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, gram-positive 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.

44 **IMPORTANCE**

45 The activation of inflammasome complexes plays a crucial role in intracellular
46 pathogen detection. NLRP11 and caspase-4 are essential for recognizing
47 lipopolysaccharide (LPS), a molecule found in gram-negative bacteria such as
48 the human pathogens *Shigella* spp., which activate both canonical NLRP3 and
49 non-canonical inflammasome pathways. Through a series of deletion mutants,
50 we demonstrate that the NACHT and LRR domains of NLPR11, but not its pyrin
51 domain, are critical for detection of *S. flexneri*. Notably, our research reveals that
52 the gram-positive bacterium *M. tuberculosis* is also detected by NLPR11 and
53 caspase-4, despite not producing LPS. These findings significantly expand the
54 range of pathogens recognized by NLRP11 and caspase-4 to now include gram-
55 positive bacteria and underscore the versatility of these innate immune
56 components in pathogen detection.

57 **INTRODUCTION**

58 Intracellular pathogens are detected by host cell cytosolic Nucleotide-binding
59 Oligomerization Domain and Leucine-rich Repeat-containing receptors (NLRs) or
60 Absent in Melanoma 2-like receptors (ALRs), which trigger the activation of an
61 inflammasome complex (1-5). In addition to the respective NLR or ALR, this
62 complex typically comprises an adaptor component (ASC) and pro-caspase-1.
63 Upon full activation, the inflammasome complex contains active caspase-1,
64 which cleaves pro-IL-1β to generate mature IL-1β. Caspase-1 also cleaves
65 gasdermin D (GSDMD), releasing an N-terminal (NT-GSDMD) fragment that
66 forms pores in the cell membrane through which IL-1β exits the cell (6-10). The
67 process culminates with the activation of NINJ1, a membrane disrupting protein
68 that leads to plasma membrane rupture (11-13). This final stage marks the
69 completion of pyroptosis, a specific and inflammatory form of 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. The non-canonical inflammasome
75 pathway relies on the recognition of lipopolysaccharide (LPS) by inflammatory
76 pro-caspase-4/5 (humans) or their murine homologue pro-caspase-11 (14-17).
77 LPS binding leads to caspase activation and subsequent cleavage of GSDMD.
78 The resulting NT-GSDMD pores facilitate K^+ efflux, which in turn triggers NLRP3
79 inflammasome activation. Humans but not mice express NLRP11, which is

80 required for efficient activation of the non-canonical inflammasome in response to
81 intracellular LPS (18), as well as canonical NLRP3 inflammasome activation (19).
82 In the current study, we determined the importance of the NLRP11 pyrin,
83 NACHT, and/or LRR domains for their function in LPS recognition, IL-1β
84 production and pyroptosis induction.

85 *Mycobacterium tuberculosis* is a human adapted, facultative intracellular
86 pathogen that activates the NLRP3 inflammasome of infected macrophages (20).
87 IL-1β is of great importance for a protective host response to *M. tuberculosis*
88 infections (20, 21). *M. tuberculosis* evolved to evade recognition by host cell
89 inflammasomes (20). 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 (22). The *M. tuberculosis* protein kinase (PknF) limits activation of the
93 NLRP3 inflammasome via an unknown mechanism (23). The Rv3364c protein
94 suppresses host cathepsin G activation, leading to reduced inflammasome
95 activation (24). 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 a gram-positive bacterium that does not
98 express LPS but is atypical because it has an outer membrane composed of
99 mycobacterial lipids and lipoproteins (25). 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 (18).
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 cell death upon treatment with the NLRP3 agonist nigericin was significantly
119 decreased compared to nigericin-treated WT macrophages (Fig. 1B). Activation
120 of caspase-1, which involves cleavage and release of its active domains, and
121 activation of GSDMD, which involves its cleavage, were significantly reduced in
122 *NLRP11*$^{-/-}$ macrophages (Fig. S2). In parallel, release of IL-1β, a pro-
123 inflammatory cytokine effector of inflammasome activation, was reduced in
124 *NLRP11*$^{-/-}$ macrophages (Fig 1C).

125 To test whether these phenotypes were specifically due to activation of NLRP3,
126 we treated cells with the NLRP3 inhibitor MCC950. Treatment with MCC950
127 rescued nigericin-induced cell death, processing of caspase-1 and GSDMD, and
128 release of IL-1β and IL-18 to the levels of mock-treated THP-1s (Fig. 1B-C, and
129 Fig. S2), indicating that the observed phenotypes were due to activation of
130 NLRP3.

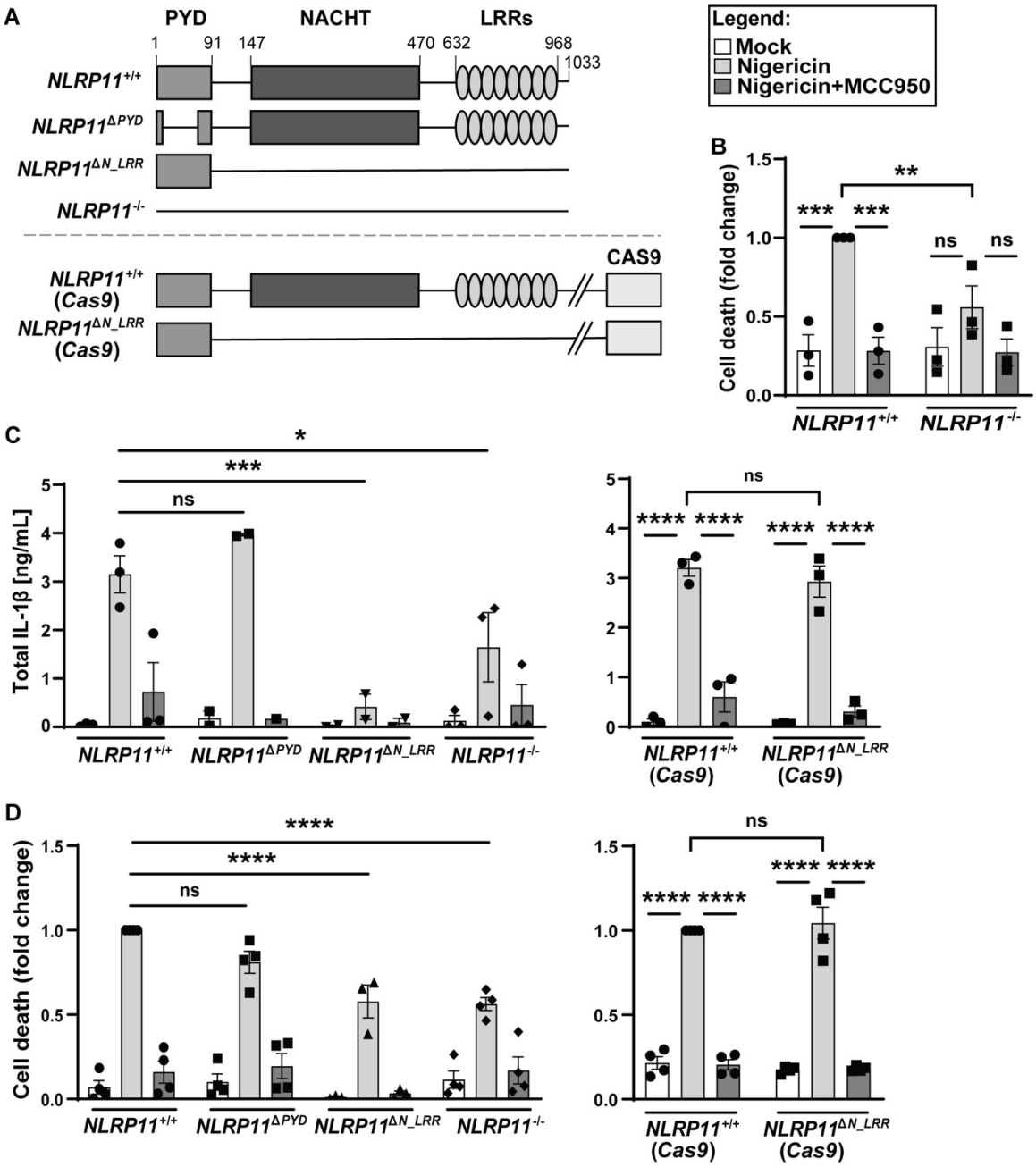


Fig. 1. Efficient activation of the canonical NLRP3 inflammasome depends on the NLRP11 NACHT and/or LRR domains. *(A) Schematic of NLRP11 mutants generated in THP-1 macrophages. (B) Activation of cell death in*

