1 **NLRP11 is required for canonical NLRP3 and non-canonical inflammasome**

2 **activation during human macrophage infection with mycobacteria**

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14 Running Head: Mycobacteria NLRP3 and NLRP11 inflammasome activation.

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ABSTRACT

The NLRP11 protein is only expressed in primates and participates in the activation of the canonical NLRP3 and non-canonical NLRP3 inflammasome activation after infection with gram-negative bacteria. Here, we generated a series of defined NLRP11 deletion mutants to further analyze the role of NLRP11 in NLRP3 inflammasome activation. Like the complete NLRP11 deletion mutant ($NLRP11^{-/-}$), the NLRP11 mutant lacking the NACHT and LRR domains ($NLRP11^{\Delta N\_LRR}$) showed reduced activation of the canonical NLRP3 inflammasome, whereas a pyrin domain mutant ($NLRP11^{\Delta PYD}$) had no effect on NLRP3 activation. The $NLRP11^{-/-}$ and $NLRP11^{\Delta N\_LRR}$ mutants but not the $NLRP11^{\Delta PYD}$ mutant also displayed reduced activation of caspase-4 during infection with the intracytosolic, gram-negative pathogen *Shigella flexneri*. We found that the human adapted, acid-fast pathogen *Mycobacterium tuberculosis* and the opportunistic pathogen *M. kansasii* both activate the non-canonical NLRP11 inflammasome in a caspase-4/5-dependent pathway. In conclusion, we show that NLRP11 functions in the non-canonical caspase-4/5 inflammasome activation pathway and the canonical NRLP3 inflammasome pathway, and that NLRP11 is required for full recognition of mycobacteria by each of these pathways. Our work extends the spectrum of bacterial pathogen recognition by the non-canonical NLRP11-caspase4/5 pathway beyond gram-negative bacteria.

43 **IMPORTANCE**

44 The activation of inflammasome complexes plays a crucial role in intracellular

45 pathogen detection. NLRP11 and caspase-4 are essential for recognizing

46 lipopolysaccharide (LPS), a molecule found in gram-negative bacteria such as

47 the human pathogens *Shigella* spp., which activate both canonical NLRP3 and

48 non-canonical inflammasome pathways. Through a series of deletion mutants,

49 we demonstrate that the NACHT and LRR domains of NLRP11, but not its pyrin

50 domain, are critical for detection of *S. flexneri*. Notably, our research reveals that

51 the acid-fast bacterium *M. tuberculosis* is also detected by NLRP11 and

52 caspase-4, despite not producing LPS. These findings significantly expand the

53 range of pathogens recognized by NLRP11 and caspase-4 to now include acid -

54 fast bacteria that do not contain LPS and underscore the versatility of these

55 innate immune components in pathogen detection.

INTRODUCTION

Intracellular pathogens are detected by host cell cytosolic Nucleotide-binding Oligomerization Domain and Leucine-rich Repeat-containing receptors (NLRs) or Absent in Melanoma 2-like receptors (ALRs), which trigger the activation of an inflammasome complex (1-5). In addition to the respective NLR or ALR, this complex typically comprises an adaptor component (ASC) and pro-caspase-1. Upon full activation, the inflammasome complex contains active caspase-1, which cleaves pro-IL-1β to generate mature IL-1β. Caspase-1 also cleaves gasdermin D (GSDMD), releasing an N-terminal (NT-GSDMD; GSDMD p30) fragment that forms pores in the cell membrane through which IL-1β exits the cell (6-10). The process culminates with the activation of NINJ1, a membrane disrupting protein that leads to plasma membrane rupture (11-13). This final stage marks the completion of pyroptosis, a specific and inflammatory form of cell death.

Activation of the canonical NLRP3 inflammasome is triggered by various intracellular stressors, including potassium efflux, which occurs with GSDMD pore formation or when cells are exposed to certain bacterial toxins that damage the cell membrane, or increased reactive oxygen species (ROS), a common cellular response to intracellular bacteria (14-21). The non-canonical inflammasome pathway relies on the recognition of lipopolysaccharide (LPS) by inflammatory pro-caspase-4/5 (humans) or their murine homologue pro-caspase-11 (22-25). LPS binding leads to caspase activation and subsequent cleavage of GSDMD. The resulting NT-GSDMD pores facilitate K^+ efflux, which in turn

79   triggers NLRP3 inflammasome activation. Humans but not mice express

80   NLRP11, which is required for efficient activation of the non-canonical

81   inflammasome in response to intracellular LPS (26), as well as canonical NLRP3

82   inflammasome activation (27). In the current study, we determined the

83   importance of the NLRP11 pyrin, NACHT, and/or LRR domains for their function

84   in LPS recognition, IL-1β production and pyroptosis induction.

85   *Mycobacterium tuberculosis* is a human adapted, facultative intracellular

86   pathogen that activates the NLRP3 inflammasome of infected macrophages (28).

87   IL-1β is of great importance for a protective host response to *M. tuberculosis*

88   infections (28, 29). *M. tuberculosis* evolved to evade recognition by host cell

89   inflammasomes (28). The *M. tuberculosis* phospholipid phosphatase (PtpB)

90   cleaves cell membrane phosphatidylinositol-4 phosphate which reduces insertion

91   of NT-GSDMD into the plasma membrane and this limits pyroptosis and IL-1β

92   secretion (30). The *M. tuberculosis* protein kinase (PknF) limits activation of the

93   NLRP3 inflammasome via an unknown mechanism (31). The Rv3364c protein

94   suppresses host cathepsin G activation, leading to reduced inflammasome

95   activation (32). Despite all these inhibitory mechanisms, the host macrophage is

96   still able to sense intracellular *M. tuberculosis* via activation of the NLRP3

97   inflammasome. *M. tuberculosis* is an acid-fast bacterium that does not express

98   LPS but is atypical because it has an outer membrane composed of

99   mycobacterial lipids and lipoproteins (33). Since *M. tuberculosis* is a human

100 adapted pathogen and NLRP11 is only expressed in primates, we hypothesized

101 the NLRP11 might participate in the recognition of mycobacteria.

102 **RESULTS**

103 **NLRP11 is required for activation of the canonical NLRP3 inflammasome in**

104 **human macrophages.** We previously found that cells lacking the coding

105 sequence of the NACHT and LRR domains of NLRP11 display defects in

106 activation of the non-canonical inflammasome in response to cytosolic LPS (26).

107 To further evaluate the contributions of NLRP11 to innate immune signaling, we

108 generated a series of defined domain deletions in NLRP11 in human THP-1

109 monocytes (Fig. 1A): using CRISPR-Cas9 technology, we deleted the *NLRP11*

110 pyrin domain [PYD] (*NLRP11$^{\Delta PYD}$*), the NACHT and leucine-rich repeat [LRR]

111 domains (*NLRP11$^{\Delta N\_LRR}$*), or the entire *NLRP11* coding sequence (*NLRP11$^{-/-}$*)

112 (Fig. S1 in the supplemental material). We generated these mutations using

113 Cas9 ribonucleoproteins, which are associated with long-term genome stability,

114 isolated clonal derivatives by limiting dilution and carried forward only those

115 clonal lineages that contained a homozygous out-of-frame deletion.

116 We assessed the impact of deletions of *NLRP11* on activation of the canonical

117 NLRP3 inflammasome. In ribonucleoprotein-generated *NLRP11$^{-/-}$* macrophages,

118 although NLRP3 levels were not reduced (Fig. S1), cell death upon treatment

119 with the NLRP3 agonist nigericin was significantly decreased compared to

120 nigericin-treated WT macrophages (Fig. 1B). Activation of caspase-1, which

121 involves cleavage and release of its active domains, and activation of GSDMD,

122 which involves its cleavage, were significantly reduced in *NLRP11$^{-/-}$*

123 macrophages (Fig. S2). In parallel, release of IL-1β, a pro-inflammatory cytokine

124   effector of inflammasome activation, was reduced in *NLRP11$^{-/-}$* macrophages (Fig

125   1C).

