Title:

Cytosolic Sensor of Bacterial Lipopolysaccharide in Human Macrophages

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Summary paragraph

Inflammasomes are cytosolic supramolecular organizing centers that, in response to pathogen-derived molecules and endogenous danger signals, assemble and activate innate immune responses. Bacterial lipopolysaccharide (LPS) is an inflammasome trigger and a major mediator of inflammation during infection, including during the potentially lethal condition sepsis. Activation of most inflammasomes is triggered by sensing of pathogen products by a specific host cytosolic nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing protein (NLRP) or other sensor protein that in turn activates a pro-inflammatory caspase. LPS that accesses the cell cytosol (cLPS) induces cell-autonomous activation of a non-canonical inflammasome that contains caspases-4/5 in humans or caspase-11 in mice[1-3]. Whereas the NLRPs that sense most pathogen triggers have been identified, no NLRP is known to sense cLPS, which together with the observation that caspases-4, -5, and -11 bind LPS *in vitro*[4], has led to the postulate that inflammasome activation by cLPS occurs independent of an NLRP. Here we show that primate-specific NLRP11 senses cLPS and promotes the activation of caspase-4. We found that in response to infection by each of several gram-negative intracellular bacterial pathogens or to LPS transfection, efficient activation of the non-canonical pathway in human-derived macrophages depends on NLRP11. Further, we found that in both immortalized human-derived macrophages and primary human macrophages, the dependence of the non-canonical pathway on NLRP11 is due to detection of cLPS. Moreover, in cell lysates, NLRP11 binds LPS independently of caspase-4 and binds caspase-4 independently of LPS. Our results demonstrate that NLRP11 senses cLPS and promotes LPS-dependent

activation of caspase-4. NLRP11 is a previously missing link in the human non-canonical inflammasome activation pathway.

44 Inflammasomes assemble in response to the presence in the cytosol of pathogen-
45 associated molecules, including dsDNA, flagellin, type 3 secretion system structural
46 proteins, pore-forming toxins, ion flux, ATP, and LPS[5]. Most inflammasomes consist of a
47 sensor protein and an inflammatory caspase, frequently linked by an adaptor protein.
48 Inflammasome assembly activates inflammatory caspases, which proteolytically
49 process the pore-forming protein gasdermin D[6-8], including caspase-1-dependent
50 proteolytically processing of the pro-inflammatory cytokines IL-18 and IL-1β[9,10]. Pores
51 formed in the plasma membrane by the N-terminal domain of gasdermin D enable the
52 release of activated pro-inflammatory cytokines and trigger pyroptotic cell death[6-8,11].
53 The presence of bacterial LPS in the cytosol of human macrophages activates a non-
54 canonical inflammasome, which in humans, contains caspases-4 and -5, and in mice,
55 caspase-11[1-3]. To date, no sensor protein of cytosolic LPS has been identified, and
56 because caspase-4, -5, and -11 bind LPS *in vitro*[4], the current paradigm is that the non-
57 canonical inflammasome lacks a sensor protein.

58 **NLRP11 required for cell death due to intracellular gram-negative pathogens**

59 In a forward genetic screen that enriched for cellular genes required for efficient cell
60 death of a human-derived myeloid lineage cell line by the gram-negative intracellular
61 bacterial pathogen *Shigella flexneri*, we identified *NLRP11*[12]. We performed targeted
62 deletion of *nlrp11* in the human-derived macrophage cell line THP-1 and found that cell
63 death during *S. flexneri* infection (as measured by lactate dehydrogenase (LDH)
64 release) was significantly reduced for cell lines that lack NLRP11. In each of two THP-1-
65 derived cell lines in which *nlrp11* was disrupted by a CRISPR-Cas9 induced indel

66 (Extended Data Fig. 1), release of LDH upon infection with *S. flexneri* was reduced by
67 more than 50% (Fig. 1a). Consistent with previous reports, *S. flexneri*-induced
68 macrophage cell death was dependent on the bacterial type 3 secretion system, which
69 mediates both bacterial uptake[13] and vacuolar escape[14,15], as a mutant that lacked
70 either the virulence plasmid that encodes this secretion system or *virB*, the
71 transcriptional activator of this system, displayed minimal cell death (Extended Data Fig.
72 2a).

73 To test whether the dependence on NLRP11 for efficient cell death was generalizable to
74 other intracellular gram-negative bacterial pathogens, we infected *NLRP11*$^{-/-}$ cells with
75 the cytosolic pathogen *Burkholderia thailandensis* and the intracellular but largely
76 vacuolar pathogen *Salmonella enterica* serovar Typhimurium. As with *S. flexneri*
77 infection, in the absence of NLRP11, cell death during infection with *B. thailandensis* or
78 *S.* Typhimurium was significantly reduced (Fig. 1b-c). In response to viral infection and
79 certain Toll-like receptor interactions, the NF-κB pathway is repressed by NLRP11-
80 dependent targeted degradation of TRAF6[16-18]; however, *S. flexneri*-induced NLRP11-
81 dependent cell death was independent of TRAF6, whether or not the *Shigella* effector
82 OspI, which suppresses TRAF6 signaling[19], was present (Extended Data Fig. 2b-d),
83 indicating that the role of NLRP11 in bacterial infection-induced cell death is distinct
84 from its role in NF-κB signaling. Together, these results indicate that NLRP11 is
85 required for efficient cell death induced by multiple intracellular gram-negative
86 pathogens and raised the possibility that the implicated pathogen-associated trigger of
87 cell death is generally present among gram-negative bacteria and depends on
88 intracellular presentation.

