NLRP11 is required for canonical NLRP3 and non-canonical inflammasome activation during human macrophage infection with mycobacteria

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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.

56 **INTRODUCTION**

57 Intracellular pathogens are detected by host cell cytosolic Nucleotide-binding
58 Oligomerization Domain and Leucine-rich Repeat-containing receptors (NLRs) or
59 Absent in Melanoma 2-like receptors (ALRs), which trigger the activation of an
60 inflammasome complex (1-5). In addition to the respective NLR or ALR, this
61 complex typically comprises an adaptor component (ASC) and pro-caspase-1.
62 Upon full activation, the inflammasome complex contains active caspase-1,
63 which cleaves pro-IL-1β to generate mature IL-1β. Caspase-1 also cleaves
64 gasdermin D (GSDMD), releasing an N-terminal (NT-GSDMD; GSDMD p30)
65 fragment that forms pores in the cell membrane through which IL-1β exits the cell
66 (6-10). The process culminates with the activation of NINJ1, a membrane
67 disrupting protein that leads to plasma membrane rupture (11-13). This final
68 stage marks the completion of pyroptosis, a specific and inflammatory form of
69 cell death.

70 Activation of the canonical NLRP3 inflammasome is triggered by various
71 intracellular stressors, including potassium efflux, which occurs with GSDMD
72 pore formation or when cells are exposed to certain bacterial toxins that damage
73 the cell membrane, or increased reactive oxygen species (ROS), a common
74 cellular response to intracellular bacteria (14-21). The non-canonical
75 inflammasome pathway relies on the recognition of lipopolysaccharide (LPS) by
76 inflammatory pro-caspase-4/5 (humans) or their murine homologue pro-caspase-
77 11 (22-25). LPS binding leads to caspase activation and subsequent cleavage of
78 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.