126   To test whether these phenotypes were specifically due to activation of NLRP3,

127   we treated cells with the NLRP3 inhibitor MCC950. Treatment with MCC950

128   rescued nigericin-induced cell death, processing of caspase-1 and GSDMD, and

129   release of IL-1β and IL-18 to the levels of mock-treated THP-1s (Fig. 1B-C, and

130   Fig. S2), indicating that the observed phenotypes were due to activation of
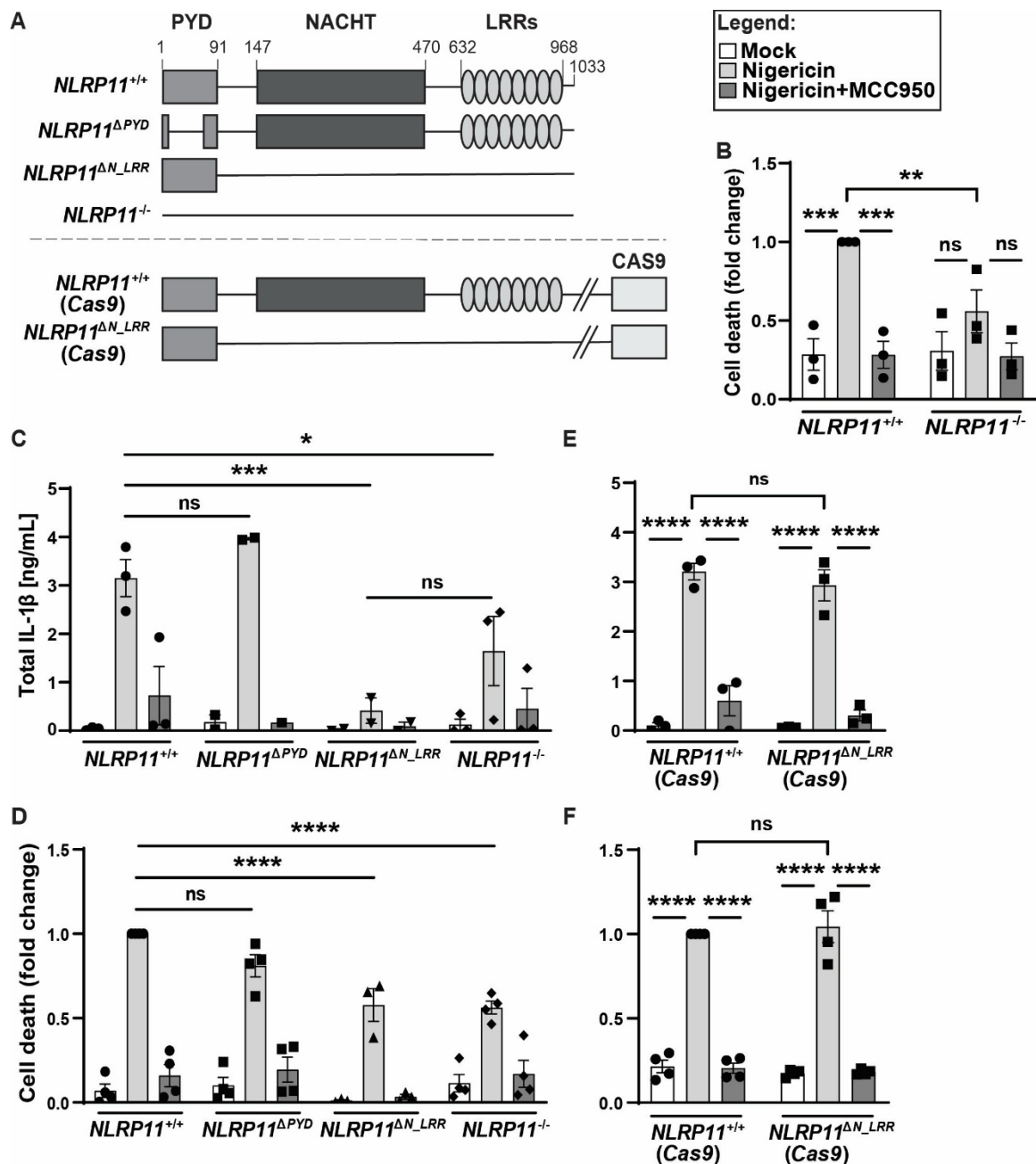
131   NLRP3.

132

133 **FIG 1** *Efficient activation of the canonical NLRP3 inflammasome depends on the*

134 *NLRP11 NACHT and/or LRR domains. (A) Schematic of NLRP11 mutants*

135 *generated in THP-1 macrophages. (B) Activation of cell death in NLRP11$^{+/+}$ and*

136   *NLRP11$^{-/-}$ macrophages by the NLRP3 agonist nigericin. Cell death, measured*

137   *via LDH release assay at 90 min, is shown normalized to nigericin-treated*

138   *NLRP11$^{+/+}$ macrophages. The level of cell death in NLRP11$^{+/+}$ macrophages*

139   *treated with nigericin approximated 30%. Where indicated, cells were pretreated*

140   *with the NLRP3 inhibitor MCC950. (C) Levels of IL-1β released into the cell*

141   *culture media at 90 min of nigericin treatment with or without addition of*

142   *MCC950. (D) Cell death, as in (B). (E-F) Levels of released IL-1β (E), as in (C),*

143   *and cell death (F), as in (B), in macrophages with stably integrated Cas9. ns, not*

144   *significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; by two-way*

145   *ANOVA. Shown are independent biological replicates. Error bars represent the*

146   *standard error of the mean. PYD, pyrin domain; NACHT, NAIP, C2TA, HET-E*

147   *and TP1 domain; LRR, leucin-rich repeat domain.*

148   **NACHT and/or LRR domains are required for NLRP11-dependent activation**

149   **of the NLRP3 inflammasome.** To determine the impact of specific domains of

150   NLRP11 on NLRP3 signaling, we assessed activation of cell death and cytokine

151   release in ribonucleoprotein-generated *NLRP11^{ΔPYD}* and *NLRP11$^{ΔN\_LRR}$*

152   macrophages. Deletion of NACHT and LRR domains, but not the PYD domain

153   alone, was associated with reduced nigericin-induced cell death (Fig. 1D) and

154   reduced release of IL-1β (Fig. 1C). In addition, in *NLRP11$^{ΔN\_LRR}$* macrophages,

155   processing of caspase-1 and GSDMD was significantly reduced (Fig. S2). In

156   contrast, in *NLRP11^{ΔPYD}* macrophages, caspase-1 was activated at levels similar

157   to those observed in *NLRP11$^{+/+}$* macrophages, and activation of GSDMD was

158   only mildly decreased. These findings indicate that the NLRP11 NACHT and/or

159   LRR domains are required for efficient NLRP3 inflammasome activation,

160   consistent with previous data demonstrating a requirement for the NLRP11

161   NACHT and LRR domains for oligomerization of NLRP3 (27). The findings also

162   suggest that the involvement of NLRP11 in NLRP3 activation does not strictly

163   depend on the NLRP11 pyrin domain.

164   As we found previously (26), nigericin activation of NLRP3 was not impacted in

165   *Cas9*-integrated *NLRP11$^{\Delta N\_LRR}$* macrophages (*NLRP11$^{\Delta N\_LRR}$* (*Cas9*), Fig. 1E-F).

166   This contrasts with what we observe in *NLRP11Δ^{N_LRR}* macrophages that lack

167   *Cas9* (*NLRP11$^{\Delta N\_LRR}$*, Fig. 1C-D). This result suggests that, as relates specifically

168   to NLRP3 signaling, the observed lack of impact of NLRP11 NACHT and LRR

169   disruption on NLRP3 in the *Cas9*-integrated cell lineage is not due to the

170   maintenance of the NLRP11 pyrin domain; rather, the lack of impact on NLRP3

171   in this lineage likely results from non-NLRP11 genetics and suggests that

172   genome-level changes resulting from the chronic presence of *Cas9* contributed

173   to this phenotype.

174   **Activation of the NLRP3 inflammasome by infection with the acid-fast**

175   **bacterium *M. tuberculosis* depends on NLRP11.** Whether NLRP11 is involved

176   in recognition of acid-fast or other gram-positive bacteria and mounting of a

177   cellular inflammatory response to them has not been studied. IL-1β produced by

178   THP-1 macrophages infected with *M. tuberculosis* is dependent on NLRP3 (34,

179   35). NLRP11 is also recruited by NLRP3 in the process of its activation (27).