NLRP11 recognizes cLPS

We tested whether LPS, which is specific to the cell envelope of gram-negative bacteria, might be required for NLRP11-dependent cell death. Infection of THP-1 macrophages with the cytosolic gram-positive pathogen *Listeria monocytogenes* induced minimal levels of cell death during the timeframe of the experiment, but when coated with *S. enterica* LPS, induced cell death that was largely dependent on NLRP11 (Fig. 2a). The observed cell death was dependent on cytosolic localization, as a *L. monocytogenes* strain lacking listeriolysin O (LLO), which is required to lyse the vacuole[20], induced minimal cell death. Introduction of LPS alone directly into the cytosol by electroporation similarly induced cell death that was significantly dependent on NLRP11, both in *NLRP11*$^{-/-}$ and NLRP11 siRNA-treated THP-1 macrophages (Fig. 2b-c), whereas cell death induced by extracellular LPS was independent of NLRP11 (Extended Data Fig. 3a). These data indicate that NLRP11 functions in a cell death pathway that is activated by LPS localized to the cytosol (cLPS). For pathogens such as *Salmonella* that do not themselves efficiently lyse the vacuole, delivery of LPS to the cytosol occurs upon guanylate-binding protein-2 (GBP2)-mediated vacuolar lysis[21], a likely explanation for the lack of differential NLRP11-dependent cell death induced by wild-type *S.* Typhimurium, which are largely vacuolar, and *sifA* mutant *S.* Typhimurium, which is predominantly cytosolic[22] (Fig. 1c).

NLRP11 required for efficient activation of non-canonical inflammasome

We tested whether NLRP11 was required for activation of the non-canonical inflammasome, which in humans, contains caspases-4 and -5. Activation of caspase-4

111 and the downstream pore-forming protein gasdermin D were dependent on NLRP11;
112 during *S. flexneri* infection, processing of caspase-4 and gasdermin D, leading to the
113 release of active domains of each, was significantly reduced in the absence of NLRP11
114 (Fig. 1d-e). In THP-1 macrophages depleted of caspase-4, the absence of NLRP11
115 caused no significant additional decrease in cell death induced by LPS transfection (Fig.
116 2d and Extended Data Fig. 4a). Depletion of *CASP1* was associated with no significant
117 decrease in cell death in response to cLPS as compared to control depletion for either
118 THP-1 macrophages containing NLRP11 or THP-1 macrophages lacking NLRP11 (Fig.
119 2d and Extended Data Fig. 4b). Despite testing several antibodies, we were unable to
120 detect native cellular NLRP11 by western blot, presumably due to very low levels of
121 native protein. These data indicate that NLRP11 is required for efficient activation of the
122 non-canonical inflammasome, likely functioning in the caspase-4 pathway, and that
123 NLRP11 function is independent of caspase-1. Consistent with NLRP11 functioning
124 specifically in the non-canonical inflammasome pathway, NLRP11 was not required for
125 activation of the canonical NLRP3 inflammasome, as cell death induced by the NLRP3
126 agonist nigericin was independent of NLRP11 (Extended Data Fig. 3b-d).

127 **NLRP11 interacts with LPS and caspase-4**

128 In contrast to NLRP3 and NLR family CARD domain containing 4 (NLRC4)
129 inflammasomes, activation of the non-canonical pathway is independent of the adaptor
130 protein ASC, as signaling in response to cytosolic LPS is normal in human or mouse-
131 derived macrophages lacking ASC[2,4] and NLRP11 does not co-localize with ASC
132 specks[16,23]. We tested whether NLRP11 interacts with other core components of the

133 non-canonical pathway by ectopic expression of selected components in HEK293T
134 cells, which lack most innate immune proteins. Biotin-conjugated LPS precipitated
135 NLRP11 in the absence of caspase-4 (Fig. 3a), indicating that NLRP11 binds LPS
136 independently of caspase-4. As previously described[4], LPS also specifically precipitated
137 caspase-4 and its catalytically inactive derivative (CASP4 C258A), including in the
138 presence of NLRP11 (Fig. 3b), whereas the triacylated lipid Pam3CSK4 did not
139 precipitate either NLRP11 or, as previously described[4], caspase-4 (Fig. 3a). In addition
140 to binding to LPS independent of caspase-4, NLRP11 specifically precipitated caspase-
141 4 (Fig. 3c). To characterize the binding relationship of NLRP11 to caspase-4, we
142 developed a plate binding assay using FLAG-NLRP11 in HEK293T cell lysates and
143 purified caspase-4 (Methods and Extended Data Fig. 5a). In this assay, FLAG-NLRP11
144 binding depended on caspase-4, as binding of FLAG-NLRP11 in the absence of
145 caspase-4 was at background levels (Fig. 3d, diamonds). Moreover, binding of NLRP11
146 to caspase-4 increased as a function of increasing concentrations of both NLRP11
147 lysate and caspase-4, resembling the asymptotic phase of a sigmoidal curve, beginning
148 to reach saturation at 8-10 μg/ml caspase-4 coating the wells (Fig. 3d).

149 **NLRP11 displays caspase specificity**

150 In addition to caspase-4, NLRP11 co-precipitated with caspase-1; of note, NLRP11 did
151 not co-precipitate with caspase-5, a paralog of caspase-4 that binds LPS and is a
152 component of at least some non-canonical inflammasomes [4,10,24], or the more divergent
153 caspase-9 (Fig. 3e and Extended Data Fig. 6a). Although caspase-1 co-precipitates
154 with NLRP11 (Fig. 3e), in THP-1 macrophages, NLRP11 function in LPS-induced cell

155 death is independent of caspase-1 (Fig. 2d), indicating that the observed interaction of
156 caspase-1 with NLRP11 does not functionally contribute to activation of the non-
157 canonical inflammasome.

158 **NLRP11 role in non-canonical inflammasome activation is independent of**
159 **interferon-gamma and guanylate-binding protein-1**

160 Host guanylate-binding proteins (GBPs) bind intracellular LPS, promote non-canonical
161 inflammasome activation[25-29], and are encoded by genes that are strongly induced by
162 interferon-gamma (IFNγ)[30,31]. Human GBP1 binds LPS on the *S. flexneri* surface,
163 whereupon it enhances caspase-4 recruitment[32]. We tested whether IFNγ stimulation
164 altered the role of NLRP11 in activation of the non-canonical inflammasome. IFNγ pre-
165 treatment of THP-1 macrophages induced GBP1, as expected, yet NLRP11 was still
166 required for efficient activation of caspase-4 and cell death (Extended Data Fig. 7a-c),
167 indicating that IFNγ and GBP1 do not bypass the requirement for NLRP11 in non-
168 canonical inflammasome induced pyroptosis.