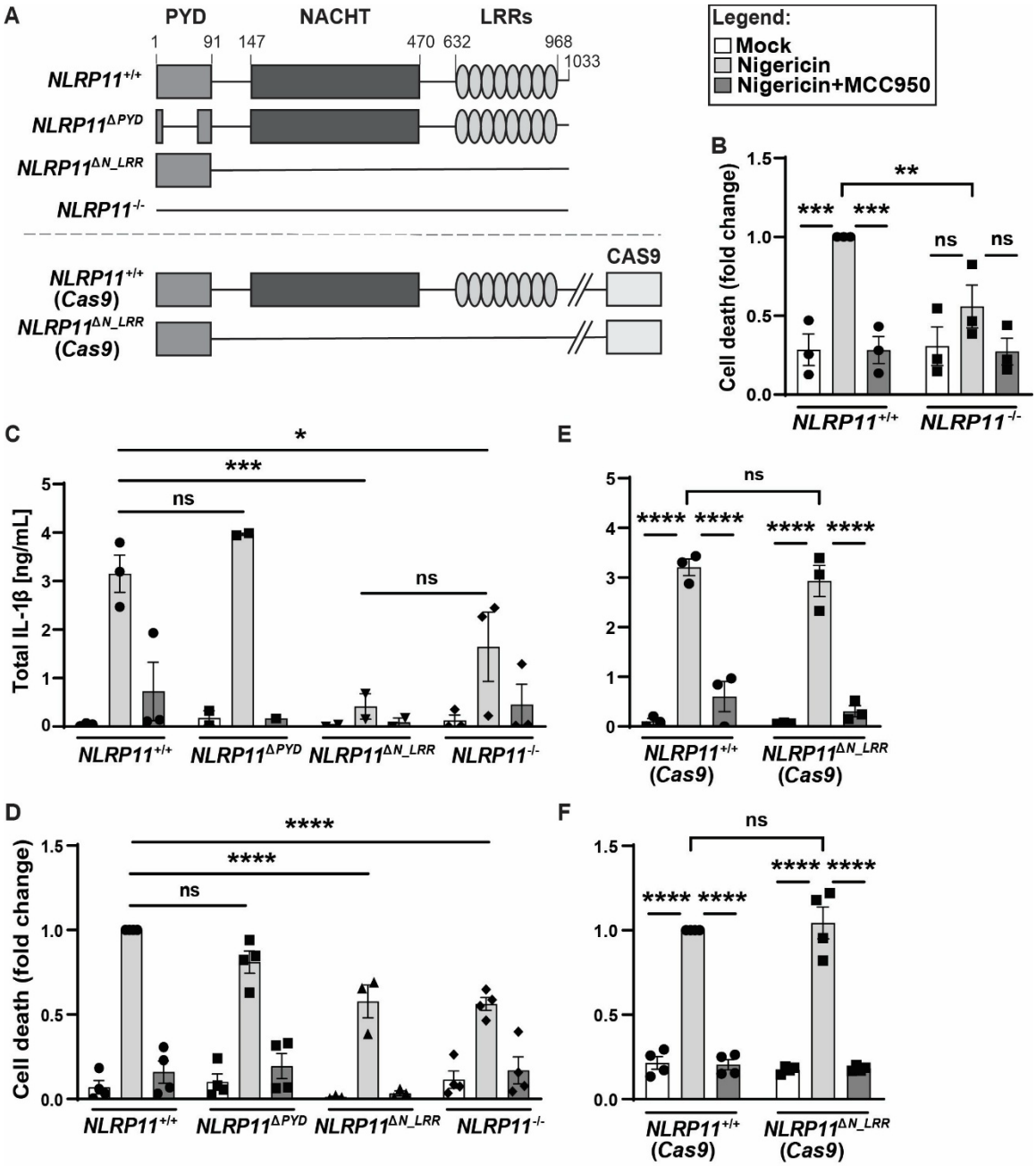
102 **RESULTS**

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$^{\Delta PYD}$* and *NLRP11$^{\Delta N_LRR}$*
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$^{\Delta N_LRR}$* macrophages,
155 processing of caspase-1 and GSDMD was significantly reduced (Fig. S2). In
156 contrast, in *NLRP11$^{\Delta PYD}$* 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

