaged by time-lapse. Representative of n>50 cells in 5 independent experiments.

(**f**) THP1 mNeonGreen-ALG-2 (green)/Gal3-mScalet (magenta)/ASC-mIRFP670 (grey) cells pulsed and chased with TCSP2a (0.4μg/mL), exposed to 405 nm blue light, and imaged for 60 minutes by simultaneous TIRF and widefield time-lapse microscopy. Representative of n>30 cells in 5 independent experiments.