169 **Requirement for NLRP11 in primary human macrophages**

170 Following treatment of primary human monocyte-derived macrophages with siRNA to
171 NLRP11, although overall levels of cell death are lower than for immortalized THP-1
172 cells, cell death induced by cLPS was significantly diminished (Fig. 2e). As in THP-1
173 macrophages, in primary macrophages, the defect in cLPS-induced cell death
174 depended on caspase-4, but not MyD88 (Figs. 2f-g and Extended Data Fig. 4c-d),
175 indicating that in these assays, the trigger was cLPS rather than extracellular LPS, and

176 demonstrating that the requirement for NLRP11 in the non-canonical inflammasome
177 pathway extends to primary human cells.

178 In summary, the data reported here identify NLRP11 as a primate-specific cytosolic
179 sensor of bacterial LPS in the activation of the caspase-4 dependent inflammasome and
180 caspase-4 dependent pyroptosis. Galectin-3, one among a family of β-galactoside–
181 binding proteins, also senses cytosolic pathogens, promotes delivery of GBPs to
182 ruptured pathogen-containing vacuoles, and enhances assembly of LPS-triggered
183 caspase-4/11 oligomerization and activation[33-35]. Whether galectin-3 activity synergizes
184 with the interaction of LPS and NLRP11 is uncertain; however, the independence of
185 NLRP11 function from GBP1 (Extended Data Fig. 7) suggests that any contribution of
186 galectin-3 to this process would be minor. Additionally, it remains uncertain whether the
187 unique presence of NLRP11 in primates and the absence of it or identifiable homologs
188 in mice contributes to the observed ~10^4-fold increased susceptibility to gram-negative
189 bacterial sepsis and LPS of humans as compared to mice[36-42]; additional insights into
190 these relationships would potentially enable much-needed improved mouse models for
191 bacterial sepsis.

192 **Methods**

193 **Bacterial strains, plasmids, antibodies, and reagents.** *Shigella flexneri* serotype 2a
194 wildtype strain 2457T and the *S. flexneri* mutant strains *virB*::Tn5, BS103, and Δ*ospC3*,
195 which are isogenic to 2457T, have been described previously[43-46]. *Salmonella enterica*
196 serovar Typhimurium and the isogenic Δ*sifA* mutant[22] were gifts from L. Knodler.
197 *Burkholderia thailandensis*[47] was a gift from P. Cotter. *Listeria monocytogenes* 10403s
198 and 10403 LLO^{-}[48] were gifts from M. Kielian and J. Miller. *S. flexneri* was grown
199 overnight in tryptic soy broth at 37°C with aeration. *L. monocytogenes* was grown
200 overnight in brain heart infusion broth at 30°C at a stationary slant. *B. thailandensis* was
201 grown overnight in low salt Luria broth (LB) at 37°C with aeration. *S. enterica*
202 Typhimurium was grown in LB broth at 37°C with aeration.

203 Caspase-1 (Abcam, ab207802) rabbit monoclonal antibody was used at 0.5 μg/mL
204 (1:1000). Caspase-4 (Santa Cruz, sc-56056), and gasdermin D (Santa Cruz,
205 GSDMDC1 64-Y) mouse monoclonal antibodies were used at 0.5 μg/mL (1:200). FLAG
206 M2 mouse monoclonal antibody (Sigma, F3165) was used at 76 ng/mL (1:5000). Myc
207 rabbit monoclonal antibody (Cell Signaling, 2278S) was used at 1:1000. GBP1 mouse
208 monoclonal antibody (Abcam, ab119236) was used at 1:1000. For western blots of
209 immunoprecipitations, the secondary antibody used was TrueBlot horseradish
210 peroxidase-conjugated anti-mouse Ig (Rockland, 18-8817-31). For detecting β-actin,
211 horseradish peroxidase-conjugated anti-β-actin antibody (Sigma, A3854) was used. For
212 all other western blots, the secondary antibody used was horseradish peroxidase-
213 conjugated goat anti-rabbit or goat anti-mouse IgG antibodies (Jackson

214 ImmunoResearch, 115-035-003 or 111-035-144). All antibodies were diluted in 5% milk
215 in TBST (0.1% Tween).

216 **Strain and plasmid construction.** The *S. flexneri ospI* deletion mutant was generated
217 in the 2457T strain background using the lambda red method by introducing a
218 chloramphenicol acetyltransferase (*cat*) cassette into the *orf169b* gene (which encodes
219 OspI), as described[49].

220 pCMV-CASP4-MYC and pCMV-CASP5-MYC were made from pCMV-CASP4-MYC-
221 FLAG and pCMV-CASP5-MYC-FLAG, gifts from S. Shin. pCMV-CASP4-MYC C258A
222 and pCMV-CASP4-MYC K19E C258A was generated by splicing by overlap extension
223 PCR (SOE-PCR). pcDNA-NLRP11 (gift from H. Wu) was subcloned into pCMV-FLAG
224 with an N-terminal FLAG tag for FLAG-NLRP11 and an N-terminal MYC tag for MYC-
225 NLRP11. All FLAG tags are 3xFLAG. All N-terminal Myc tags are 6xMyc; all C-terminal
226 Myc tags are 1xMyc. All DNA used for transfection was prepared LPS-free (Macherey-
227 Nagel, 740410.50).