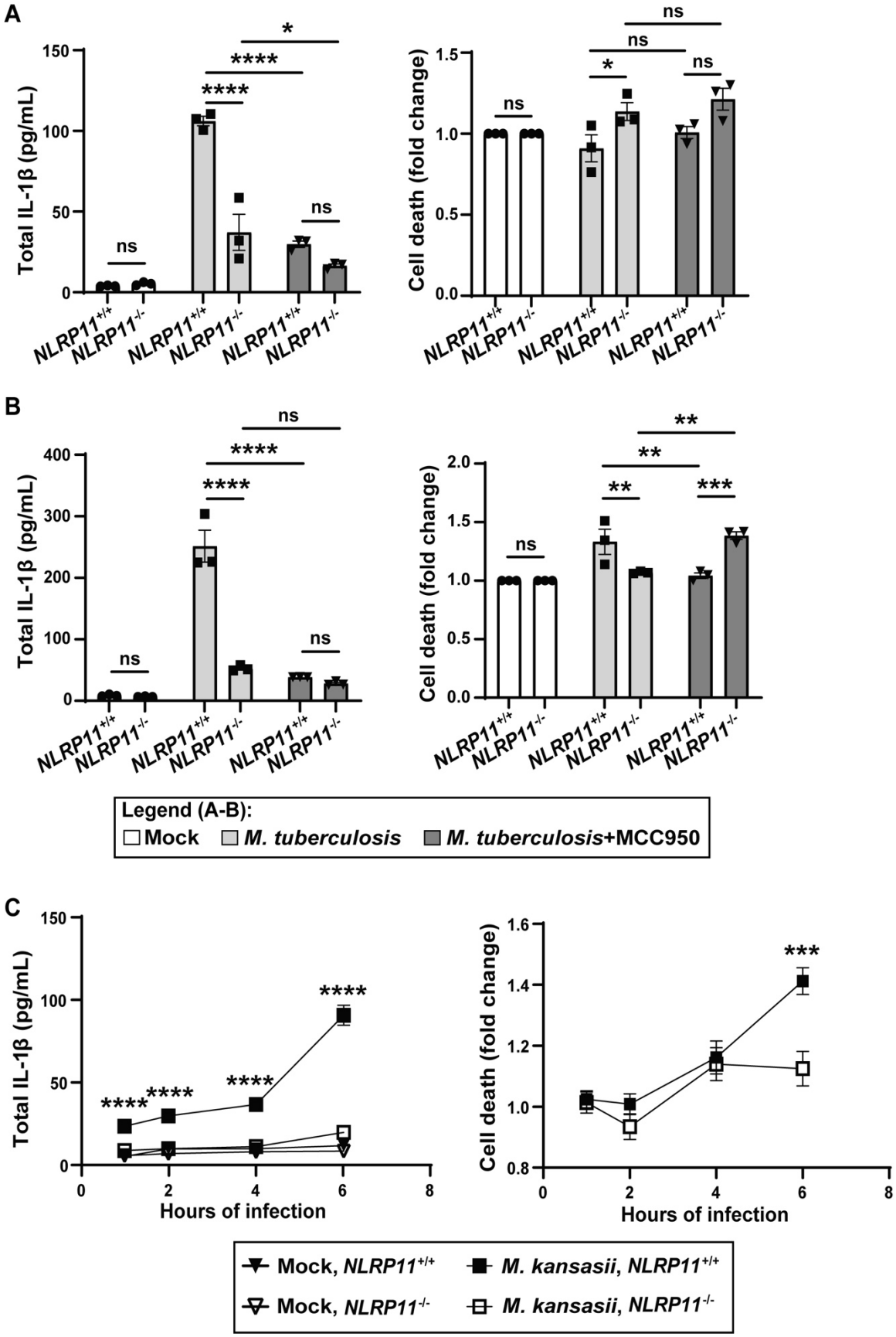
159 LRR domains are required for efficient NLRP3 inflammasome activation,
160 consistent with previous data demonstrating a requirement for the NLRP11
161 NACHT and LRR domains for oligomerization of NLRP3 (27). The findings also
162 suggest that the involvement of NLRP11 in NLRP3 activation does not 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 *NLRP11*Δ^{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).


A
Total IL-1β (pg/mL)
Cell death (fold change)
NLRP11+/+
NLRP11-/-
ns
*
****
B
**
***
Legend (A-B):
Mock
M. tuberculosis
M. tuberculosis+MCC950
C
Hours of infection
Mock, NLRP11+/+
Mock, NLRP11-/-
M. kansasii, NLRP11+/+
M. kansasii, NLRP11-/-

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$^{\Delta N_LRR}$*) or lacking all of NLRP11 (*NLRP11$^{-/-}$*),
223 but not in macrophages lacking only the PYD (*NLRP11$^{\Delta PYD}$*) (Fig. 3B).

224 To test whether the observed defect in activation of pyroptosis in *NLRP11$^{\Delta N_LRR}$*
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*$^{\Delta N_LRR}$ and
228 *NLRP11*$^{-/-}$ macrophages but not reduced in *NLRP11*$^{\Delta PYD}$ 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*$^{\Delta N_LRR}$ and
231 *NLRP11*$^{-/-}$ macrophages was not due to changes in its expression or stability.
232 Similarly, processing of GSDMD was significantly reduced in *NLRP11*$^{\Delta N_LRR}$ and
233 *NLRP11*$^{-/-}$ macrophages but not in *NLRP11*$^{\Delta PYD}$ 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*$^{\Delta N_LRR}$ 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.