135 *NLRP11$^{+/+}$ and NLRP11$^{-/-}$ macrophages by the NLRP3 agonist nigericin. Cell*
136 *death, measured via LDH release assay at 90 min, is shown normalized to*
137 *nigericin-treated NLRP11$^{+/+}$ macrophages. Where indicated, cells were*
138 *pretreated with the NLRP3 inhibitor MCC950. (C) Levels of IL-1β released into*
139 *the cell culture media at 90 min of nigericin treatment with or without addition of*
140 *MCC950. (D) Cell death, as in (B). ns, not significant; *, P < 0.05; **, P < 0.01;*
141 ****, P < 0.001; ****, P < 0.0001; by two-way ANOVA. Shown are independent*
142 *biological replicates. Error bars represent the standard error of the mean. PYD,*
143 *pyrin domain; NACHT, NAIP, C2TA, HET-E and TP1 domain; LRR, leucin-rich*
144 *repeat domain.*

145 **NACHT and/or LRR domains are required for NLRP11-dependent activation**
146 **of the NLRP3 inflammasome.** To determine the impact of specific domains of
147 NLRP11 on NLRP3 signaling, we assessed activation of cell death and cytokine
148 release in ribonucleoprotein-generated *NLRP11$^{\Delta PYD}$* and *NLRP11$^{\Delta N_LRR}$*
149 macrophages. Deletion of NACHT and LRR domains, but not the PYD domain
150 alone, was associated with reduced nigericin-induced cell death (Fig. 1D) and
151 reduced release of IL-1β (Fig. 1C). In addition, in *NLRP11$^{\Delta N_LRR}$* macrophages,
152 processing of caspase-1 and GSDMD was significantly reduced (Fig. S2). In
153 contrast, in *NLRP11$^{\Delta PYD}$* macrophages, caspase-1 was activated at levels similar
154 to those observed in *NLRP11$^{+/+}$* macrophages, and activation of GSDMD was
155 only mildly decreased. These findings indicate that the NLRP11 NACHT and/or
156 LRR domains are required for efficient NLRP3 inflammasome activation,
157 consistent with previous data demonstrating a requirement for the NLRP11

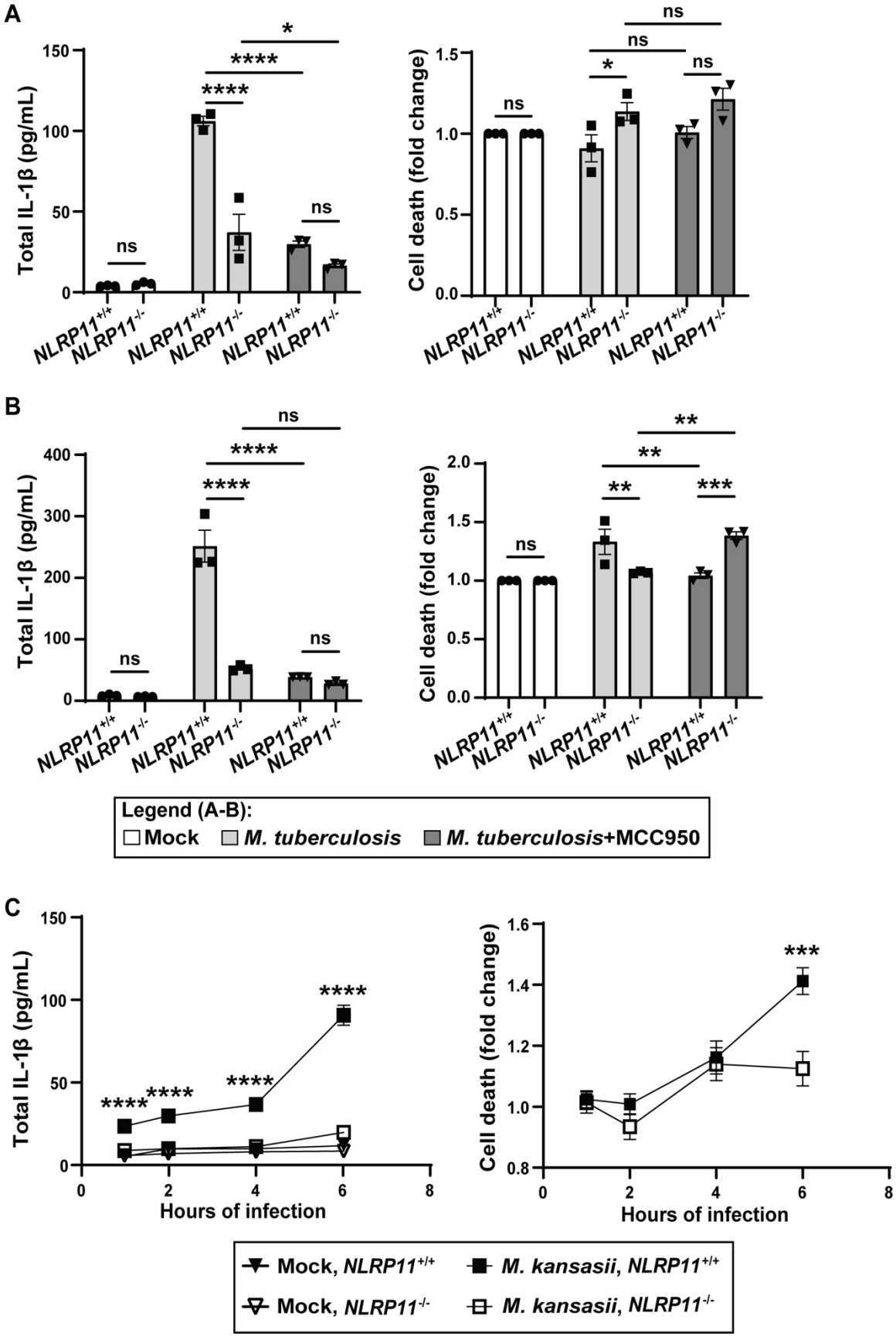
158 NACHT and LRR domains for oligomerization of NLRP3 (19). The findings also
159 suggest that the involvement of NLRP11 in NLRP3 activation does not strictly
160 depend on the NLRP11 pyrin domain.

161 As we found previously (18), nigericin activation of NLRP3 was not impacted in
162 *Cas9*-integrated *NLRP11*$^{\Delta N_LRR}$ macrophages (*NLRP11*$^{\Delta N_LRR}$ (*Cas9*), Fig. 1C-D).
163 This result suggests that, as relates specifically to NLRP3 signaling, the lack of
164 impact of NLRP11 NACHT and LRR disruption on NLRP3 in this *Cas9*-integrated
165 cell lineage is not due to the maintenance of the NLRP11 pyrin domain; rather, it
166 likely results from non-NLRP11 genetics of the lineage and suggests that
167 genome-level changes resulting from the chronic presence of *Cas9* contributed
168 to this phenotype.

169 **Activation of the NLRP3 inflammasome by infection with the gram-positive**
170 **bacterium *M. tuberculosis* depends on NLRP11.** Whether NLRP11 is involved
171 in recognition of gram-positive bacteria and mounting of a cellular inflammatory
172 response to them has not been studied. IL-1β produced by THP-1 macrophages
173 infected with *M. tuberculosis* is dependent on NLRP3 (26, 27). NLRP11 is also
174 recruited by NLRP3 in the process of its activation (19). Therefore, we
175 hypothesized that in *NLRP11*$^{-/-}$ THP-1 macrophages infected by *M. tuberculosis*,
176 IL-1β production might be lower than in WT macrophages. To test this
177 hypothesis, we determined levels of IL-1β release during infection with *M.*
178 *tuberculosis* of macrophages containing or lacking NLRP11. As a readout for
179 inflammasome activation during *M. tuberculosis* infection, cell death is less useful
180 than IL-1β release because few *M. tuberculosis*-infected macrophages die. At 8

181 and 24 hours of infection, significantly less IL-1β was released from *NLRP11*$^{-/-}$
182 macrophages than from WT macrophages (Fig. 2A-B). To test whether the
183 observed IL-1β release is dependent on activation of NLRP3, infected cells were
184 treated with MCC950. *M. tuberculosis*-infected *NLRP11*$^{-/-}$ macrophages treated
185 with MCC950 display a decrease of IL-1β compared with those not treated with
186 MCC950 (Fig. 2A-B), indicating a requirement for NLRP3, which could be direct
187 or indirect.