180   Therefore, we hypothesized that in *NLRP11$^{-/-}$* THP-1 macrophages infected by

181   *M. tuberculosis*, IL-1β production might be lower than in WT macrophages. To

182   test this hypothesis, we determined levels of IL-1β release during infection with

183   *M. tuberculosis* of macrophages containing or lacking NLRP11. As a readout for

184   inflammasome activation during *M. tuberculosis* infection, cell death is less useful

185   than IL-1β release because few *M. tuberculosis*-infected macrophages die. At 8

186   and 24 hours of infection, significantly less IL-1β was released from *NLRP11$^{-/-}$*

187   macrophages than from WT macrophages (Fig. 2A-B). To test whether the

188   observed IL-1β release is dependent on activation of NLRP3, infected cells were

189   treated with MCC950. *M. tuberculosis*-infected *NLRP11$^{-/-}$* macrophages treated

190   with MCC950 display a decrease of IL-1β compared with those not treated with

191   MCC950 (Fig. 2A-B), indicating a requirement for NLRP3, which could be direct

192   or indirect.

193   As for *M. tuberculosis*, IL-1β produced by THP-1 macrophages infected with *M.*

194   *kansasii*, an opportunistic pathogen that is a close relative of *M. tuberculosis*, is

195   dependent on NLRP3 (36). Release of IL-1β during infection with *M. kansasii*

196   was dependent on NLRP11 (Fig. 2C, left graph), similar to the phenotype

197   observed for *M. tuberculosis*-infected cells. At late times of *M. kansasii* infection,

198   cell death was also partially dependent on NLRP11 (Fig. 2C, right graph).

199   Whereas IL-1β release from *NLRP11$^{-/-}$* macrophages was significantly reduced at

200   1, 2, 4 and 6 h of infection, cell death was significantly lower only at 6 h of
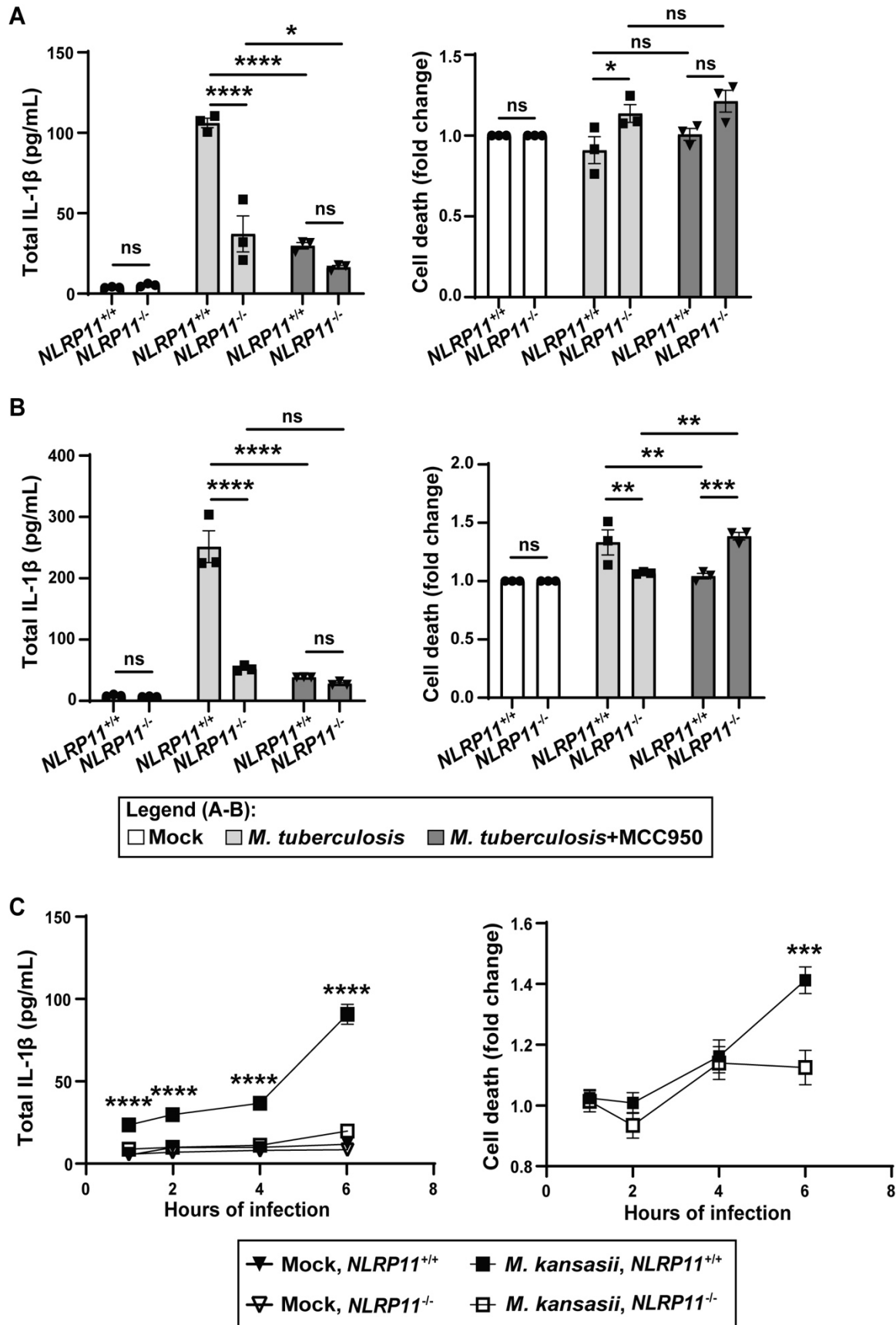
201   infection (Fig. 2C).

203   *FIG 2 NLRP3 activation by M. tuberculosis and M. kansasi in THP-1*

204   *macrophages is dependent on NLRP11. (A and B) IL-1β release and cell death*

205   *of NLRP11$^{-/-}$ and WT THP-1 macrophages at 8 h (A) or 24 h (B) of infection with*

206   *M. tuberculosis (MOI 10) with or without addition of MCC950. (C) IL-1β release*

207   *and cell death of NLRP11$^{-/-}$ and WT THP-1 macrophages over 1-6 h of infection*

208   *with M. kansasii. Cell death was normalized to cell lineage-specific uninfected*

209   *group. ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P <*

210   *0.0001; by two-way ANOVA (A-B) or paired parametric t-tests (C). Shown are*

211   *independent biological replicates. Error bars represent the standard error of*

212   *mean.*

213   **NLRP11 NACHT and/or LRR domains are required for activation of the non-**

214   **canonical inflammasome by the gram-negative bacterium *Shigella flexneri*.**

215   We previously found that NLRP11 is required for activation of the non-canonical

216   inflammasome during infection with a variety of gram-negative bacteria, including

217   *S. flexneri* (26). Cell death during infection with *S. flexneri* was unaffected by

218   deletion of the pyrin domain (PYD), whereas deletion of the NACHT and LRR

219   domains or deletion of the entire *NLRP11* locus resulted in 40-50% decreases in

220   macrophage cell death (Fig. 3A). Furthermore, release of pro-inflammatory

221   cytokines IL-1β and IL-18 was reduced up to 10-fold in macrophages lacking the

222   NACHT and LRR domains (*NLRP11$^{ΔN\_LRR}$*) or lacking all of NLRP11 (*NLRP11$^{-/-}$*),

223   but not in macrophages lacking only the PYD (*NLRP11^{ΔPYD}*) (Fig. 3B).

224   To test whether the observed defect in activation of pyroptosis in *NLRP11$^{ΔN\_LRR}$*

225   and *NLRP11$^{-/-}$* macrophages resulted from a defect in activation of the non-

226   canonical inflammasome, we examined processing of caspase-4 and GSDMD.

227   Activation of caspase-4 was reduced approximately 2-fold in *NLRP11*$^{ΔN\_LRR}$ and

228   *NLRP11*$^{-/-}$ macrophages but not reduced in *NLRP11*^{ΔPYD} macrophages (Fig. 3C

229   and Fig. S3). Levels of full-length pro-caspase-4 in cell lysates were unaffected,

230   indicating that the decrease in caspase-4 released from *NLRP11*$^{ΔN\_LRR}$ and

231   *NLRP11*$^{-/-}$ macrophages was not due to changes in its expression or stability.