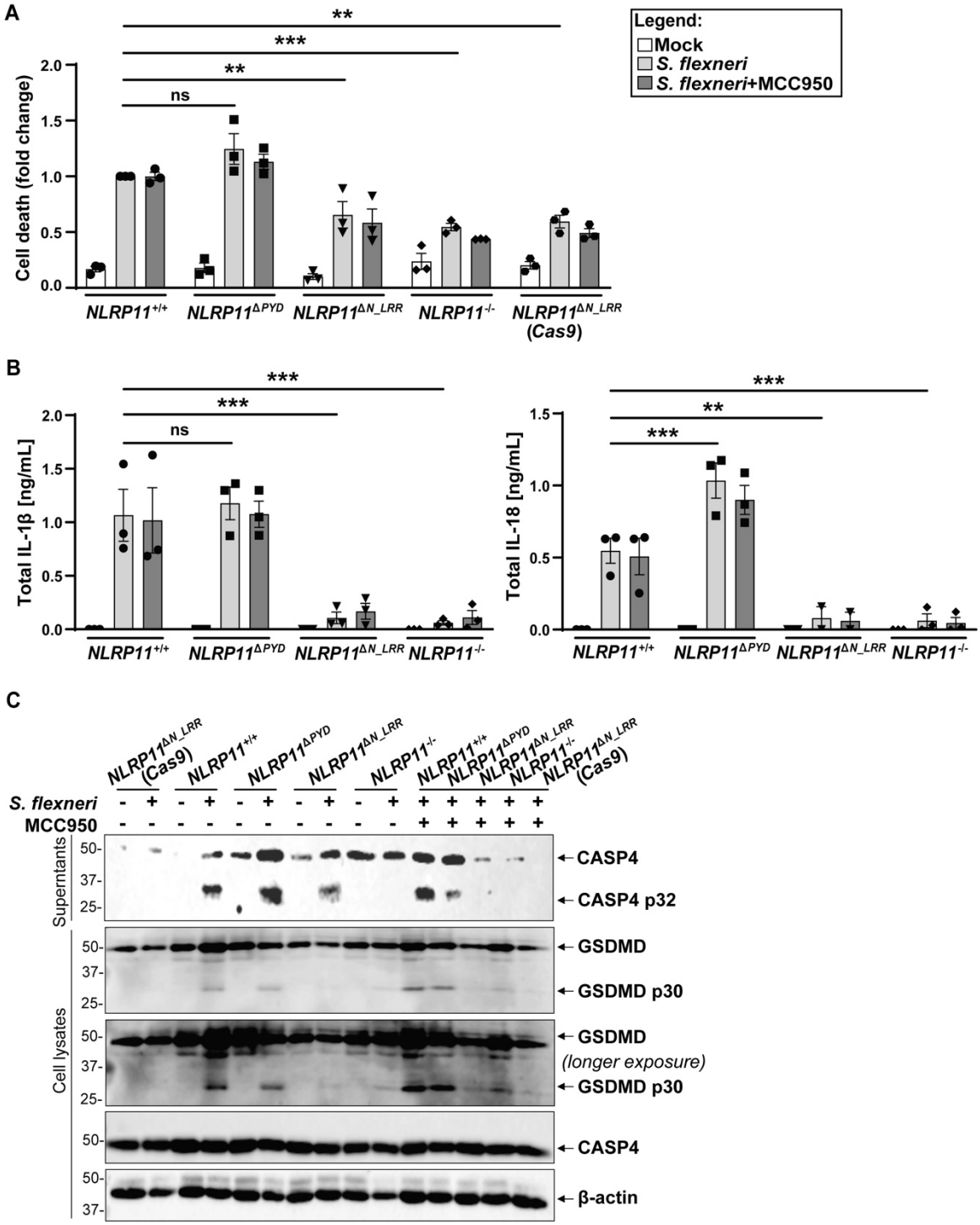


FIG 3 *NACHT and/or LRR domains are required for NLRP11-dependent non-canonical inflammasome activation during S. flexneri infection. (A) Activation of cell death in indicated NLRP11 mutant cell lines at 3 h of infection with S. flexneri*