188 As for *M. tuberculosis*, IL-1β produced by THP-1 macrophages infected with *M.*
189 *kansasii*, an opportunistic pathogen that is a close relative of *M. tuberculosis*, is
190 dependent on NLRP3 (28). Release of IL-1β during infection with *M. kansasii*
191 was dependent on NLRP11 (Fig. 2C, left graph), similar to the phenotype
192 observed for *M. tuberculosis*-infected cells. At late times of *M. kansasii* infection,
193 cell death was also partially dependent on NLRP11 (Fig. 2C, right graph).
194 Whereas IL-1β release from *NLRP11*$^{-/-}$ macrophages was significantly reduced at
195 1, 2, 4 and 6 h of infection, cell death was significantly lower only at 6 h of
196 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-/-

198 ***Fig. 2. NLRP3 activation by M. tuberculosis and M. kansasi in THP-1***
199 ***macrophages is dependent on NLRP11.*** *(A and B) IL-1β release and cell death*
200 *of NLRP11$^{-/-}$ and WT THP-1 macrophages at 8 h (A) or 24 h (B) of infection with*
201 *M. tuberculosis (MOI 10) with or without addition of MCC950. (C) IL-1β release*
202 *and cell death of NLRP11$^{-/-}$ and WT THP-1 macrophages over 1-6 h of infection*
203 *with M. kansasii. Cell death was normalized to cell lineage-specific uninfected*
204 *group. ns, not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P <*
205 *0.0001; by two-way ANOVA (A-B) or paired parametric t-tests (C). Shown are*
206 *independent biological replicates. Error bars represent the standard error of*
207 *mean.*

208 **NLRP11 NACHT and/or LRR domains are required for activation of the non-**
209 **canonical inflammasome by the gram-negative bacterium *Shigella flexneri*.**
210 We previously found that NLRP11 is required for activation of the non-canonical
211 inflammasome during infection with a variety of gram-negative bacteria, including
212 *S. flexneri* (18). Cell death during infection with *S. flexneri* was unaffected by
213 deletion of the pyrin domain (PYD), whereas deletion of the NACHT and LRR
214 domains or deletion of the entire *NLRP11* locus resulted in 40-50% decreases in
215 macrophage cell death (Fig. 3A). Furthermore, release of pro-inflammatory
216 cytokines IL-1β and IL-18 was reduced up to 10-fold in macrophages lacking the
217 NACHT and LRR domains (*NLRP11$^{\Delta N_LRR}$*) or lacking all of NLRP11 (*NLRP11$^{-/-}$*),
218 but not in macrophages lacking only the PYD (*NLRP11$^{\Delta PYD}$*) (Fig. 3B).

219 To test whether the observed defect in activation of pyroptosis in *NLRP11$^{\Delta N_LRR}$*
220 and *NLRP11$^{-/-}$* macrophages resulted from a defect in activation of the non-

221 canonical inflammasome, we examined processing of caspase-4 and GSDMD.
222 Activation of caspase-4 was significantly reduced in *NLRP11*$^{\Delta N_LRR}$ and *NLRP11*$^{-}$
223 $^{/-}$ macrophages but not *NLRP11*$^{\Delta PYD}$ macrophages (Fig. 3C and Fig. S3). Levels
224 of full-length pro-caspase-4 in cell lysates were unaffected, indicating that the
225 decrease in caspase-4 released from *NLRP11*$^{\Delta N_LRR}$ and *NLRP11*$^{-/-}$
226 macrophages was not due to changes in its expression or stability. Similarly,
227 processing of GSDMD was significantly reduced in *NLRP11*$^{\Delta N_LRR}$ and *NLRP11*$^{-/-}$
228 macrophages but not in *NLRP11*$^{\Delta PYD}$ macrophages, yet levels of full-length
229 GSDMD were unaltered (Fig. 3C and Fig. S3). Thus, the NACHT and/or LRR
230 domains are required for efficient activation of the NLRP11-dependent non-
231 canonical inflammasome during infection with *S. flexneri*.

232 **Activation of the non-canonical inflammasome by *S. flexneri* is**
233 **independent of NLRP3.** To test whether the NLRP11-dependent defect in
234 activation of the non-canonical inflammasome is dependent on NLRP3, we
235 examined inflammasome activation in *S. flexneri*-infected *NLRP11*$^{\Delta N_LRR}$
236 macrophages pre-treated with MCC950. Treatment with MCC950 did not impact
237 the levels of cell death, cytokine release, or processing of caspase-4 and
238 GSDMD (Fig. 3A-C, and Fig. S3). Therefore, NLRP3 is not required for activation
239 of the NLRP11-dependent non-canonical inflammasome during *S. flexneri*
240 infection.

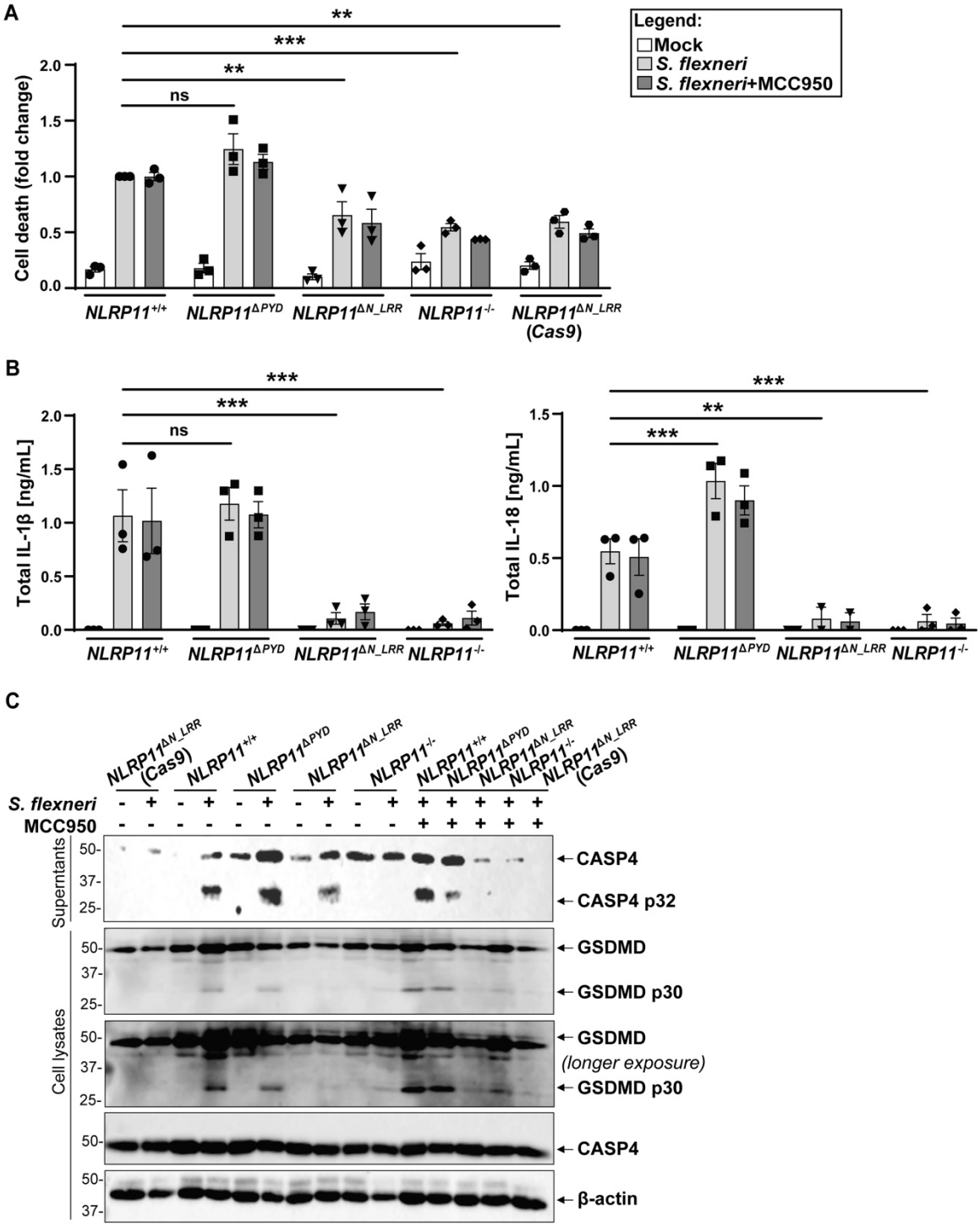


Fig. 3. NACHT and/or LRR domains are required for NLRP11-dependent non-canonical inflammasome activation during S. flexneri infection. *(A) Activation of cell death in indicated NLRP11 mutant cell lines at 3 h of infection*