232   Similarly, processing of GSDMD was significantly reduced in *NLRP11*$^{ΔN\_LRR}$ and

233   *NLRP11*$^{-/-}$ macrophages but not in *NLRP11*^{ΔPYD} macrophages, yet levels of full-

234   length GSDMD were unaltered (Fig. 3C and Fig. S3). Thus, the NACHT and/or

235   LRR domains are required for efficient activation of the NLRP11-dependent non-

236   canonical inflammasome during infection with *S. flexneri*.

237   **Activation of the non-canonical inflammasome by *S. flexneri* is**

238   **independent of NLRP3.** To test whether the NLRP11-dependent activation of

239   the non-canonical inflammasome is dependent on NLRP3, we examined

240   inflammasome activation in *S. flexneri*-infected *NLRP11*$^{ΔN\_LRR}$ macrophages pre-

241   treated with MCC950. Treatment with MCC950 did not impact the levels of cell

242   death, cytokine release, or processing of caspase-4 and GSDMD (Fig. 3A-C, and

243   Fig. S3). Therefore, NLRP3 is not required for activation of the NLRP11-

244   dependent non-canonical inflammasome during *S. flexneri* infection.
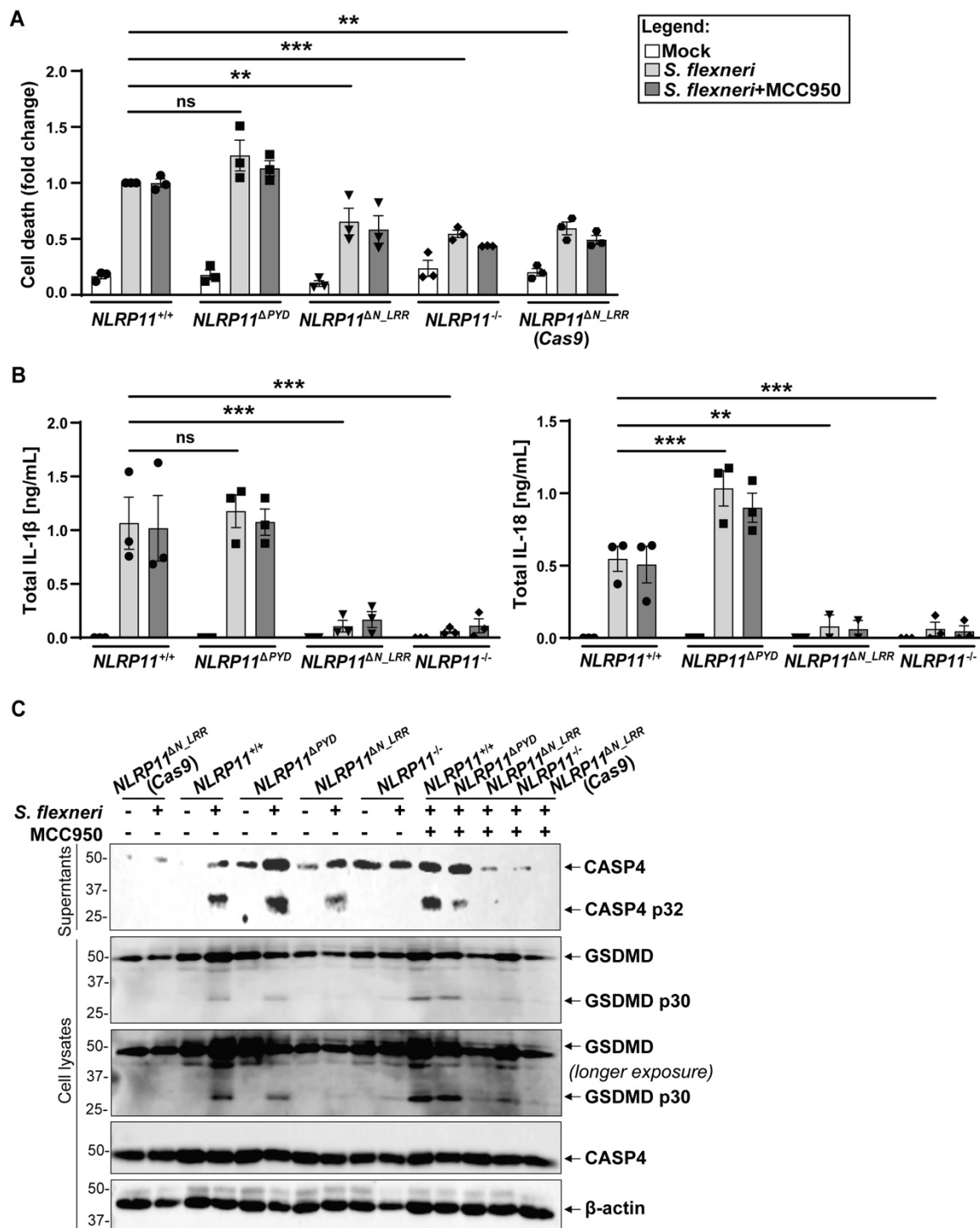
FIG 3 NACHT and/or LRR domains are required for NLRP11-dependent non-

canonical inflammasome activation during S. flexneri infection. (A) Activation of

cell death in indicated NLRP11 mutant cell lines at 3 h of infection with S. flexneri

249 *(MOI 10). Cell death is shown normalized to S. flexneri-infected NLRP11$^{+/+}$*

250 *macrophages. The level of cell death in infected NLRP11$^{+/+}$ macrophages*

251 *approximated 15-20%. Where indicated, cells were pretreated with the NLRP3*

252 *inhibitor MCC950. (B) Levels of IL-1β and IL-18 released into the cell culture*

253 *media from (A). (C) Cleaved caspase-4 (CASP4 p32) released into the cell*

254 *culture media and intracellular cleaved GSDMD (p30) under same conditions.*

255 *Representative western blots. ns, not significant; **, P < 0.01; ***, P < 0.001; by*

256 *two-way ANOVA. Shown are independent biological replicates (A-B). Error bars*

257 *represent the standard error of the mean.*

258 **Activation of the non-canonical inflammasome by *M. tuberculosis* is**

259 **dependent on NLRP11.** The *NLRP11$^{\Delta N\_LRR}$* (*Cas9*) cell line accurately reports

260 on the role of NLRP11 in non-canonical caspase-4 activity, while displaying

261 normal activation of NLRP3 in response to nigericin (26). Therefore, to determine

262 whether NLRP11 is required for non-canonical caspase-4 inflammasome activity

263 during mycobacterial infection, we compared IL-1β release from mycobacteria-

264 infected WT (*Cas9*) to that from mycobacteria-infected *NLRP11$^{\Delta N\_LRR}$* (*Cas9*)

265 macrophages. *NLRP11$^{\Delta N\_LRR}$* cells infected with *M. tuberculosis* or *M. kansasii*

266 showed significantly diminished IL-1β release at all time points (Fig. 4A-B),

267 whereas those infected with the non-pathogenic *M. smegmatis* showed increases

268 in IL-1β release over time that were similar to *M. kansasii* but which were not

269 reduced in the *NLRP11$^{\Delta N\_LRR}$* cells at any time point (Fig. 4C). These data

270 indicate that IL-1β released during *M. tuberculosis* and *M. kansasii* infection is

271 dependent, at least partially, on the NACHT and/or LRR domains of NLRP11.

272   Cell death was not significantly different between cell lines during infection with

273   any of the three mycobacterial species.

274   To further examine the kinetics of NLRP11 dependent activation of the non-

275   canonical inflammasome, we assayed IL-1β release in *M. kansasii*-infected WT

276   and *NLRP11*$^{ΔN\_LRR}$ (*Cas9*) macrophages at 1, 2, 4 and 6 hours of infection. At

277   every time point, significantly lower levels of IL-1β were released from

278   *NLRP11*$^{ΔN\_LRR}$ macrophages than their WT counterparts (Fig. 4D), indicating that

279   NLRP11 is required for IL-1β release even early during infection. As above (Fig.

280   4B), at early times, NLRP11-dependent differences in cell death are minimal (Fig.

281   4D).