228 **Generation of CRISPR knockouts.** THP-1 cells were transduced with lentivirus
229 packaged with pLentiCas9-Blast (Addgene, 52962) generated from HEK293T cells.
230 THP-1 cell suspensions were treated with 8 μg/mL polybrene, combined with lentivirus
231 and centrifuged in 12-well plates for 2 h at 1000 x g at 30°C. After centrifugation, cells
232 were incubated for 2 h and washed once with fresh media. After 48 h, blasticidin
233 (10 μg/mL) was added to the transduced cells. Guide RNA sequence ("sgRNA3",
234 AGGCATGCAAAGCTGTCATG) was subcloned into pLentiGuide-Puro (Addgene,
235 59702). Guide RNA expressing plasmids were packaged into lentivirus using HEK293T

236 cells. Polybrene treated THP-1 cells were transduced and selected with 1 μg/mL
237 puromycin. Cells were limiting diluted and single cell clones were screened by
238 SURVEYOR (IDT, 706020) and Sanger sequencing coupled with TIDE analysis
239 (Brinkman et. al 2014).

240 **Generation of *TRAF6* depletion cell lines.** pLKO.1-shTRAF6 ([50], gift of A. Goldfeld),
241 which encodes shRNA to *TRAF6*, was packaged into lentivirus in HEK293T cells.
242 Lentivirus were introduced into NLRP11+/+ and NLRP11-/- THP-1 cell lines and cells
243 containing pLKO.1-shTRAF6 were selected, expanded by maintenance in 0.8 μg/mL
244 puromycin, as described[50], and stable clones were isolated by limiting dilution.

245 **Mammalian cell culture.** All cell lines were obtained from ATCC. HEK293T cells were
246 maintained in Dulbecco's Modified Eagle's medium (Gibco, 11965118). THP1 cells were
247 maintained in RPMI-1640 (Gibco, 21870092). All media was supplemented with 10%
248 (vol/vol) fetal bovine serum (heat inactivated, sterile filtered, R&D Systems). For THP-1
249 cells, RPMI-1640 media was also supplemented with 10 mM HEPES (Gibco,15630080)
250 and 2 mM L-glutamine (Gibco, 25030081). All cells were grown at 37°C in 5% CO_2. To
251 differentiate monocyte cell lines into macrophage-like cells, 50 ng/mL phorbol-12-
252 myristrate-13-acetate (PMA) was added 48 h before experiments. Where indicated,
253 cells were pretreated with 10 ng/mL IFNγ 16-20 h prior to infection.

254 **Precipitation experiments.** HEK293T cells were transiently transfected using Fugene6
255 (Promega). Stock solutions of 10x Buffer A (100 mM HEPES, 1500 mM NaCl, 10 mM
256 EGTA, 1 mM $MgCl_2$, pH 7.4) and 20% Triton X-100 were prepared as described[51]. The
257 cell culture medium was removed 24-48 h after transfection. Cells were washed with

258 cold PBS and lysed in 1x Buffer A, 0.5% Triton-X. Cell lysates were incubated on ice for
259 15 min, vortexed for 15 s and centrifuged at 13,200 rpm for 10 min at 4°C. The pellet
260 was discarded, and Bradford assay was used to determine protein concentration. 30 μL
261 of FLAG M2 Magnetic Beads (Millipore-Sigma, M8823) were washed twice in 1x Buffer
262 A, 0.1% Triton-X and equilibrated in the same buffer at 4°C for 2 h. 500 μg of protein
263 lysate was incubated with the beads for 2 h at 4°C. Beads were washed 3 times with 1x
264 Buffer A, 0.1% Triton-X and eluted with 50 μL of 2x Laemmli buffer.

265 Streptavidin pulldown was largely as described[4], except that 4 μg biotinylated *E. coli*
266 O111:B4 LPS was loaded onto 8 μg of Streptavidin Mag sepharose (GE, 28985738, or
267 Pierce, 88816). Cell lysates were diluted to 4 mg/mL before a 2 h incubation with beads.
268 For co-precipitation experiments, separate cells were transfected with each DNA
269 construct, and equivalent amounts of lysates were combined with each other or with
270 untransfected lysate, such that the amount of target protein and the total amount of
271 lysate were similar in each experimental condition; these lysates were then incubated at
272 37°C overnight. Lysates were cleared for non-specific binding with streptavidin beads
273 and then incubated for 2 h at 30°C with 4 μg biotinylated *E. coli* O111:B4 LPS loaded
274 onto Streptavidin Mag sepharose.

275 **Plate binding assays.** Clear flat bottom Nunc Maxisorp ELISA plates (Thermo Fisher,
276 442404) were coated with various concentrations of purified recombinant caspase-4
277 (Origene, TP760359) overnight at 4°C. The plates were washed 3x with PBS then
278 blocked for 2 h at RT with 1% bovine serum albumin (Fisher, BP9700100) in PBS.
279 Excess blocking solution was removed. HEK 293T cells transiently expressing FLAG-

280 NLRP11 and control untransfected cells were lysed into 50 mM Tris-HCl pH 7.4, 150
281 mM NaCl, 5 mM $MgCl_2$, 100 µg/ml digitonin (Thermo Fisher Scientific, BN2006) and
282 complete Mini EDTA-free protease inhibitor (Roche), as described [28]. Total protein
283 concentration of lysates was normalized to 2 mg/mL. Serial dilutions were in
284 untransfected HEK293T cell lysate. Varying concentrations of cell lysates were
285 incubated with pre-blocked ELISA plates for 1 h at RT. Excess lysate was removed by
286 washing 3 times with PBS. For detection of FLAG-NLRP11, wells were incubated with
287 0.8 µg/mL anti-FLAG rabbit (Sigma, F7425-.2MG) was incubated for 1 h at RT, and
288 after washing with PBS, 0.16 µg/mL goat-anti-rabbit conjugated to horseradish
289 peroxidase (HRP) (Jackson, 111-035-144). To detect caspase-4 bound to the plate,
290 wells were incubated with 0.5 µg/mL anti-caspase-4 (Santa Cruz, sc-56056) and, after
291 washing with PBS, 0.16 µg/mL goat-anti-mouse conjugated to HRP (Jackson, 115-035-
292 003), each for 1 hr. After 6 washes with PBS, 50 µL of One Step Ultra-TMB (Fisher
293 Scientific, PI34029) was added to each well and incubated for 5 min before stopping
294 with 4 N sulfuric acid and reading in a BioTek plate reader.