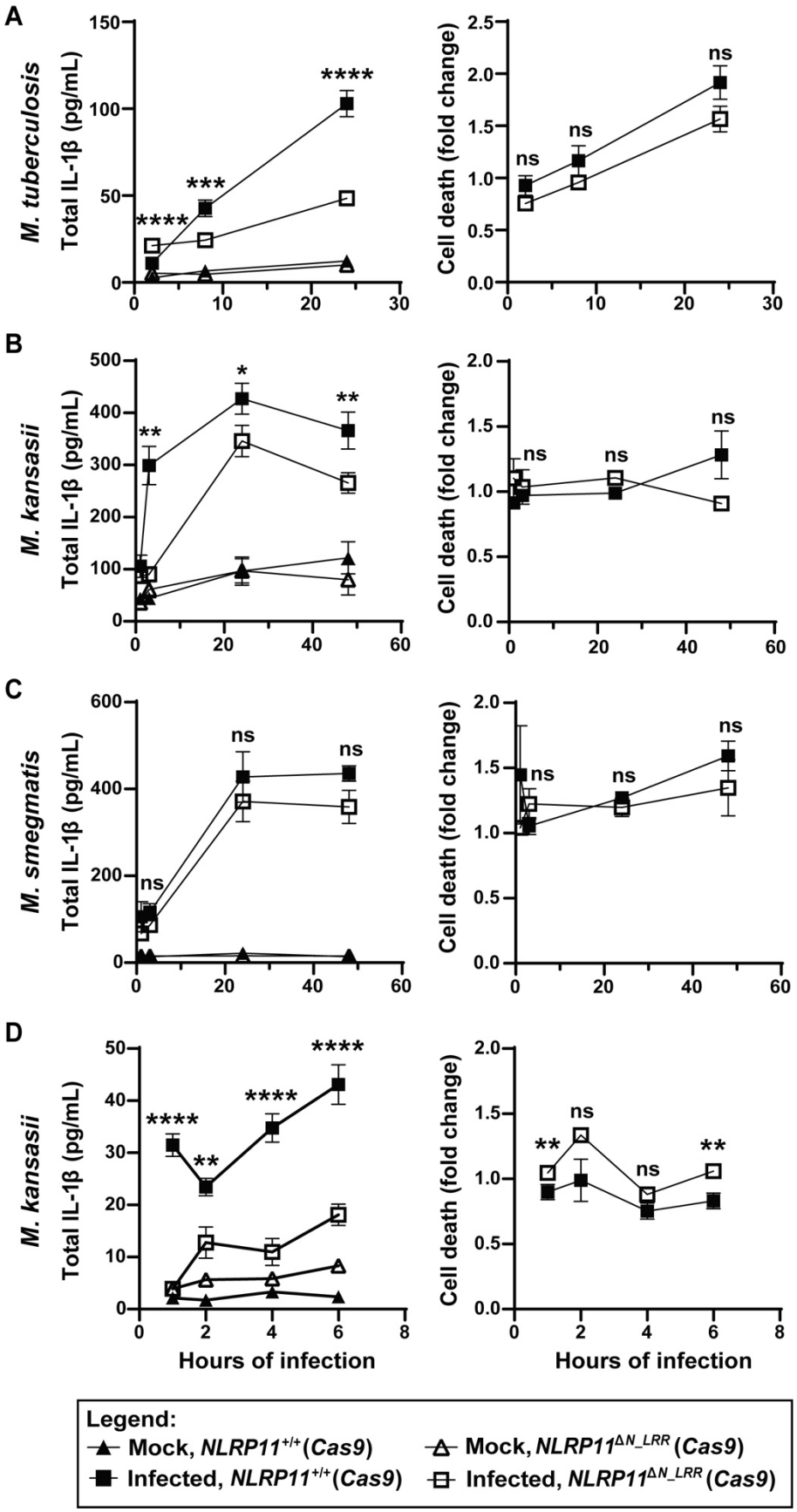
245 *with S. flexneri (MOI 10). Cell death is shown normalized to S. flexneri-infected*
246 *NLRP11$^{+/+}$ macrophages. Where indicated, cells were pretreated with the NLRP3*
247 *inhibitor MCC950. (B) Levels of IL-1β and IL-18 released into the cell culture*
248 *media from (A). (C) Cleaved caspase-4 released into the cell culture media and*
249 *intracellular cleaved GSDMD under same conditions. Ns, not significant; **, P <*
250 *0.01; ***, P < 0.001; by two-way ANOVA. Shown are independent biological*
251 *replicates. Error bars represent the standard error of the mean.*

252 **Activation of the non-canonical inflammasome by *M. tuberculosis* is**
253 **dependent on NLRP11.** The *NLRP11$^{\Delta N_LRR}$* (*Cas9*) cell line accurately reports
254 on the role of NLRP11 in non-canonical caspase-4 activity, while displaying
255 normal activation of NLRP3 in response to nigericin (18). Therefore, to determine
256 whether NLRP11 is required for non-canonical caspase-4 inflammasome activity
257 during mycobacterial infection, we compared IL-1β release from mycobacteria-
258 infected WT (*Cas9*) to that from mycobacteria-infected *NLRP11$^{\Delta N_LRR}$* (*Cas9*)
259 macrophages. *NLRP11$^{\Delta N_LRR}$* cells infected with *M. tuberculosis* or *M. kansasii*
260 showed significantly diminished IL-1β release at all time points (Fig. 4A-B),
261 whereas those infected with the non-pathogenic *M. smegmatis* showed increases
262 in IL-1β release over time that were similar to *M. kansasii* but which were not
263 reduced in the *NLRP11$^{\Delta N_LRR}$* cells at any time point (Fig. 4C). These data
264 indicate that IL-1β released during *M. tuberculosis* and *M. kansasii* infection is
265 dependent, at least partially, on the NACHT and/or LRR domains of NLRP11.
266 Cell death was not significantly different between cell lines during infection with
267 any of the three mycobacterial species.

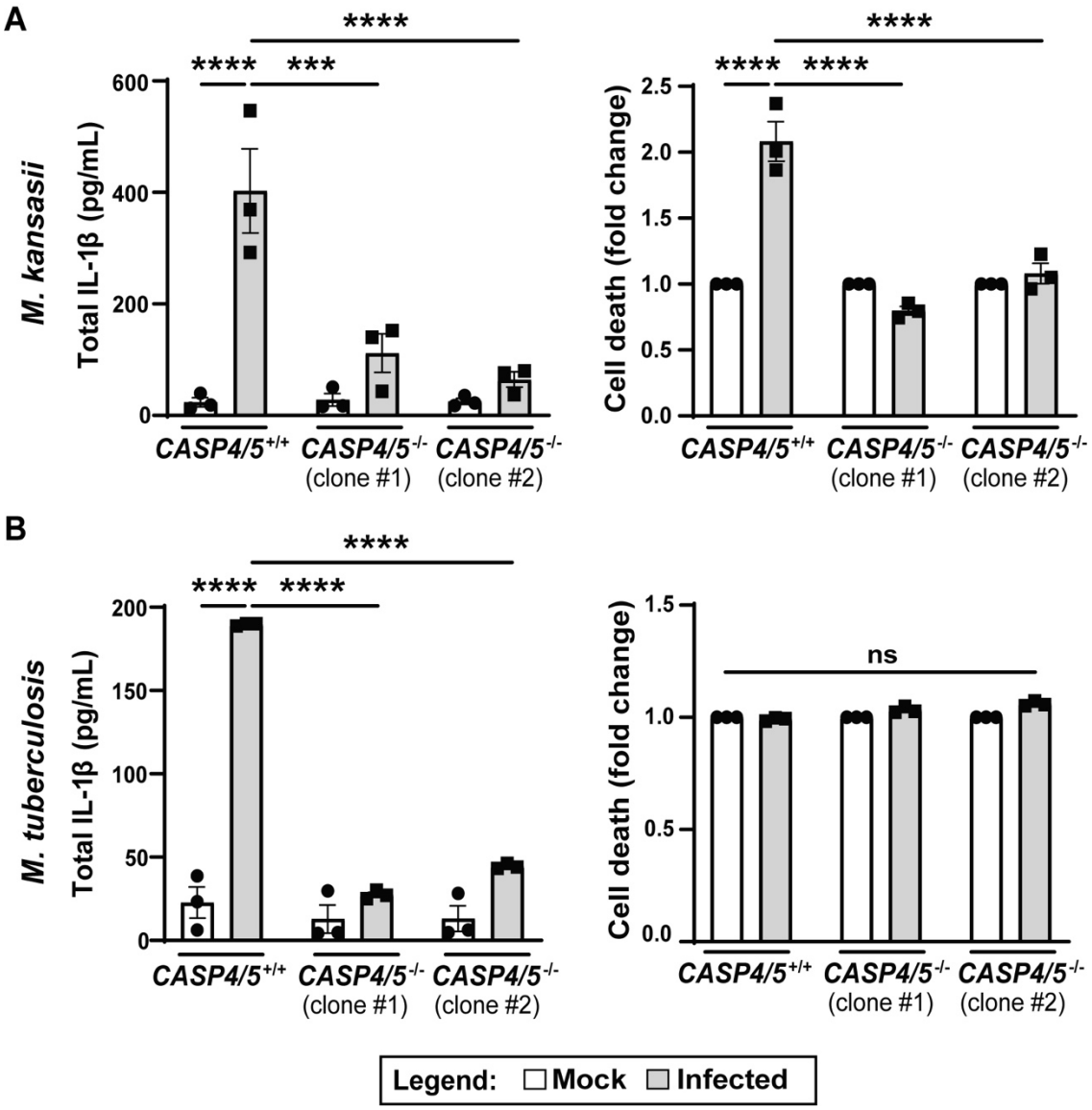
268 To further examine the kinetics of NLRP11 dependent activation of the non-
269 canonical inflammasome, we assayed IL-1β release in *M. kansasii*-infected WT
270 and *NLRP11*$^{\Delta N_LRR}$ (*Cas9*) macrophages at 1, 2, 4 and 6 hours of infection. At
271 every time point, significantly lower levels of IL-1β was released from
272 *NLRP11*$^{\Delta N_LRR}$ macrophages than their WT counterparts (Fig. 4D), indicating that
273 NLRP11 is required for IL-1β release even early during infection. As above (Fig.
274 4B), at early times, NLRP11-dependent differences in cell death are minimal (Fig.
275 4D).