282   The use of the NLRP11$^{ΔN\_LRR}$ (*Cas9*) mutant THP-1 cells is consistent with but

283   not proof that *M. tuberculosis* and *M. kansasii* infection induces activation of the

284   non-canonical inflammasome. In humans, the non-canonical inflammasome

285   involves either or both of caspase-4 and caspase-5. During NLRP11-dependent

286   activation of the non-canonical inflammasome, NLRP11 is required for

287   downstream activation of at least caspase-4 (26); in this study, caspase-5 was

288   not examined. To directly test whether mycobacteria activate the non-canonical

289   inflammasome, we tested whether macrophages that lack both caspase-4 and

290   caspase-5 support mycobacterium-induced IL-1β release and cell death. At 4

291   hours of infection with *M. kansasii* (Fig. 5A, left graph) and 24 hours of infection

292   with *M. tuberculosis* (Fig. 5B, left graph), the absence of caspases-4 and -5 was

293   associated with a complete absence of IL-1β release. Paralleling the impact of

294   caspases-4 and -5 on IL-1β release, during infection with *M. kansasii*, their

295   absence was associated with a two-fold reduction in cell death to baseline (Fig.

296   5A, right graph). During infection with *M. tuberculosis*, the impact of their

297   absence on cell death was minimal (Fig. 5B, right graph). In WT THP-1

298   macrophages, specific to infection with *M. tuberculosis* was a small amount of

299   caspase-4 processing and release that was not blocked by inhibition of NLRP3

300   with MCC950 (Fig. S4). These data indicate that IL-1β secretion during *M.*

301   *tuberculosis* and *M. kansasii* infection is dependent on the non-canonical

302   caspase-4/5 inflammasome.
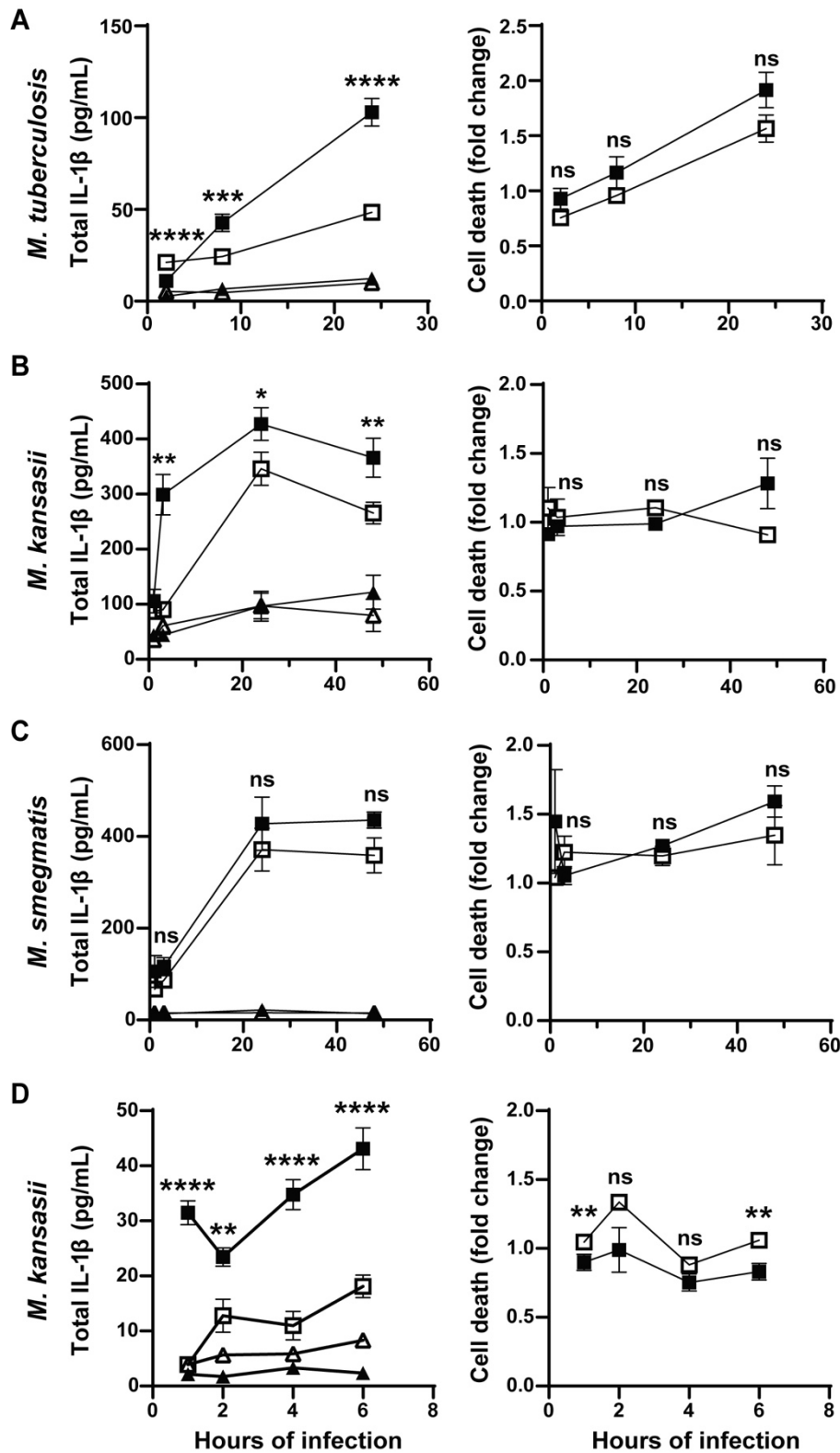
Legend:
- ▲ Mock, $NLRP11^{+/+}$ (*Cas9*)
- △ Mock, $NLRP11^{\Delta N\_LRR}$ (*Cas9*)
- ■ Infected, $NLRP11^{+/+}$ (*Cas9*)
- ☐ Infected, $NLRP11^{\Delta N\_LRR}$ (*Cas9*)

303

304   **FIG 4** *Mycobacteria activate the non-canonical inflammasome pathway. IL-1β*

305   *release and cell death of NLRP11$^{+/+}$ (Cas9) and NLRP11$^{\Delta N\_LRR}$ (Cas9) mutant*

306   *THP-1 macrophages infected with M. tuberculosis (A), M. kansasii (B, D), or M.*

307   *smegmatis (C) (MOI 10), at the indicated timepoints. Cell death was normalized*

308   *to cell lineage-specific uninfected group. The level of cell death in NLRP11$^{\Delta N\_LRR}$*

309   *(Cas9) cells approximated 10-20%. ns, not significant; **, P < 0.01; ***, P <*

310   *0.001; ****, P < 0.0001; by paired parametric t-tests, comparing infected*

311   *NLRP11$^{+/+}$ (Cas9) to infected NLRP11$^{\Delta N\_LRR}$ (Cas9). Shown are independent*