295 **Bacterial infection.** Overnight cultures were started from a single colony taken from a
296 freshly streaked plate. Infections with *S. flexneri* were performed with exponential phase
297 bacteria, after back-dilution from overnight cultures. Infections with *L. monocytogenes*
298 were performed with stationary phase bacteria from overnight cultures. Infections were
299 with a multiplicity of infection (MOI) of 10 for *S. flexneri* cultures and 1 for *L.*
300 *monocytogenes*. Bacteria were centrifuged onto cells at 2000 rpm for 10 min, followed
301 by incubation at 37°C. After 30 min, the cells were washed twice, and then 25 µg/mL

302 gentamicin was added to the media to kill extracellular bacteria. Where appropriate *S.*
303 *enterica* serovar Minnesota LPS was added to 10 μg/mL at the time of infection and
304 MCC950 to 1 μM 30 min prior to infection and maintained during infection. For LDH
305 assays of *S. flexneri* infected cells, 100 μL of cell culture supernatant was collected at 2
306 h and 30 min and at 5 h and/or 30 min of infection; for detection of caspases and/or
307 gasdermin D by western blot, 100 μL of cell culture supernatant was collected at the
308 same time points and was mixed with 35 μL 4x Laemmli buffer supplemented with
309 cOmplete EDTA-free protease inhibitors (Roche, 5056489001). For parallel analyses of
310 *L. monocytogenes* infected cells, gentamicin was used at 15 μg/mL, and samples were
311 collected at 6 h of infection. For western blot analyses, whole cell lysates were collected
312 in 2x Laemmli buffer.

313 Infections with *B. thailandensis* and *S.* Typhimurium were performed with exponential
314 phase bacteria at a multiplicity of infection (MOI) of 10. Bacteria were centrifuged onto
315 cells at 2000 rpm for 10 min, followed by incubation at 37°C. For *S.* Typhimurium, after
316 30 min incubation, the cells were washed twice, and then 25 μg/mL gentamicin was
317 added to the media to kill extracellular bacteria. For *B. thailandensis*, after 2 h
318 incubation, the cells were washed twice with cRPMI. For LDH assays with *S.*
319 Typhimurium infection, 100 μL of cell culture supernatant was collected at 2 h and 30
320 min and at 5 h and 30 min of infection and, for LDH assays with *B. thailandensis*, 100
321 μL of cell culture supernatant was collected at 24 h.

322 **Isolation of primary human peripheral blood mononuclear cells (PBMCs) and**
323 **CD14 monocytes.** Cells were isolated from leukopaks using density-gradient

324 centrifugation. Briefly, blood was diluted 1:2 with RPMI-1640 (Gibco), layered on top of
325 Ficoll-Paque Plus (GE Healthcare) using SepMate™-50 (IVD) (StemCell Technologies),
326 and centrifuged at 1,200g for 20 min. The PBMC layer was collected and resuspended
327 in 50 ml RPMI-1640 (Gibco) and centrifuged again at 300 g for 10 min. The cells were
328 counted, resuspended in Cryostor CS10 (StemCell Technologies), and aliquoted in
329 cryopreservation tubes at a concentration of 1×10^8 cells per mL. The tubes were
330 placed at −80°C overnight, then transferred to liquid nitrogen for long-term storage. To
331 isolate CD14+ cells, EasySep™ Human CD14 Positive Selection Kit II (StemCell
332 Technologies) was used, following the manufacturer's instructions. Cells were cultured
333 in complete RPMI, differentiated with 25 ng/mL m-CSF (StemCell Technologies), and
334 maintained at 37°C in 5% CO_2.

335 **LPS transfection.** Electroporation of cells with LPS was performed using the Neon
336 Transfection System (Invitrogen) according to the manufacturer's instructions. THP-1
337 cells were seeded in low-attachment 10 cm dishes with 50 ng/mL PMA in RPMI. After
338 48 h, the cells were lifted in PBS (without calcium or magnesium). For electroporation,
339 1.5×10^6 THP-1 cells were pelleted, resuspended in Resuspension Buffer R with 2
340 μg/mL of LPS or water control. Using a 10 μL Neon tip, cells were pulsed once at 1400
341 V and 10 ms pulse width. For primary human macrophage cells, 7.5×10^5 CD14
342 macrophages were pelleted, then resuspended in Resuspension Buffer T with 2 μg/mL
343 of LPS or water control. Using a 10 μL Neon tip, cells were pulsed 2 times at 2250 V
344 and 20 ms pulse width. Electroporated cells were immediately resuspended in 2 mL of

345 RPMI and placed into wells of a 24 well plate. Cells were incubated for 2.5 h at 37°C
346 before measuring LDH release.

347 **siRNA transient knockdowns.** The siRNAs used for NLRP11 knockdowns were
348 Hs_NALP11_1 FlexiTube siRNA and Hs_NALP11_5 FlexiTube siRNA at 10 nM total (5
349 nM each siRNA); for Caspase-1 Hs_CASP1_14 FlexiTube siRNA and Hs_CASP1_13
350 FlexiTube siRNA at 30 nM total (15 nM each siRNA); and for MyD88 Hs_MYD88_5
351 FlexiTube siRNA and Hs_MYD88_11 FlexiTube siRNA at 30 nM total (15 nM each
352 siRNA) (Qiagen). AllStars Negative Control siRNAs (Qiagen) were used as negative
353 controls at 10 nM or 30 nM, accordingly. The siRNAs used for caspase-4 knockdowns
354 were 3941, 4036 and 4130 at 30 nM total (10 nM each siRNA) (ThermoFisher). siRNAs
355 were transfected using the HiPerfect reagent (Qiagen) into primary human monocyte-
356 derived macrophages 72 h before or into THP-1 cells 48 h before experimental assays,
357 following the manufacturer's protocol for "Transfection of Differentiated Macrophage
358 Cell Lines." After transfection, cells were maintained at 37°C in 5% CO_2.