276 The use of the NLRP11$^{\Delta N_LRR}$ (*Cas9*) mutant THP-1 cells is consistent with but
277 not proof of *M. tuberculosis* and *M. kansasii* infection being dependent on the
278 non-canonical inflammasome. In humans, the non-canonical inflammasome
279 involves either or both of caspase-4 and caspase-5. During NLRP11-dependent
280 activation of the non-canonical inflammasome, NLRP11 is required for
281 downstream activation of at least caspase-4 (18); in this study, caspase-5 was
282 not examined. To directly test whether mycobacteria activate the non-canonical
283 inflammasome, we tested whether macrophages that lack both caspase-4 and
284 caspase-5 support mycobacterium-induced IL-1β release and cell death. At 4
285 hours of infection with *M. kansasii* (Fig. 5A, left graph) and 24 hours of infection
286 with *M. tuberculosis* (Fig. 5B, left graph), the absence of caspases-4 and -5 was
287 associated with a complete absence of IL-1β release, indicating that caspase-4
288 and/or caspase-5 are required for mycobacteria activation of the non-canonical
289 inflammasome. Paralleling the impact of caspases-4 and -5 on IL-1β release,
290 during infection with *M. kansasii*, their absence was associated with a two-fold

291 reduction in cell death to baseline (Fig. 5A, right graph), whereas during infection
292 with *M. tuberculosis*, the impact of their absence on cell death was minimal (Fig.
293 5B, right graph). These data indicate that IL-1β released during *M. tuberculosis*
294 and *M. kansasii* infection is dependent on the non-canonical caspase-4/5
295 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)

297 ***Fig. 4. Mycobacteria activate the non-canonical inflammasome pathway.*** *IL-*
298 *1β release and cell death of NLRP11$^{+/+}$ (Cas9) and NLRP11$^{\Delta N_LRR}$ (Cas9) mutant*
299 *THP-1 macrophages infected with M. tuberculosis (A), M. kansasii (B, D), or M.*
300 *smegmatis (C) (MOI 10), at the indicated timepoints. Cell death was normalized*
301 *to cell lineage-specific uninfected group. ns, not significant; **, P < 0.01; ***, P <*
302 *0.001; ****, P < 0.0001; by paired parametric t-tests. Shown are independent*
303 *biological replicates. Error bars represent the standard error of mean.*



304

305 ***Fig. 5. Caspase-4/5 are required for recognition of mycobacteria in human***

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

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

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

309 *infection (A), and for M. tuberculosis, at 24 h of infection (B). ns, not significant;*

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

311 *biological replicates. Error bars represent the standard error of mean.*

312 **DISCUSSION**

313 Two recent studies establish that NLRP11 is required for efficient activation of
314 the non-canonical caspase-4/5 inflammasome in macrophage-like THP-1 cells
315 and primary human macrophages (18, 19). These studies demonstrated that
316 NLRP11 is required for non-canonical inflammasome activation in response to
317 cytosolic LPS (18, 19) and infection with gram-negative bacteria (18). Our
318 findings herein extend our understanding of NLRP11 function by demonstrating
319 that NLRP11 is required for efficient activation of the non-canonical
320 inflammasome by the gram-positive bacteria *M. tuberculosis* and *M. kansasii*.

321 The present study offers clarification on previously conflicting findings regarding
322 the role of NLRP11 role in activation of the canonical NLRP3 inflammasome:
323 Gangopadhyay et al. reported that NLRP11 is required for NLRP3 activation (19),
324 while Rojas-Lopez et al. found no significant involvement of NLRP11 in this
325 process (18). Our results confirm a role for NLRP11 in activation of the non-
326 canonical inflammasome and demonstrate that the NACHT and/or LRR domains
327 are required for NLRP3 activation but that NLRP3 activation does not strictly
328 depend on the NLRP11 pyrin domain (Fig. 1C-D). With respect to a role for
329 NLRP11 in activation of the NLRP3 inflammasome specifically, we speculate that
330 the difference in the results reported herein compared with those reported in
331 Rojas-Lopez et al. is a result of some genomic instability of the cell lineage in
332 Rojas-Lopez et al. due to the integration of *Cas9*. The new constructs described
333 in the present study were generated by transfection of Cas9 protein together with
334 guide RNAs (Materials and Methods). The constitutive presence of *Cas9* may

335 have resulted in off-target effects that masked the role of NLRP11 specifically in
336 the canonical NLRP3 inflammasome pathway. Our new results thus unite the
337 findings in the field to support the consensus that NRLP11 is required for
338 activation of both the non-canonical and the canonical NLRP3 inflammasome.

339 The murine homologue of human caspases-4 and -5, caspase-11, is required for
340 the detection of cytosolic LPS. *caspase-11*$^{-/-}$ mice are more resistant to death
341 from *Escherichia coli*-induced or LPS-induced septic shock (14, 16). As
342 described above, in humans, caspase-4 and/or caspase-5 (17, 29, 30) and
343 NLRP11 (18, 19), which is absent in mice, are required for recognition of
344 cytosolic LPS. Indirect evidence suggests that lipoteichoic acid (LTA) of gram-
345 positive bacteria can also be detected by caspase-4 (31, 32). Here, we
346 demonstrate for the first time that NLRP11 and the non-canonical caspase-4
347 inflammasome are required for detection of intracellular *M. tuberculosis* (Fig. 2,
348 Fig. 4, and Fig. 5). Our results indicate that, for infection with *M. tuberculosis* or
349 *M. kansasii*, sensing of the infection by the non-canonical pathway is essential for
350 host inflammasome responses, since caspase-4/5 are required for IL-1β release
351 (Fig. 5). Interestingly, a 2010 shRNA screen of NOD proteins required for IL-1β
352 release from *M. tuberculosis*-infected THP-1 cells identified *NLRP11* as a hit
353 (33).

354 *M. tuberculosis* contains neither LPS nor LTA, and so what could the ligand be
355 for caspase-4 and/or NLRP11? Lipoarabinomannans (LAM) are complex
356 glycolipids similar to LPS that, like lipid A of LPS, are anchored in the inner
357 membrane via a lipid anchor (25). The sugar composition of LAMs differs among

358 mycobacterial species (34), which could explain why *M. tuberculosis* and *M.*
359 *kansasii* but not *M. smegmatis* induce non-canonical inflammasome activation
360 (Fig. 4). The Pro-Glu motif-containing and Pro-Pro-Glu motif-containing (PE/PPE)
361 domain containing family of proteins are abundant in mycobacterial species such
362 as *M. tuberculosis* and *M. kansasii* but absent in *M. smegmatis* (35, 36). The
363 PPE13 protein interacts with the NACHT or LRR domains of NLRP3, leading to
364 its activation (37). Nevertheless, according to our data, such a mechanism of
365 action is unlikely, since we found an almost complete absence of IL-1β secretion
366 in caspase-4/5 deficient THP-1 cells (Fig. 5), and the non-canonical pathway
367 should not be required if PPE13 directly activates the canonical NLRP3
368 inflammasome. In conclusion, our results expand the range of pathogens
369 recognized by NLRP11 and caspase-4 to now include gram-positive bacteria and
370 underscore the versatility of these innate immune components in pathogen
371 detection.