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.*

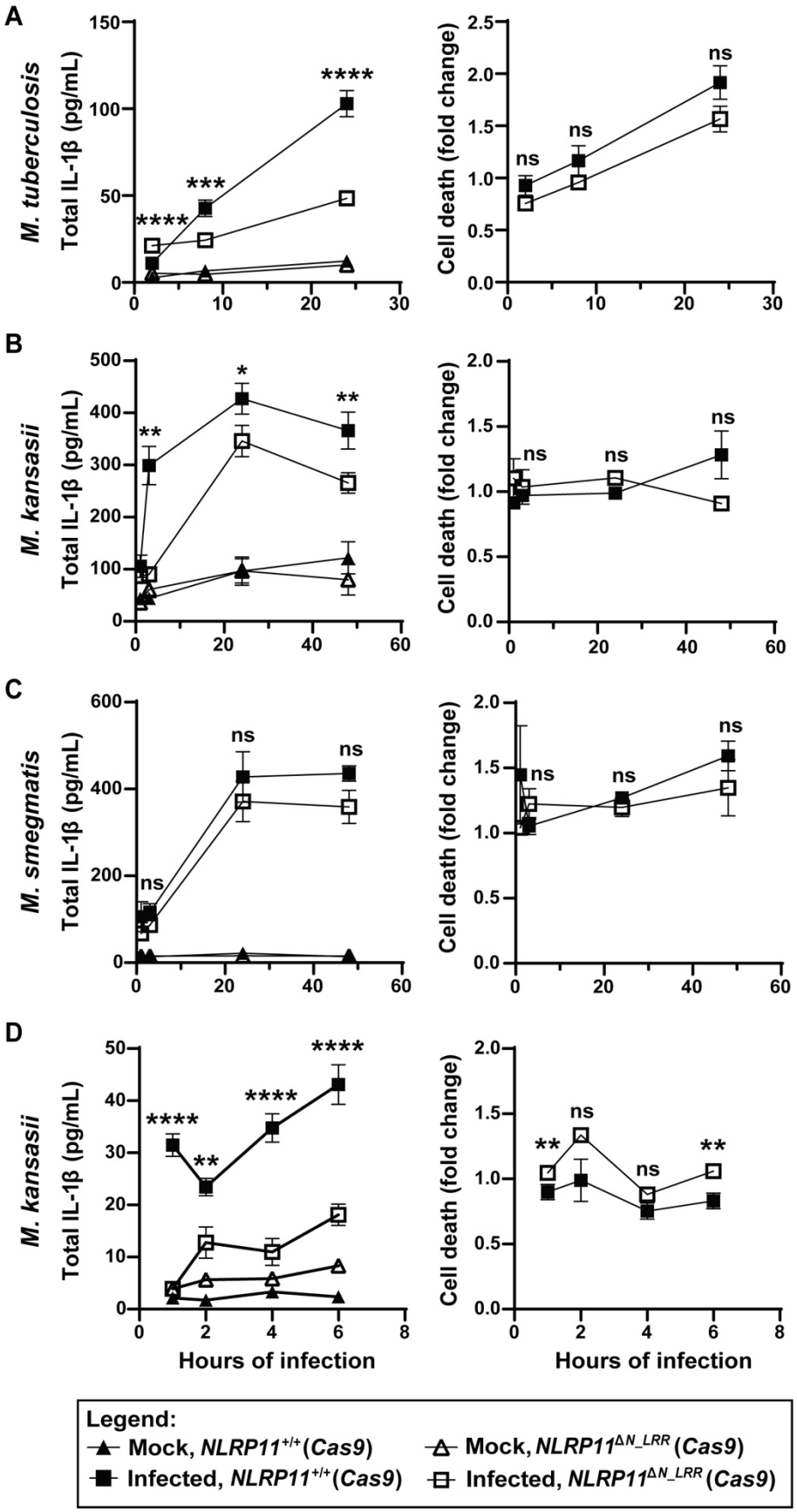
258 **Activation of the non-canonical inflammasome by *M. tuberculosis* is**
259 **dependent on NLRP11.** The *NLRP11$^{\Delta N_LRR}$* (*Cas9*) cell line accurately reports
260 on the role of NLRP11 in non-canonical caspase-4 activity, while displaying
261 normal activation of NLRP3 in response to nigericin (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*$^{\Delta N_LRR}$ (*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*$^{\Delta N_LRR}$ 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$^{\Delta N_LRR}$ (*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.