312   *biological replicates. Error bars represent the standard error of mean.*
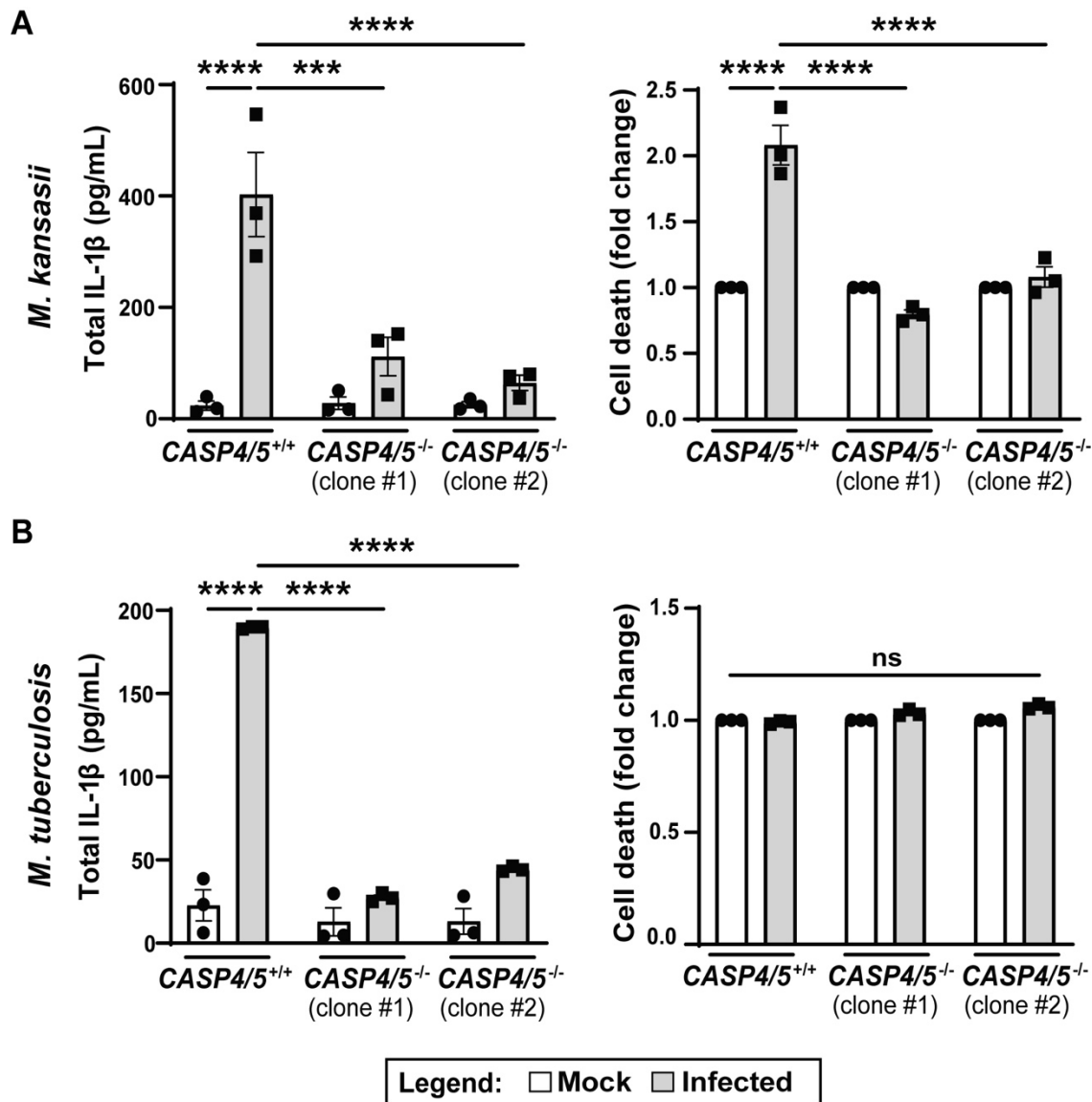
FIG 5 *Caspase-4/5 are required for recognition of mycobacteria in human macrophages. IL-1β release and cell death of WT and two distinct clones of caspase-4/5$^{-/-}$ (CASP4/5$^{-/-}$) THP-1 macrophages infected with M. kansasii (A) or M. tuberculosis (B) (MOI 10). For M. kansasii, assays were performed at 4 h of infection (A), and for M. tuberculosis at 24 h of infection (B). Cell death in infected CASP4/5$^{-/-}$ macrophages approximated 20-60%. ns, not significant; ***, P <*

320   *0.001; ****, P < 0.0001; by two-way ANOVA. Shown are independent biological*

321   *replicates. Error bars represent the standard error of mean.*

DISCUSSION

Two recent studies establish that NLRP11 is required for efficient activation of the non-canonical caspase-4/5 inflammasome in macrophage-like THP-1 cells and primary human macrophages (26, 27). These studies demonstrated that NLRP11 is required for non-canonical inflammasome activation in response to cytosolic LPS (26, 27) and infection with gram-negative bacteria (26). Our findings herein extend our understanding of NLRP11 function by demonstrating that NLRP11 is required for efficient activation of the non-canonical inflammasome by the acid-fast bacteria *M. tuberculosis* and *M. kansasii* (Fig. 6).

The present study offers clarification on previously conflicting findings regarding the role of NLRP11 role in activation of the canonical NLRP3 inflammasome: Gangopadhyay et al. reported that NLRP11 is required for NLRP3 activation (27), while Rojas-Lopez et al. found no significant involvement of NLRP11 in this process (26). Our results confirm a role for NLRP11 in activation of the non-canonical inflammasome and demonstrate that the NACHT and/or LRR domains are required for NLRP3 activation but that NLRP3 activation does not strictly depend on the NLRP11 pyrin domain (Fig. 1C-D). With respect to a role for NLRP11 in activation of the NLRP3 inflammasome specifically, we speculate that the difference in the results reported herein compared with those reported in Rojas-Lopez et al. is a result of some genomic instability of the cell lineage in Rojas-Lopez et al. due to the integration of *Cas9*. The new constructs described in the present study were generated by transfection of Cas9 protein together with guide RNAs (Materials and Methods). The constitutive presence of *Cas9* may

FIG 6 *Role of NLRP11 in inflammasome activation during mycobacterial infection. NLRP11 displays dual function in activation of inflammasome responses in human macrophages infected with mycobacteria. NLRP11 and caspase-4 are required for efficient mycobacterial activation of the non-canonical inflammasome. Active caspase-4 cleaves GSDMD to release the N-terminal fragment (GSDMD-N) which forms pores in the plasma membrane. These pores lead to K^+ efflux, a known trigger for activation of NLRP3. NLRP11 is required for efficient activation of the NLRP3 inflammasome, which leads to activation of caspase-1. Caspase-1 efficiently cleaves pro-IL-1β and GSDMD leading to more pore formation and release of mature IL-1β.*

have resulted in off-target effects that masked the role of NLRP11 specifically in the canonical NLRP3 inflammasome pathway. Our new results thus unite the findings in the field and establish a consensus that NLRP11 is required for activation of both the non-canonical and the canonical NLRP3 inflammasome.

359   Although the mechanisms by which NLRP11 promotes NLRP3 activation remain

360   unclear, we speculate that NLRP11 may function as a helper NLR, as has been

361   described for both mammalian and plant NLRs. In some cases, as NLR that has

362   evolved to recognize the pathogen-associated molecular pattern interacts with a

363   second NLR that executes immune signaling (37-41). In other cases, two NLRs

364   that interact biochemically function more efficiently as a hetero-complex (37, 42,

365   43). The observation that NLRP11 is present in human macrophages at very low

366   levels (26) suggests that NLRP11 may act upstream of NLRP3, which is

367   abundant, rather than forming a hetero-complex. However, future investigations

368   are required to clarify the mechanisms of NLRP11 function in NLRP3 activation.

369   The murine homologue of human caspases-4 and -5, caspase-11, is required for

370   the detection of cytosolic LPS in mice. *Caspase-11*$^{-/-}$ mice are more resistant to

371   death from *Escherichia coli*-induced or LPS-induced septic shock (22, 24). As

372   described above, in humans, caspase-4 and/or caspase-5 (25, 44, 45) and

373   NLRP11 (26, 27), which is absent in mice, are required for recognition of

374   cytosolic LPS. Indirect evidence suggests that lipoteichoic acid (LTA) of gram-

375   positive bacteria can also be detected by caspase-4 (46, 47). Here, we

376   demonstrate for the first time that NLRP11 and the non-canonical caspase-4

377   inflammasome are required for detection of intracellular *M. tuberculosis* (Fig. 2,

378   Fig. 4, and Fig. 5). Our results indicate that, for infection with *M. tuberculosis* or

379   *M. kansasii*, sensing of the infection by the non-canonical pathway is essential for

380   host inflammasome responses, since caspase-4/5 are required for IL-1β release

381   (Fig. 5). Interestingly, a 2010 shRNA screen of NOD proteins required for IL-1β

382   release from *M. tuberculosis*-infected THP-1 cells identified *NLRP11* as a hit

383   (48).