372 **MATERIALS AND METHODS**

373 **Bacterial strains and growth conditions.** *M. tuberculosis* (H37Rv), *M. kansasii*
374 (ATCC1278), and *M. smegmatis* (mc2155) strains were grown in liquid
375 Middlebrook 7H9 medium supplemented with 10% oleic acid-albumin-dextrose-
376 catalase (OADC) growth supplement (B12351), 0.2% glycerol, and 0.05% Tween
377 80. *S. flexneri* wild-type serotype 2a strain 2457T was used in this study (38). *S.*
378 *flexneri* was grown in tryptic soy broth at 37ºC with aeration.

379 **Generation of CRISPR deletions in NLRP11.** We previously created a deletion
380 of the coding sequences of the NACHT and LRR domains in the *NLRP11* gene
381 of THP-1 monocytes using a lentivirus-based system to deliver guide RNAs into
382 cells in which *Cas9* had previously been stably integrated, such that the
383 generated *NLRP11* knockout carried an integrated copy of *Cas9* gene (18). In
384 the current study, to generate knockout lineages that lack chronic *Cas9*,
385 chemically modified sgRNAs (Synthego) were electroporated as
386 ribonucleoprotein (RNP) complexes into freshly cultured THP-1 monocytes using
387 the Neon Transfection System (Invitrogen, MPK5000) following the
388 manufacturer's instructions. Specifically, to create the editing RNP complexes,
389 100 pmol of each sgRNA (Fig. S1E) were mixed with 25 pmol of SpCas9 2NLS
390 nuclease (Synthego) (4:1 ratio) in Resuspension Buffer R (Invitrogen,
391 MPK1069B) and incubated for 15 min at room temperature. THP-1 monocytes
392 were washed in PBS and resuspended in Buffer R. The resulting RNP
393 complexes were electroporated by pulsing twice $1x10^6$ cells in the 10 µL Neon
394 Tip at 1700 V and 2 ms pulse width. Electroporated cells were immediately

395 resuspended in 2.5 mL complete growth medium and placed into a 6-well plate.
396 Cells were incubated for 72 hours at 37ºC, after which 1.5 mL was transferred to
397 a 75 cm^2 culture flask, and 1 mL was used to purify genomic DNA using DNeasy
398 Blood and Tissue kit (Qiagen). Following PCR and sequencing, editing efficiency
399 was determined using ICE Analyzer (Synthego). The remaining cells were
400 limiting-diluted and expanded as single clones in a 96-well dish. Using
401 sequencing, isolated single clones were validated for homozygous out-of-frame
402 deletions (Fig. S1).

403 **Mammalian cell culture.** Human-derived THP-1 monocytes (American Type
404 Culture Collection, TIB-202) were maintained in RPMI 1640 medium (Gibco,
405 11875119) supplemented with 10% (v/v) fetal bovine serum (heat-inactivated,
406 sterile-filtered, R&D systems) and 10 mM HEPES (Gibco, 15630-080). Cells
407 were grown in 5% CO_2 in a humidified incubator at 37ºC. For use in experiments,
408 cells were seeded in 48-well plates at 2×10^5 cells per well or in 96-well plates at
409 5×10^4 cells per well. For all non-mycobacterial experiments, to induce
410 differentiation into macrophages, cells were incubated with 100 ng/mL phorbol
411 12-myristate 13-acetate (PMA) for 24 h, after which they were rested in media
412 without PMA overnight prior to use in experiments. For mycobacterial infections,
413 cells were differentiated into macrophages in 50 ng/mL PMA for 48 h, after which
414 they were rested in media without PMA for 48 h prior to use in experiments.

415 **Cell treatments.** In experiments with nigericin treatment, PMA-differentiated
416 THP-1 macrophages were primed with 500 ng/mL ultrapure LPS from
417 *Escherichia coli* O111:B4 (InvivoGen, tlrl-3pelps) for 4 hours followed by

418 treatment with 10 μM nigericin (InvivoGen, tlrl-nig) for 90 min. In experiments
419 involving nigericin treatment or *S. flexneri* infection, 2 μM MCC950 (NLRP3
420 inhibitor, InvivoGen, inh-mcc) was added where appropriate one hour prior to
421 addition of nigericin or infection and maintained in the medium for the duration of
422 the experiment. In cells infected with *M. tuberculosis*, MCC950 was added at 4 h
423 of infection and maintained in the medium for the duration of the experiment.

424 **Bacterial infections.** For infections with mycobacterial strains, bacteria grown to
425 an exponential phase were centrifuged at 2000 g for 7 min. Bacterial pellets were
426 resuspended in 1X phosphate-buffered saline (PBS) containing 0.05% Tween 80
427 and centrifuged at 80 g for 3 min to pellet clumped bacteria. The supernatants
428 (single cell bacterial suspensions) were used to determine the multiplicity of
429 infection (MOI) by optical density. PMA differentiated THP-1 macrophages were
430 infected at the MOI of 10 and incubated for 2 h (*M. smegmatis*) or for 4 h (*M.*
431 *tuberculosis* and *M. kansasii*) in 5% CO_2 in a humidified incubator at 37°C. After
432 this period of phagocytosis, infected macrophages were gently washed twice with
433 prewarmed 1X PBS. Gentamicin, to kill extracellular bacteria, was added at 100
434 μg/mL in complete media containing RPMI1640 supplemented with 5% human
435 AB serum. Samples for LDH release assay and IL-1β ELISA were collected at
436 the indicated times.

437 For infections with *S. flexneri*, PMA-differentiated macrophages were infected at
438 an MOI of 10 with bacteria grown to exponential phase after back-dilution from
439 an overnight culture. Bacteria were centrifuged onto macrophages at 800 g for 10
440 min at room temperature, followed by incubation in 5% CO_2 in a humidified

441 incubator at 37ºC. After 30 min, cells were washed twice with Hanks' Balanced
442 Salt Solution (HBSS, Gibco, 14025-092), and gentamicin was added to 25 µg/mL
443 in RPMI 1640 medium supplemented with 10 mM HEPES without serum.
444 Samples for LDH release assay, cytokine ELISAs, and western immunoblot were
445 collected at 2 h 40 min. of infection.

446 **LDH cytotoxicity assay.** Macrophage cell death was assessed by release of
447 lactate dehydrogenase (LDH) due to loss of cellular membrane integrity. To
448 measure LDH activity, 50 µL of cell culture supernatants from a 96-well plate
449 were collected at indicated times and a CytoTox 96® Non-Radioactive
450 Cytotoxicity Assay (experiments with mycobacteria; Promega, G1780) or a
451 CyQUANT™ LDH Cytotoxicity Assay (experiments with *S. flexneri* and nigericin;
452 ThermoFisher Scientific, C20301) was used following the manufacturer's
453 instructions.

454 **Cytokine ELISAs.** Cell culture supernatants harvested from mycobacteria-
455 infected cells were assayed for levels of secreted IL-1β using Human Total IL-1β
456 DuoSet ELISA kit (biotechne, DY201) following the manufacturer's instructions.
457 Cell culture supernatants harvested from cells infected with *S. flexneri* or treated
458 with nigericin were assayed for levels of secreted cytokines using Human Total
459 IL-18 DuoSet ELISA kit (biotechne, DY318-05) and ELISA MAX™ Deluxe Set
460 Human IL-1β kit (BioLegend, 437004) following the manufacturers' instructions.

461 **Protein extraction and western immunoblot analysis.** For detection of
462 released caspases, 100 µL of cell culture supernatants from a 48-well plate were

463 collected at the indicated times and mixed with 35 µL of 4x Laemmli buffer [250
464 mM Tris (pH 6.8), 8% SDS, 50% glycerol, 0,04% bromophenol blue]
465 supplemented with cOmplete EDTA-free protease inhibitor (Roche,
466 11873580001). For detection of cell-associated caspases and full-length and
467 cleaved GSDMD, cells in the same plate were lysed directly in 100 µL of 4x
468 Laemmli buffer supplemented with the same protease inhibitor. Following SDS–
469 polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred onto
470 a nitrocellulose membrane.