A
M. tuberculosis
Total IL-1β (pg/mL)
Cell death (fold change)
B
M. kansasii
C
M. smegmatis
D
M. kansasii
Hours of infection
Legend:
Mock, NLRP11+/+(Cas9)
Mock, NLRP11ΔN_LRR(Cas9)
Infected, NLRP11+/+(Cas9)
Infected, NLRP11ΔN_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.*

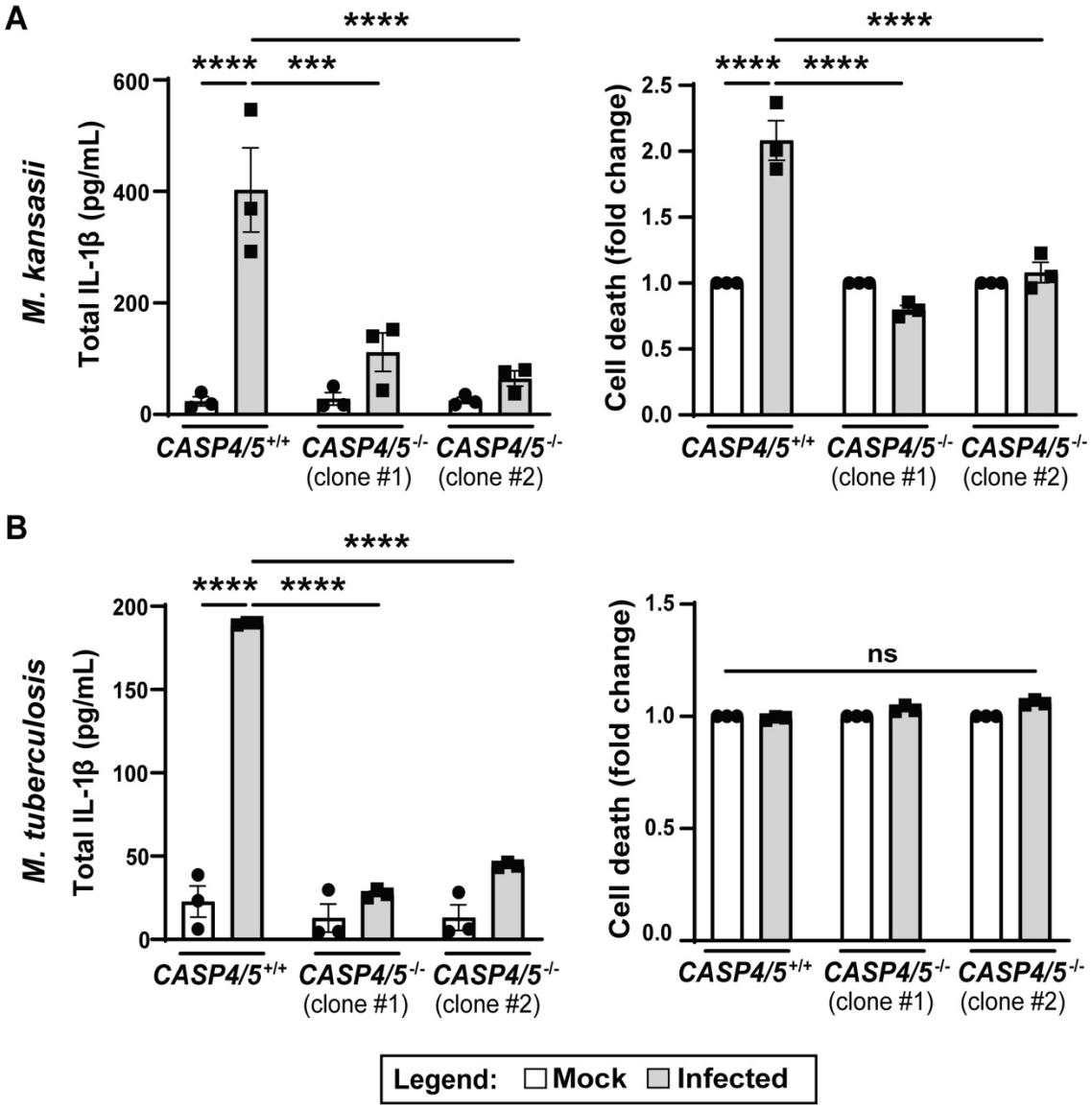


FIG 5 *Caspase-4/5 are required for recognition of mycobacteria in human macrophages. IL-1β release and cell death of WT and two distinct clones of caspase-4/5$^{-/-}$ (CASP4/5$^{-/-}$) THP-1 macrophages infected with M. kansasii (A) or M. tuberculosis (B) (MOI 10). For M. kansasii, assays were performed 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 <*

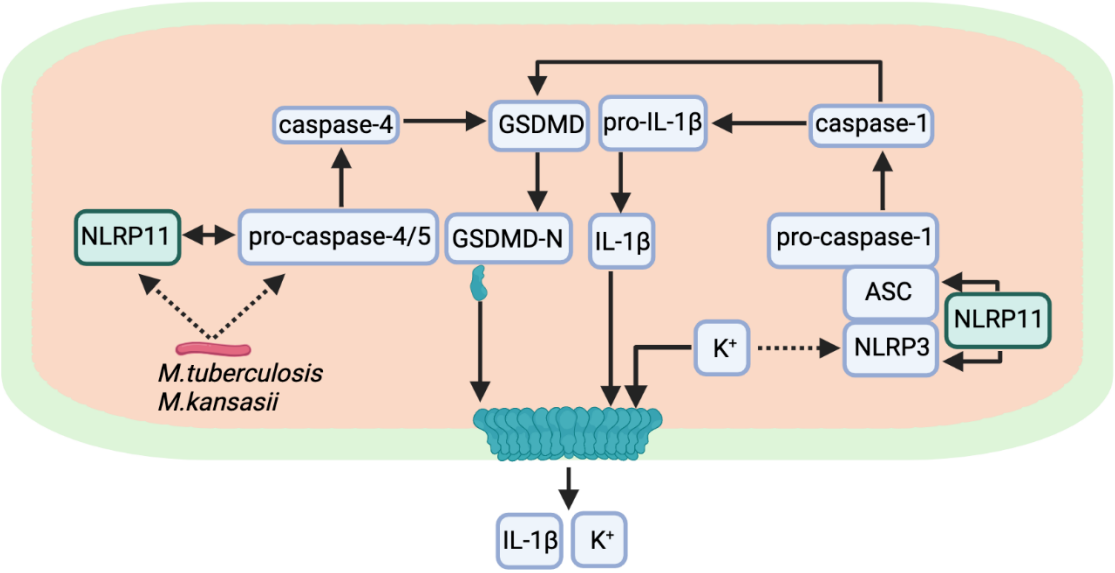
320 *0.001; ****, P < 0.0001; by two-way ANOVA. Shown are independent biological*

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

322 **DISCUSSION**

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

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



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

355 have resulted in off-target effects that masked the role of NLRP11 specifically in
356 the canonical NLRP3 inflammasome pathway. Our new results thus unite the
357 findings in the field and establish a consensus that NLRP11 is required for
358 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 $1x10^6$ 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 37ºC. 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.

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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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