384   *M. tuberculosis* contains neither LPS nor LTA, and so what could the ligand be

385   for caspase-4 and/or NLRP11? Potential ligands include the lipoarabinomannans

386   (LAM), which are complex glycolipids similar to LPS and, like lipid A of LPS, are

387   anchored in the inner membrane via a lipid anchor (33). The sugar composition

388   of LAMs differs among mycobacterial species (49), which could explain why *M.*

389   *tuberculosis* and *M. kansasii* but not *M. smegmatis* induce non-canonical

390   inflammasome activation (Fig. 4). Future investigation of the role of LAM in

391   inflammasome activation is needed. A second possible group of mycobacterial

392   ligands for caspase-4 and/or NLRP11 are the Pro-Glu motif-containing and Pro-

393   Pro-Glu motif-containing (PE/PPE) domain containing family of proteins, which

394   are abundant in mycobacterial species such as *M. tuberculosis* and *M. kansasii*

395   but absent in *M. smegmatis* (50, 51). However, the PPE protein PPE13 interacts

396   with the NACHT or LRR domains of NLRP3, leading to its activation (52),

397   whereas our data indicate that direct activation of NLRP3 is unlikely the

398   mechanism of action for *M. tuberculosis* and *M. kansasii*, since we found an

399   almost complete absence of IL-1β secretion in caspase-4/5 deficient THP-1 cells

400   (Fig. 5). In conclusion, our results expand the range of pathogens recognized by

401   NLRP11 and caspase-4 to now include acid-fast bacteria and underscore the

402   versatility of these innate immune components in pathogen detection.

403 **MATERIALS AND METHODS**

404 **Bacterial strains and growth conditions.** *M. tuberculosis* (H37Rv), *M. kansasii*

405 (ATCC1278), and *M. smegmatis* (mc2155) strains were grown in liquid

406 Middlebrook 7H9 medium supplemented with 10% oleic acid-albumin-dextrose-

407 catalase (OADC) growth supplement (B12351), 0.2% glycerol, and 0.05% Tween

408 80. *S. flexneri* wild-type serotype 2a strain 2457T was used in this study (53). *S.*

409 *flexneri* was grown in tryptic soy broth at 37ºC with aeration.

410 **Generation of CRISPR deletions in NLRP11.** We previously created a deletion

411 of the coding sequences of the NACHT and LRR domains in the *NLRP11* gene

412 of THP-1 monocytes using a lentivirus-based system to deliver guide RNAs into

413 cells in which *Cas9* had previously been stably integrated, such that the

414 generated *NLRP11* knockout carried an integrated copy of *Cas9* gene (26). In

415 the current study, to generate knockout lineages that lack chronic *Cas9*,

416 chemically modified sgRNAs (Synthego) were electroporated as

417 ribonucleoprotein (RNP) complexes into freshly cultured THP-1 monocytes using

418 the Neon Transfection System (Invitrogen, MPK5000) following the

419 manufacturer's instructions. Specifically, to create the editing RNP complexes,

420 100 pmol of each sgRNA (Fig. S1E) were mixed with 25 pmol of SpCas9 2NLS

421 nuclease (Synthego) (4:1 ratio) in Resuspension Buffer R (Invitrogen,

422 MPK1069B) and incubated for 15 min at room temperature. THP-1 monocytes

423 were washed in PBS and resuspended in Buffer R. The resulting RNP

424 complexes were electroporated by pulsing twice 1×10^6 cells in the 10 μL Neon

425 Tip at 1700 V and 2 ms pulse width. Electroporated cells were immediately

426   resuspended in 2.5 mL complete growth medium and placed into a 6-well plate.

427   Cells were incubated for 72 hours at 37ºC, after which 1.5 mL was transferred to

428   a 75 cm^2 culture flask, and 1 mL was used to purify genomic DNA using DNeasy

429   Blood and Tissue kit (Qiagen). Following PCR and sequencing, editing efficiency

430   was determined using ICE Analyzer (Synthego). The remaining cells were

431   limiting-diluted and expanded as single clones in a 96-well dish. Using

432   sequencing, isolated single clones were validated for homozygous out-of-frame

433   deletions (Fig. S1).

434   **Mammalian cell culture.** WT human-derived THP-1 monocytes (American Type

435   Culture Collection, TIB-202) were obtained by ATCC. Caspase-4 and -5 deficient

436   THP-1 cells were generated as described (54, 55) and kindly provided by Dr.

437   C.Y. Taabazuing (University of Pennsylvania). Cells were maintained in RPMI

438   1640 medium (Gibco, 11875119) supplemented with 10% (v/v) fetal bovine

439   serum (heat-inactivated, sterile-filtered, R&D systems) and 10 mM HEPES

440   (Gibco, 15630-080). Cells were grown in 5% CO_2 in a humidified incubator at

441   37ºC. For use in experiments, cells were seeded in 48-well plates at 2×10^5 cells

442   per well or in 96-well plates at 5×10^4 cells per well. For all non-mycobacterial

443   experiments, to induce differentiation into macrophages, cells were incubated

444   with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) for 24 h, after which they

445   were rested in media without PMA overnight prior to use in experiments. For

446   mycobacterial infections, cells were differentiated into macrophages in 50 ng/mL

447   PMA for 48 h, after which they were rested in media without PMA for 48 h prior to

448   use in experiments.

449 **Cell treatments.** In experiments with nigericin treatment, PMA-differentiated

450 THP-1 macrophages were primed with 500 ng/mL ultrapure LPS from

451 *Escherichia coli* O111:B4 (InvivoGen, tlrl-3pelps) for 4 hours followed by

452 treatment with 10 µM nigericin (InvivoGen, tlrl-nig) for 90 min. In experiments

453 involving nigericin treatment or *S. flexneri* infection, 2 µM MCC950 (NLRP3

454 inhibitor, InvivoGen, inh-mcc) was added where appropriate one hour prior to

455 addition of nigericin or infection and maintained in the medium for the duration of

456 the experiment. In cells infected with *M. tuberculosis*, MCC950 was added at 4 h

457 of infection and maintained in the medium for the duration of the experiment.

458 **Bacterial infections.** For infections with mycobacterial strains, bacteria grown to

459 an exponential phase were centrifuged at 2000 g for 7 min. Bacterial pellets were

460 resuspended in 1X phosphate-buffered saline (PBS) containing 0.05% Tween 80

461 and centrifuged at 80 g for 3 min to pellet clumped bacteria. The supernatants

462 (single cell bacterial suspensions) were used to determine the multiplicity of

463 infection (MOI) by optical density. PMA differentiated THP-1 macrophages were

464 infected at the MOI of 10 and incubated for 2 h (*M. smegmatis*) or for 4 h (*M.*

465 *tuberculosis* and *M. kansasii*) in 5% CO_2 in a humidified incubator at 37°C. After

466 this period of phagocytosis, infected macrophages were gently washed twice with

467 prewarmed 1X PBS. Gentamicin, to kill extracellular bacteria, was added at 100

468 µg/mL in complete media containing RPMI1640 supplemented with 5% human

469 AB serum. Samples for LDH release assay and IL-1β ELISA were collected at

470 the indicated times.

471   For infections with *S. flexneri*, PMA-differentiated macrophages were infected at

472   an MOI of 10 with bacteria grown to exponential phase after back-dilution from

473   an overnight culture. Bacteria were centrifuged onto macrophages at 800 g for 10

474   min at room temperature, followed by incubation in 5% CO_2 in a humidified

475   incubator at 37°C. After 30 min, cells were washed twice with Hanks' Balanced

476   Salt Solution (HBSS, Gibco, 14025-092), and gentamicin was added to 25 μg/mL

477   in RPMI 1640 medium supplemented with 10 mM HEPES without serum.

478   Samples for LDH release assay, cytokine ELISAs, and western immunoblot were

479   collected at 2 h 40 min. of infection.

480   **LDH cytotoxicity assay.** Macrophage cell death was assessed by release of

481   lactate dehydrogenase (LDH) due to loss of cellular membrane integrity. To

482   measure LDH activity, 50 μL of cell culture supernatants from a 96-well plate

483   were collected at indicated times and a CytoTox 96® Non-Radioactive

484   Cytotoxicity Assay (experiments with mycobacteria; Promega, G1780) or a

485   CyQUANT™ LDH Cytotoxicity Assay (experiments with *S. flexneri* and nigericin;

486   ThermoFisher Scientific, C20301) was used following the manufacturer's

487   instructions.