471 Antibodies used for immunoblotting were as follows: caspase-1 (Abcam,
472 ab207802) rabbit monoclonal antibody at 0.5 µg/mL (1:1000), caspase-4 (Santa
473 Cruz, sc-56056) and GSDMD (Santa Cruz, sc-81868) mouse monoclonal
474 antibodies at 0.5 µg/mL (1:200). Secondary antibodies were goat anti-rabbit
475 immunoglobulin G (IgG) or goat anti-mouse IgG, each conjugated to horseradish
476 peroxidase (HRP) (Jackson ImmunoResearch). Antibodies specific for β-actin
477 were HRP-conjugated (Sigma, A3854). Immunoreactive bands were visualized
478 by chemiluminescence with SuperSignal West Pico PLUS or SuperSignal West
479 Femto Maximum Sensitivity substrates (Thermo Fisher Scientific, 34580 and
480 34096).

481 **Statistical analysis.** GraphPad Prism software was used for graphing data and
482 statistical analyses. Statistical significance was determined using the ordinary
483 two-way ANOVA with Tukey's multiple comparisons test, with a single pooled
484 variance. For Figure 4. statistical significance was determined using a paired
485 parametric t-test. Data were graphed so that each data point represents the

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

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

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

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494 **FIGURE LEGENDS**

495 **Fig. 1. Efficient activation of the canonical NLRP3 inflammasome depends**
496 **on the NLRP11 NACHT and/or LRR domains.** (A) Schematic of NLRP11
497 mutants generated in THP-1 macrophages. (B) Activation of cell death in
498 *NLRP11*$^{+/+}$ and *NLRP11*$^{-/-}$ macrophages by the NLRP3 agonist nigericin. Cell
499 death, measured via LDH release assay at 90 min, is shown normalized to
500 nigericin-treated *NLRP11*$^{+/+}$ macrophages. Where indicated, cells were
501 pretreated with the NLRP3 inhibitor MCC950. (C) Levels of IL-1β released into
502 the cell culture media at 90 min of nigericin treatment with or without addition of
503 MCC950. (D) Cell death, as in (B). ns, not significant; *, $P < 0.05$; **, $P < 0.01$;
504 ***, $P < 0.001$; ****, $P < 0.0001$; by two-way ANOVA. Shown are independent
505 biological replicates. Error bars represent the standard error of the mean. PYD,
506 pyrin domain; NACHT, NAIP, C2TA, HET-E and TP1 domain; LRR, leucin-rich
507 repeat domain.

508 **Fig. 2. NLRP3 activation by *M. tuberculosis* and *M. kansasi* in THP-1**
509 **macrophages is dependent on NLRP11.** (A and B) IL-1β release and cell death
510 of *NLRP11*$^{-/-}$ and WT THP-1 macrophages at 8 h (A) or 24 h (B) of infection with
511 *M. tuberculosis* (MOI 10) with or without addition of MCC950. (C) IL-1β release
512 and cell death of *NLRP11*$^{-/-}$ and WT THP-1 macrophages over 1-6 h of infection
513 with *M. kansasii*. Cell death was normalized to cell lineage-specific uninfected
514 group. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P <$
515 0.0001; by two-way ANOVA (A-B) or paired parametric t-tests (C). Shown are

516 independent biological replicates. Error bars represent the standard error of
517 mean.

518 **Fig. 3. NACHT and/or LRR domains are required for NLRP11-dependent**
519 **non-canonical inflammasome activation during *S. flexneri* infection.** (A)
520 Activation of cell death in indicated NLRP11 mutant cell lines at 3 h of infection
521 with *S. flexneri* (MOI 10). Cell death is shown normalized to *S. flexneri*-infected
522 *NLRP11*$^{+/+}$ macrophages. Where indicated, cells were pretreated with the NLRP3
523 inhibitor MCC950. (B) Levels of IL-1β and IL-18 released into the cell culture
524 media from (A). (C) Cleaved caspase-4 released into the cell culture media and
525 intracellular cleaved GSDMD under same conditions. Ns, not significant; **, $P <$
526 0.01; ***, $P < 0.001$; by two-way ANOVA. Shown are independent biological
527 replicates. Error bars represent the standard error of the mean.

528 **Fig. 4. Mycobacteria activate the non-canonical inflammasome pathway.** IL-
529 1β release and cell death of *NLRP11*$^{+/+}$ (*Cas9*) and *NLRP11*$^{\Delta N_LRR}$ (*Cas9*) mutant
530 THP-1 macrophages infected with *M. tuberculosis* (A), *M. kansasii* (B, D), or *M.*
531 *smegmatis* (C) (MOI 10), at the indicated timepoints. Cell death was normalized
532 to cell lineage-specific uninfected group. ns, not significant; **, $P < 0.01$; ***, $P <$
533 0.001; ****, $P < 0.0001$; by paired parametric t-tests. Shown are independent
534 biological replicates. Error bars represent the standard error of mean.

535 **Fig. 5. Caspase-4/5 are required for recognition of mycobacteria in human**
536 **macrophages.** IL-1β release and cell death of WT and two distinct clones of
537 caspase-4/5$^{-/-}$ THP-1 macrophages infected with *M. kansasii* (A) or *M.*

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

539 infection (A), and for *M. tuberculosis*, at 24 h of infection (B). ns, not significant;

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

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

542 **REFERENCES**

543 1. Brewer SM, Brubaker SW, Monack DM. 2019. Host inflammasome
544 defense mechanisms and bacterial pathogen evasion strategies. Curr
545 Opin Immunol 60:63-70.

546 2. Broz P, Dixit VM. 2016. Inflammasomes: mechanism of assembly,
547 regulation and signalling. Nat Rev Immunol 16:407-20.

548 3. Jorgensen I, Rayamajhi M, Miao EA. 2017. Programmed cell death as a
549 defence against infection. Nat Rev Immunol 17:151-164.

550 4. Wright SS, Vasudevan SO, Rathinam VA. 2021. Mechanisms and
551 Consequences of Noncanonical Inflammasome-Mediated Pyroptosis. J
552 Mol Biol doi:10.1016/j.jmb.2021.167245:167245.

553 5. Deets KA, Vance RE. 2021. Inflammasomes and adaptive immune
554 responses. Nat Immunol 22:412-422.

555 6. Kayagaki N, Stowe IB, Lee BL, O'Rourke K, Anderson K, Warming S,
556 Cuellar T, Haley B, Roose-Girma M, Phung QT, Liu PS, Lill JR, Li H, Wu
557 J, Kummerfeld S, Zhang J, Lee WP, Snipas SJ, Salvesen GS, Morris LX,
558 Fitzgerald L, Zhang Y, Bertram EM, Goodnow CC, Dixit VM. 2015.
559 Caspase-11 cleaves gasdermin D for non-canonical inflammasome
560 signalling. Nature 526:666-71.

561 7. Shi J, Zhao Y, Wang K, Shi X, Wang Y, Huang H, Zhuang Y, Cai T, Wang
562 F, Shao F. 2015. Cleavage of GSDMD by inflammatory caspases
563 determines pyroptotic cell death. Nature 526:660-5.

564 8. Ding J, Wang K, Liu W, She Y, Sun Q, Shi J, Sun H, Wang DC, Shao F.
565 2016. Pore-forming activity and structural autoinhibition of the gasdermin
566 family. Nature 535:111-6.

567 9. He WT, Wan H, Hu L, Chen P, Wang X, Huang Z, Yang ZH, Zhong CQ,
568 Han J. 2015. Gasdermin D is an executor of pyroptosis and required for
569 interleukin-1beta secretion. Cell Res 25:1285-98.

570 10. Liu X, Zhang Z, Ruan J, Pan Y, Magupalli VG, Wu H, Lieberman J. 2016.
571 Inflammasome-activated gasdermin D causes pyroptosis by forming
572 membrane pores. Nature 535:153-8.

573 11. Kayagaki N, Kornfeld OS, Lee BL, Stowe IB, O'Rourke K, Li Q, Sandoval
574 W, Yan D, Kang J, Xu M, Zhang J, Lee WP, McKenzie BS, Ulas G,
575 Payandeh J, Roose-Girma M, Modrusan Z, Reja R, Sagolla M, Webster
576 JD, Cho V, Andrews TD, Morris LX, Miosge LA, Goodnow CC, Bertram
577 EM, Dixit VM. 2021. NINJ1 mediates plasma membrane rupture during
578 lytic cell death. Nature 591:131-136.