359 **Nigericin.** PMA differentiated THP-1 cells were washed once with fresh RPMI and
360 primed with 1 μM O55:B5 *E. coli* LPS for 3 h, at which time 10 μM nigericin or vehicle
361 control was added. LDH release was assayed at indicated times after treatment.

362 **LDH assay.** LDH was measured using an LDH Cytotoxicity Kit (Pierce, 88953) or
363 Cytotoxicity Detection Kit (Sigma, 11644793001) per the manufacturer's instructions.
364 Measurements at 490 nm and 680 nm were taken immediately after addition of Stop
365 Solution using a BioTek Plate Reader. To determine LDH activity, the 680 nm
366 absorbance value (background) was subtracted from the 490-nm absorbance value,

367 and cytotoxicity was calculated for each sample as LDH activity as a fraction of the total
368 LDH activity for cells treated with the Triton X-100 lysis buffer provided by the
369 manufacturer. For experiments involving primary cells, a different donor was used for
370 each biological replicate; due to inherent variability from donor to donor in the baseline
371 levels of death in cell death assays, within-donor data were normalized to the negative
372 control condition before compilation of the biological replicates.

373 **Statistics and reproducibility.** All results are data from at least 3 independent
374 biological replicates. Statistical analysis was performed with Prism 9.

375 **Data availability.** All relevant data are included in the manuscript and will be made
376 available by request to the corresponding author, marcia.goldberg@mgh.harvard.edu.

377 **Supplementary information**

378 The online version contains supplementary material.

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385 **Author contributions**

386 M.B.G. conceived the study, supervised the project, and wrote the manuscript with input
387 from all authors; M.R.L. K.A.M., A.C.H., and V.K. performed cell-based experiments;
388 A.S.Z., T.E.W., and M.L.G.M. performed biochemical experiments. All authors
389 organized and analyzed data and edited the manuscript.

390 **Competing interests**

391 The authors declare no competing interests.

392 **Materials and correspondence**

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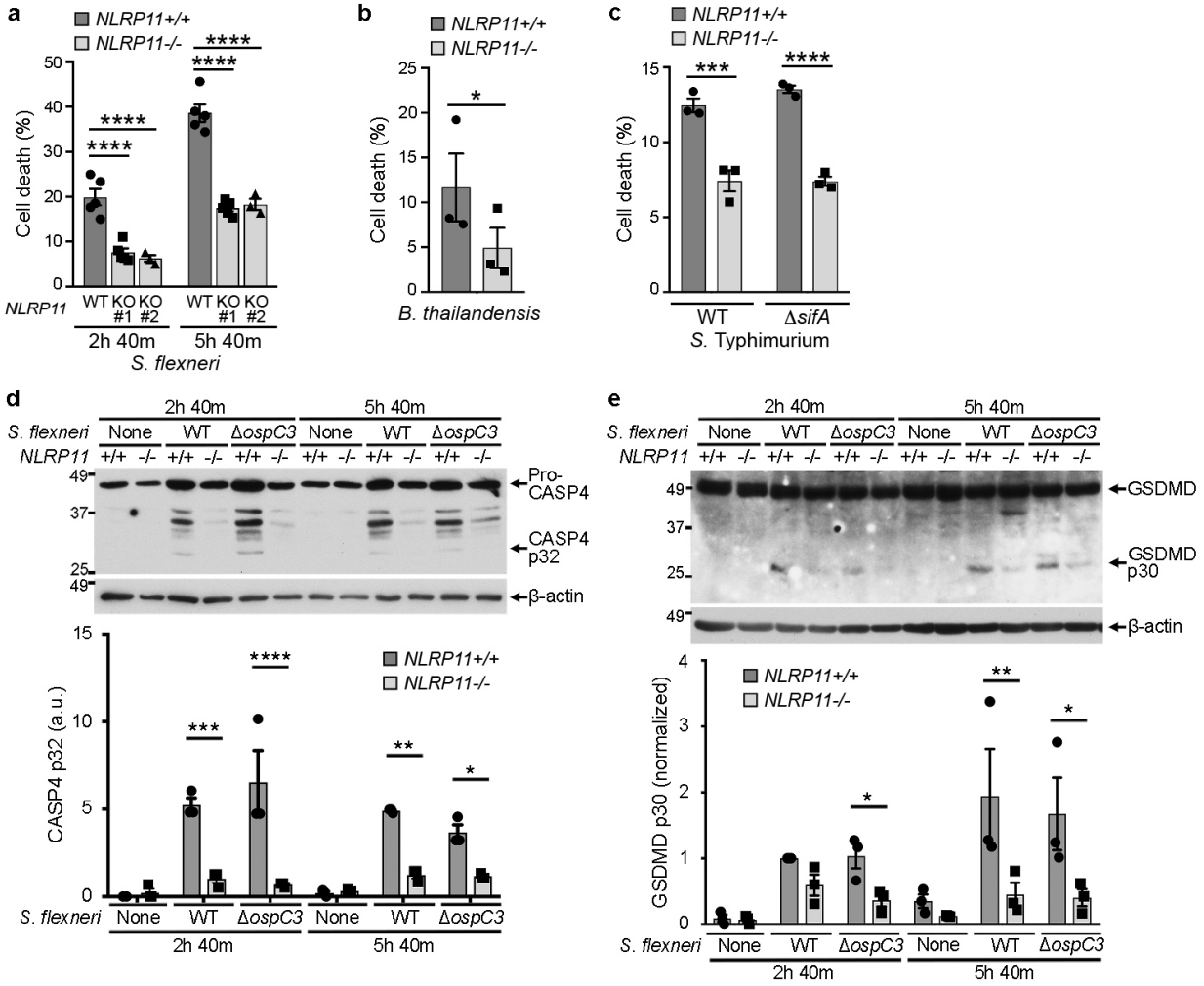
550 **Figures and figure legends**