488   **Cytokine ELISAs.** Cell culture supernatants harvested from mycobacteria-

489   infected cells were assayed for levels of secreted IL-1β using Human Total IL-1β

490   DuoSet ELISA kit (biotechne, DY201) following the manufacturer's instructions.

491   Cell culture supernatants harvested from cells infected with *S. flexneri* or treated

492   with nigericin were assayed for levels of secreted cytokines using Human Total

493   IL-18 DuoSet ELISA kit (biotechne, DY318-05) and ELISA MAX™ Deluxe Set

494   Human IL-1β kit (BioLegend, 437004) following the manufacturers' instructions.

495   **Protein extraction and western immunoblot analysis.** For detection of

496   released caspases, 100 µL of cell culture supernatants from a 48-well plate were

497   collected at the indicated times and mixed with 35 µL of 4x Laemmli buffer [250

498   mM Tris (pH 6.8), 8% SDS, 50% glycerol, 0,04% bromophenol blue]

499   supplemented with cOmplete EDTA-free protease inhibitor (Roche,

500   11873580001). For detection of cell-associated caspases and full-length and

501   cleaved GSDMD, cells in the same plate were lysed directly in 100 µL of 4x

502   Laemmli buffer supplemented with the same protease inhibitor. Following SDS–

503   polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred onto

504   a nitrocellulose membrane.

505   Antibodies used for immunoblotting were as follows: caspase-1 (Abcam,

506   ab207802) rabbit monoclonal antibody at 0.5 µg/mL (1:1000), caspase-4 (Santa

507   Cruz, sc-56056) and GSDMD (Santa Cruz, sc-81868) mouse monoclonal

508   antibodies at 0.5 µg/mL (1:200). Secondary antibodies were goat anti-rabbit

509   immunoglobulin G (IgG) or goat anti-mouse IgG, each conjugated to horseradish

510   peroxidase (HRP) (Jackson ImmunoResearch). Antibodies specific for β-actin

511   were HRP-conjugated (Sigma, A3854). Immunoreactive bands were visualized

512   by chemiluminescence with SuperSignal West Pico PLUS or SuperSignal West

513   Femto Maximum Sensitivity substrates (Thermo Fisher Scientific, 34580 and

514   34096).

515 **Statistical analysis.** GraphPad Prism software was used for graphing data and

516 statistical analyses. Statistical significance was determined using the ordinary

517 two-way ANOVA with Tukey's multiple comparisons test, with a single pooled

518 variance. For Fig. 4, statistical significance was determined using a paired

519 parametric t-test. Data were graphed so that each data point represents the

520 mean of technical triplicate wells for one experiment, and all results are data from

521 at least three independent biological replicates, unless noted otherwise.

522 Differences were considered statistically significant if the P value was <0.05.

523 **ACKNOWLEDGMENTS**

528 **FIGURE LEGENDS**

529 **FIG 1** Efficient activation of the canonical NLRP3 inflammasome depends on the

530 NLRP11 NACHT and/or LRR domains. (A) Schematic of *NLRP11* mutants

531 generated in THP-1 macrophages. (B) Activation of cell death in *NLRP11*^{+/+} and

532 *NLRP11*^{-/-} macrophages by the NLRP3 agonist nigericin. Cell death, measured

533 via LDH release assay at 90 min, is shown normalized to nigericin-treated

534 *NLRP11*^{+/+} macrophages. The level of cell death in *NLRP11*^{+/+} macrophages

535 treated with nigericin approximated 30%. Where indicated, cells were pretreated

536 with the NLRP3 inhibitor MCC950. (C) Levels of IL-1β released into the cell

537 culture media at 90 min of nigericin treatment with or without addition of

538 MCC950. (D) Cell death, as in (B). (E-F) Levels of released IL-1β (E), as in (C),

539 and cell death (F), as in (B), in macrophages with stably integrated Cas9. ns, not

540 significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; by two-way

541 ANOVA. Shown are independent biological replicates. Error bars represent the

542 standard error of the mean. PYD, pyrin domain; NACHT, NAIP, C2TA, HET-E

543 and TP1 domain; LRR, leucin-rich repeat domain.

544 **FIG 2** NLRP3 activation by *M. tuberculosis* and *M. kansasi* in THP-1

545 macrophages is dependent on NLRP11. (A and B) IL-1β release and cell death

546 of *NLRP11*^{-/-} and WT THP-1 macrophages at 8 h (A) or 24 h (B) of infection with

547 *M. tuberculosis* (MOI 10) with or without addition of MCC950. (C) IL-1β release

548 and cell death of *NLRP11*^{-/-} and WT THP-1 macrophages over 1-6 h of infection

549 with *M. kansasii*. Cell death was normalized to cell lineage-specific uninfected

550 group. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P <$

551 0.0001; by two-way ANOVA (A-B) or paired parametric t-tests (C). Shown are

552 independent biological replicates. Error bars represent the standard error of

553 mean.

554 **FIG 3** NACHT and/or LRR domains are required for NLRP11-dependent non-

555 canonical inflammasome activation during *S. flexneri* infection. (A) Activation of

556 cell death in indicated *NLRP11* mutant cell lines at 3 h of infection with *S. flexneri*

557 (MOI 10). Cell death is shown normalized to *S. flexneri*-infected NLRP11$^{+/+}$

558 macrophages. The level of cell death in infected *NLRP11*$^{+/+}$ macrophages

559 approximated 15-20%. Where indicated, cells were pretreated with the NLRP3

560 inhibitor MCC950. (B) Levels of IL-1β and IL-18 released into the cell culture

561 media from (A). (C) Cleaved caspase-4 (CASP4 p32) released into the cell

562 culture media and intracellular cleaved GSDMD (p30) under same conditions.

563 Representative western blots. ns, not significant; **, P < 0.01; ***, P < 0.001; by

564 two-way ANOVA. Shown are independent biological replicates (A-B). Error bars

565 represent the standard error of the mean.

566 **FIG 4** Mycobacteria activate the non-canonical inflammasome pathway. IL-1β

567 release and cell death of *NLRP11*$^{+/+}$ (*Cas9*) and *NLRP11*$^{\Delta N\_LRR}$ (*Cas9*) mutant

568 THP-1 macrophages infected with *M. tuberculosis* (A), *M. kansasii* (B, D), or *M.*

569 *smegmatis* (C) (MOI 10), at the indicated timepoints. Cell death was normalized

570 to cell lineage-specific uninfected group. The level of cell death in *NLRP11*$^{\Delta N\_LRR}$

571 (*Cas9*) cells approximated 10-20%. ns, not significant; **, *P* < 0.01; ***, *P* <

572 0.001; ****, *P* < 0.0001; by paired parametric t-tests, comparing infected

573   *NLRP11$^{+/+}$* (*Cas9*) to infected *NLRP11$^{\Delta N\_LRR}$* (*Cas9*). Shown are independent

574   biological replicates. Error bars represent the standard error of mean.

575   **FIG 5** Caspase-4/5 are required for recognition of mycobacteria in human

576   macrophages. IL-1β release and cell death of WT and two distinct clones of

577   *caspase-4/5$^{-/-}$* (*CASP4/5$^{-/-}$*) THP-1 macrophages infected with *M. kansasii* (A) or

578   *M. tuberculosis* (B) (MOI 10). For *M. kansasii*, assays were performed at 4 h of

579   infection (A), and for *M. tuberculosis* at 24 h of infection (B). Cell death in infected

580   *CASP4/5$^{-/-}$* macrophages approximated 20-60%. ns, not significant; ***, *P* <

581   0.001; ****, *P* < 0.0001; by two-way ANOVA. Shown are independent biological

582   replicates. Error bars represent the standard error of mean.

583   **FIG 6** Role of NLRP11 in inflammasome activation during mycobacterial

584   infection. NLRP11 displays dual function in activation of inflammasome

585   responses in human macrophages infected with mycobacteria. NLRP11 and

586   caspase-4 are required for efficient mycobacterial activation of the non-canonical

587   inflammasome. Active caspase-4 cleaves GSDMD to release the N-terminal

588   fragment (GSDMD-N) which forms pores in the plasma membrane. These pores

589   lead to K$^+$ efflux, a known trigger for activation of NLRP3. NLRP11 is required for

590   efficient activation of the NLRP3 inflammasome, which leads to activation of

591   caspase-1. Caspase-1 efficiently cleaves pro-IL-1β and GSDMD leading to more

592   pore formation and release of mature IL-1β.

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