579 12. David L, Borges JP, Hollingsworth LR, Volchuk A, Jansen I, Garlick E,
580 Steinberg BE, Wu H. 2024. NINJ1 mediates plasma membrane rupture by
581 cutting and releasing membrane disks. Cell 187:2224-2235 e16.

582 13. Pourmal S, Truong ME, Johnson MC, Yang Y, Zhou L, Alegre K, Stowe
583 IB, Gupta S, Chen PA, Zhang Y, Rohou A, Newton K, Kayagaki N, Dixit
584 VM, Deshpande I. 2024. Autoinhibition of dimeric NINJ1 prevents plasma
585 membrane rupture. Nature doi:10.1038/s41586-024-08273-4.

586 14. Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J,
587 Newton K, Qu Y, Liu J, Heldens S, Zhang J, Lee WP, Roose-Girma M,
588 Dixit VM. 2011. Non-canonical inflammasome activation targets caspase-
589 11. Nature 479:117-21.

590 15. Kayagaki N, Wong MT, Stowe IB, Ramani SR, Gonzalez LC, Akashi-
591 Takamura S, Miyake K, Zhang J, Lee WP, Muszynski A, Forsberg LS,
592 Carlson RW, Dixit VM. 2013. Noncanonical inflammasome activation by
593 intracellular LPS independent of TLR4. Science 341:1246-9.

594 16. Hagar JA, Powell DA, Aachoui Y, Ernst RK, Miao EA. 2013. Cytoplasmic
595 LPS activates caspase-11: implications in TLR4-independent endotoxic
596 shock. Science 341:1250-3.

597 17. Shi J, Zhao Y, Wang Y, Gao W, Ding J, Li P, Hu L, Shao F. 2014.
598 Inflammatory caspases are innate immune receptors for intracellular LPS.
599 Nature 514:187-92.

600 18. Rojas-Lopez M, Gil-Marques ML, Kharbanda V, Zajac AS, Miller KA,
601 Wood TE, Hachey AC, Egger KT, Goldberg MB. 2023. NLRP11 is a

602 pattern recognition receptor for bacterial lipopolysaccharide in the cytosol
603 of human macrophages. Sci Immunol 8:eabo4767.

604 19. Gangopadhyay A, Devi S, Tenguria S, Carriere J, Nguyen H, Jager E,
605 Khatri H, Chu LH, Ratsimandresy RA, Dorfleutner A, Stehlik C. 2022.
606 NLRP3 licenses NLRP11 for inflammasome activation in human
607 macrophages. Nat Immunol 23:892-903.

608 20. Rastogi S, Briken V. 2022. Interaction of Mycobacteria With Host Cell
609 Inflammasomes. Front Immunol 13:791136.

610 21. Mayer-Barber KD, Barber DL, Shenderov K, White SD, Wilson MS,
611 Cheever A, Kugler D, Hieny S, Caspar P, Nunez G, Schlueter D, Flavell
612 RA, Sutterwala FS, Sher A. 2010. Caspase-1 independent IL-1beta
613 production is critical for host resistance to mycobacterium tuberculosis and
614 does not require TLR signaling in vivo. J Immunol 184:3326-30.

615 22. Chai Q, Yu S, Zhong Y, Lu Z, Qiu C, Yu Y, Zhang X, Zhang Y, Lei Z,
616 Qiang L, Li BX, Pang Y, Qiu XB, Wang J, Liu CH. 2022. A bacterial
617 phospholipid phosphatase inhibits host pyroptosis by hijacking ubiquitin.
618 Science 378:eabq0132.

619 23. Rastogi S, Ellinwood S, Augenstreich J, Mayer-Barber KD, Briken V.
620 2021. Mycobacterium tuberculosis inhibits the NLRP3 inflammasome
621 activation via its phosphokinase PknF. PLoS Pathog 17:e1009712.

622 24. Danelishvili L, Everman JL, McNamara MJ, Bermudez LE. 2011. Inhibition
623 of the Plasma-Membrane-Associated Serine Protease Cathepsin G by
624 Mycobacterium tuberculosis Rv3364c Suppresses Caspase-1 and
625 Pyroptosis in Macrophages. Front Microbiol 2:281.

626 25. Dulberger CL, Rubin EJ, Boutte CC. 2020. The mycobacterial cell
627 envelope - a moving target. Nat Rev Microbiol 18:47-59.

628 26. Beckwith KS, Beckwith MS, Ullmann S, Saetra RS, Kim H, Marstad A,
629 Asberg SE, Strand TA, Haug M, Niederweis M, Stenmark HA, Flo TH.
630 2020. Plasma membrane damage causes NLRP3 activation and
631 pyroptosis during Mycobacterium tuberculosis infection. Nat Commun
632 11:2270.

633 27. Wong KW, Jacobs WR, Jr. 2011. Critical role for NLRP3 in necrotic death
634 triggered by Mycobacterium tuberculosis. Cell Microbiol 13:1371-84.

635 28. Chen CC, Tsai SH, Lu CC, Hu ST, Wu TS, Huang TT, Said-Sadier N,
636 Ojcius DM, Lai HC. 2012. Activation of an NLRP3 inflammasome restricts
637 Mycobacterium kansasii infection. PLoS One 7:e36292.

638 29. Casson CN, Yu J, Reyes VM, Taschuk FO, Yadav A, Copenhaver AM,
639 Nyguyen HT, Collman RG, Shin S. 2015. Human caspase-4 mediates
640 noncanonical inflammasome activation against gram-negative bacterial
641 pathogens. Proc Natl Acad Sci U S A 112:6688-6693.

642 30. Lagrange B, Benaoudia S, Wallet P, Magnotti F, Provost A, Michal F,
643 Martin A, Di Lorenzo F, Py BF, Molinaro A, Henry T. 2018. Human
644 caspase-4 detects tetra-acylated LPS and cytosolic Francisella and
645 functions differently from murine caspase-11. Nat Commun 9:242.

646 31. Hara H, Seregin SS, Yang D, Fukase K, Chamaillard M, Alnemri ES,
647 Inohara N, Chen GY, Nunez G. 2018. The NLRP6 Inflammasome
648 Recognizes Lipoteichoic Acid and Regulates Gram-Positive Pathogen
649 Infection. Cell 175:1651-1664 e14.

650 32. Yamagishi R, Kamachi F, Nakamura M, Yamazaki S, Kamiya T, Takasugi
651 M, Cheng Y, Nonaka Y, Yukawa-Muto Y, Thuy LTT, Harada Y, Arai T, Loo
652 TM, Yoshimoto S, Ando T, Nakajima M, Taguchi H, Ishikawa T, Akiba H,
653 Miyake S, Kubo M, Iwakura Y, Fukuda S, Chen WY, Kawada N, Rudensky
654 A, Nakae S, Hara E, Ohtani N. 2022. Gasdermin D-mediated release of
655 IL-33 from senescent hepatic stellate cells promotes obesity-associated
656 hepatocellular carcinoma. Sci Immunol 7:eabl7209.

657 33. Mishra BB, Moura-Alves P, Sonawane A, Hacohen N, Griffiths G, Moita
658 LF, Anes E. 2010. Mycobacterium tuberculosis protein ESAT-6 is a potent
659 activator of the NLRP3/ASC inflammasome. Cell Microbiol 12:1046-63.

660 34. Briken V, Porcelli SA, Besra GS, Kremer L. 2004. Mycobacterial
661 lipoarabinomannan and related lipoglycans: from biogenesis to modulation
662 of the immune response. Mol Microbiol 53:391-403.

663 35. Groschel MI, Sayes F, Simeone R, Majlessi L, Brosch R. 2016. ESX
664 secretion systems: mycobacterial evolution to counter host immunity. Nat
665 Rev Microbiol 14:677-691.

666 36. Shah S, Briken V. 2016. Modular Organization of the ESX-5 Secretion
667 System in Mycobacterium tuberculosis. Front Cell Infect Microbiol 6:49.

668 37. Yang Y, Xu P, He P, Shi F, Tang Y, Guan C, Zeng H, Zhou Y, Song Q,
669 Zhou B, Jiang S, Shao C, Sun J, Yang Y, Wang X, Song H. 2020.
670 Mycobacterial PPE13 activates inflammasome by interacting with the
671 NATCH and LRR domains of NLRP3. FASEB J 34:12820-12833.

672 38. Labrec EH, Schneider H, Magnani TJ, Formal SB. 1964. Epithelial Cell
673 Penetration as an Essential Step in the Pathogenesis of Bacillary
674 Dysentery. J Bacteriol 88:1503-18.

675