551

552 **Fig. 1. In the absence of *NLRP11*, cell death of human-derived macrophage-like**

553 **cells induced by intracellular gram-negative bacteria is defective.**

554 (**a**-**b**) Death of human-derived THP-1 macrophage-like cells upon infection with the

555 cytosolic gram-negative pathogens *S. flexneri* (**a**), and *B. thailandensis* (**b**). Cell death in

556 response to *S. flexneri* infection is defective in each of two independent *NLRP11*$^{-/-}$ THP-

557 1 cell lines (**a**). (**c**) *NLRP11* is required for efficient cell death of THP-1 cells induced by

558 the pathogen *S.* Typhimurium. Cell death induced by the *sifA* mutant, which is largely

559 cytosolic, was similar to that of WT *S.* Typhimurium, which is largely vacuolar. (**e-f**)
560 During *S. flexneri* infection of THP-1 macrophage-like cells, *NLRP11* is required for
561 processing of caspase-4 (**d**) and gasdermin D (**e**); western blot of culture supernatants.
562 In the absence of OspC3, processing was similar to induced by WT *S. flexneri*; in
563 HaCaT cells and SiHa cells, OspC3 inhibits caspase-4 activation, and in mouse bone
564 marrow-derived macrophages, OspC3 inhibits caspase-11 activation[52,53]. Cell death is
565 by LDH release, reported as the percent of total cell lysis induced by Triton X-100.
566 Mean ± S.E.M. Two-way ANOVA with Dunnett *post hoc* test (**a**, **c**) or with Sidak *post*
567 *hoc* test (**d-e**), or student T-test (**b**). *, $p<0.05$; **, $p<0.01$; ***; $p<0.001$; ****, $p<0.0001$;
568 a.u., arbitrary units.

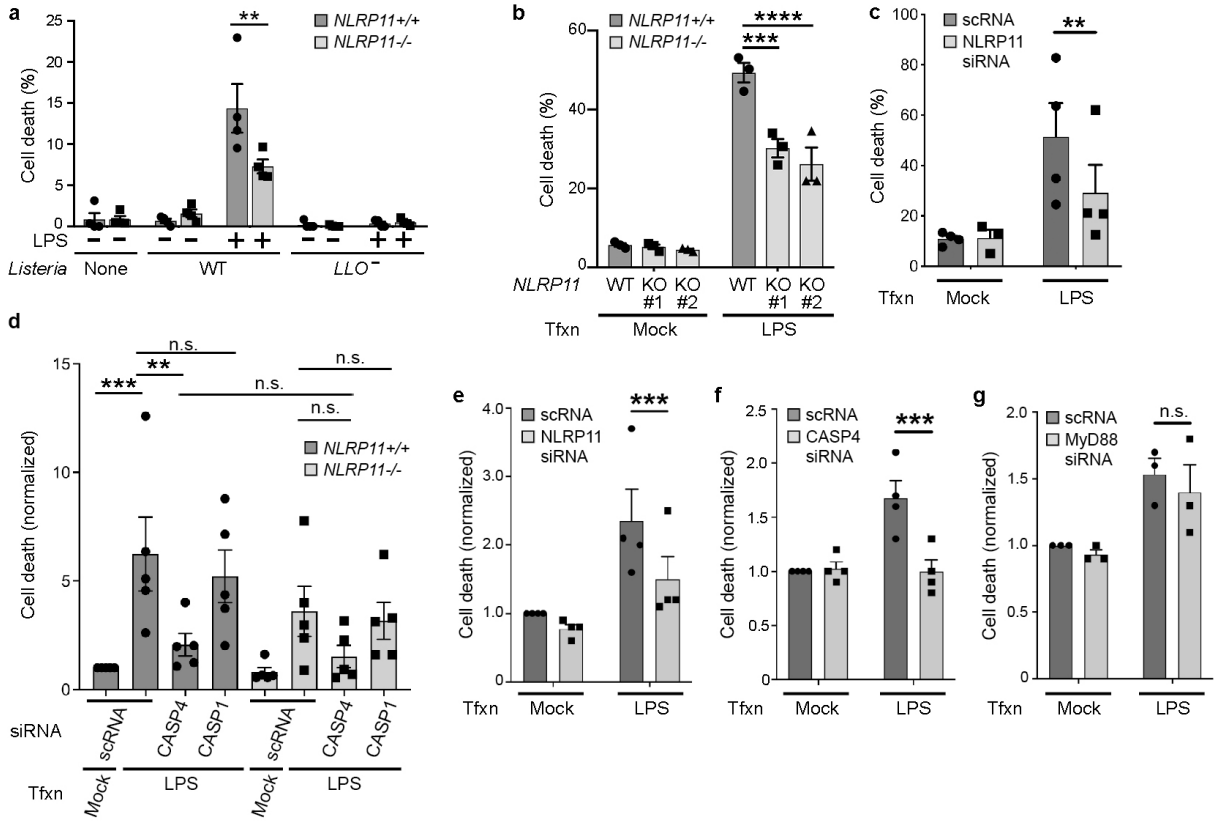


Fig. 2. *NLRP11* potentiates cell death in human macrophages in response to cytosolic LPS.

(**a**) *NLRP11* potentiates death of THP-1 macrophage-like cells induced by infection with cytosolic gram-positive pathogen *L. monocytogenes* that is coated with *S. enterica* serovar Minnesota LPS. Cell death depends on coating of *L. monocytogenes* with LPS and on escape from the vacuole, as *L. monocytogenes LLO*$^{-}$ is restricted to the phagosome. (**b-c**) In the absence of *NLRP11* (**b**) or following NLRP11 siRNA (**c**), death of THP-1 macrophage-like cells in response to electroporated LPS is defective. (**d**) NLRP11-dependent cell death of THP-1 macrophage-like cells depends on caspase-4, but not caspase-1. Cell death following siRNA depletion of *CASP4* or *CASP1*. (**e-g**) NLRP11 is required for efficient cell death of primary human monocyte-derived

581 macrophages in response to cLPS. Cell death in isolated CD14+ cells differentiated to
582 macrophages, treated with siRNA to *NLRP11* (**e**), *caspase-4* (**f**), or *MyD88* (**g**), and
583 subsequently electroporated with LPS. Cell death measured as LDH release relative to
584 complete lysis control; in (**e-g**), normalized to mock transfection following scRNA
585 treatment. Tfxn, transfection; mock, carrier alone; scRNA, scrambled siRNA; CASP4,
586 caspase-4, CASP1, caspase-1. Two-way ANOVA with Sidak *post hoc* test (**a**) or Tukey
587 *post hoc* test (**b-d**); mixed-effects analyses (**e-g**). Mean ± S.E.M. **, $p<0.01$; ***;
588 $p<0.001$; ****, $p<0.0001$; n.s., not significant.

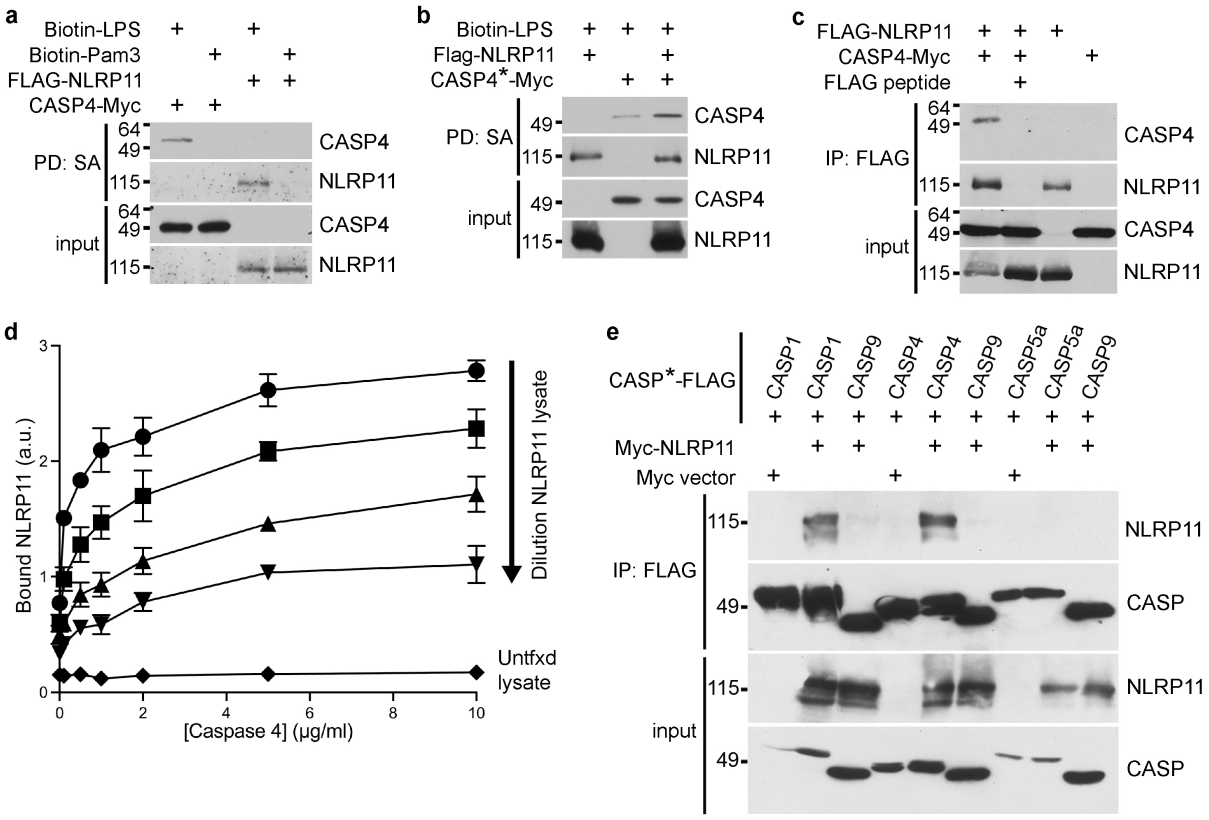


Fig. 3. NLRP11 interactions with caspases in reconstituted HEK293T lysates.

(**a-b**) Precipitation of NLRP11 and wildtype caspase-4 (CASP4) by biotin-LPS, separately (**a**) and together (**b**), and not by biotin-PAM3CSK4 (Pam3) (**a**). PD: SA, pull down with streptavidin beads. (**c**) NLRP11 precipitates wildtype CASP4. (**d**) Interaction between NLRP11 and CASP4 increases as a function of the concentration of each protein. Binding of FLAG-NLRP11 in transfected HEK293T cells to plates coated with purified wildtype CASP4 with detection of bound FLAG. Dilutions of NLRP11 lysates: circles, undiluted; squares, 1:2 dilution; triangles, 1:4 dilution; inverted triangles, 1:8 dilution. Diamonds, untransfected (untfxd) lysate. Two-way ANOVA with Tukey *post hoc* test. a.u., arbitrary units. (**e**) Specificity of NLRP11 for caspase-4 and caspase-1. FLAG-

600 tagged catalytically inactive caspases (CASP1[C285A]-FLAG, CASP5a[C315A]-FLAG,
601 CASP9[C287S]-FLAG) precipitation of Myc-NLRP11. CASP*, catalytically inactive
602 caspase. IP: FLAG, pull down with beads coated with FLAG antibody. Detection by
603 western blot with anti-FLAG or anti-Myc antibody.