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 responses¹⁻⁴. Bacterial lipopolysaccharide (LPS) is an inflammasome trigger and a major mediator of inflammation during infection. Activation of most inflammasomes is triggered by sensing of pathogen products by a specific host cytosolic NLRP or other sensor protein that then 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 mice⁵⁻⁷. 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*⁸, has led to the postulate that inflammasome activation by cLPS occurs independent of an NLRP. Here we show that in response to infection by gram-negative intracellular bacterial pathogens or to LPS transfection, efficient activation of the non-canonical pathway in human-derived macrophages depends on primate-specific NLRP11. Further, in both immortalized human-derived macrophages and primary human macrophages, NLRP11-dependent cell death is specifically due to detection of cLPS. In cell lysates, NLRP11 binds LPS independently of caspase-4 and 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 and a component of the human non-canonical inflammasome activation pathway.

Introduction

Inflammasomes assemble in response to the presence in the cytosol of pathogen-associated molecules, including dsDNA, flagellin, type 3 secretion system structural proteins, pore-forming toxins, ion flux, ATP, and LPS¹⁻⁴. Canonical inflammasomes consist of a sensor protein and an inflammatory caspase, frequently linked by an adaptor protein. Inflammasome assembly activates inflammatory caspases, which proteolytically process the pore-forming protein gasdermin D⁹⁻¹². In the caspase-1 inflammasome, caspase-1 proteolytically processes the pro-inflammatory cytokines IL-18 and IL-1 β ^{13,14}, which are released through pores formed in the plasma membrane by the N-terminal domain of gasdermin D; these pores are required both for the release of activated pro-inflammatory cytokines and for pyroptotic cell death^{9-12,15}. The presence of bacterial LPS in the cytosol of human macrophages activates a non-canonical inflammasome, which in humans, contains caspases-4 and -5, and in mice, caspase-11⁵⁻⁷, and which also leads to caspase-mediated processing of gasdermin D, pore formation, and pyroptotic cell death. To date, no sensor protein of cytosolic LPS has been identified, and because caspase-4, -5, and -11 bind LPS *in vitro*⁸, the current paradigm is that the non-canonical inflammasome lacks a sensor protein (Fig. 1a).

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

In a forward genetic screen that enriched for genes required for death during infection with the gram-negative intracellular bacterial pathogen *Shigella flexneri*, we identified the primate-specific protein NLRP11¹⁶ (Extended Data Fig. 1); the screen was conducted using a human-derived myeloid lineage cell line. The NLRP (nucleotide-

binding oligomerization domain, leucine rich repeat and pyrin domain containing protein) family includes proteins such as NLRP1 and NLRP3 that sense a range of pathogen-associated molecular patterns or damage-associated molecular patterns in the cell cytosol. We performed targeted deletion of *nlrp11* in the human-derived monocyte cell line THP-1 and found that cell death during *S. flexneri* infection (as measured by lactate dehydrogenase (LDH) release) was significantly reduced for macrophage-like cells that lack NLRP11. In each of two THP-1-derived cell lines in which *nlrp11* was disrupted by a CRISPR-Cas9 induced indel (Extended Data Fig. 2), release of LDH upon infection with *S. flexneri* was reduced by more than 50% (Fig. 1b). Consistent with previous reports, *S. flexneri*-induced macrophage cell death was dependent on the bacterial type 3 secretion system, which mediates both bacterial uptake¹⁷ and vacuolar escape^{18,19}, as a mutant that lacked either the virulence plasmid that encodes this secretion system or *virB*, the transcriptional activator of this system, displayed minimal cell death (Extended Data Fig. 3a).

To test whether the dependence on NLRP11 for efficient cell death was generalizable to other intracellular gram-negative bacterial pathogens, we infected *NLRP11*^{-/-} cells with the cytosolic pathogen *Burkholderia thailandensis* and the intracellular but largely vacuolar pathogen *Salmonella enterica* serovar Typhimurium. As with *S. flexneri* infection, in the absence of NLRP11, cell death during infection with *B. thailandensis* or *S. Typhimurium* was significantly reduced (Fig. 1c-d). In response to viral infection and certain Toll-like receptor interactions, the NF-κB pathway is repressed by NLRP11-dependent targeted degradation of TRAF6²⁰⁻²²; however, *S. flexneri*-induced NLRP11-dependent cell death was independent of TRAF6, whether or not the *Shigella* effector

Ospl, which suppresses TRAF6 signaling²³, was present (Extended Data Fig. 3b-d), indicating that the role of NLRP11 in bacterial infection-induced cell death is distinct from its role in NF- κ B signaling. Together, these results indicate that NLRP11 is required for efficient cell death induced by multiple intracellular gram-negative pathogens and raised the possibility that the implicated pathogen-associated trigger of cell death is generally present among gram-negative bacteria and depends on 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 vacuole²⁴, 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. 4a). 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 (GBP) 2-mediated vacuolar lysis²⁵, a potential explanation for the lack of differential NLRP11-dependent cell death induced by wild-type *S. Typhimurium*, which is largely vacuolar, and *sifA* mutant *S. Typhimurium*, which is predominantly cytosolic²⁶ (Fig. 1d).

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 and the downstream pore-forming protein gasdermin D were dependent on NLRP11; during *S. flexneri* infection, processing of caspase-4 and gasdermin D, leading to the release of active domains of each, was significantly reduced in the absence of NLRP11 (Fig. 1e-f). In THP-1 macrophages depleted of caspase-4, the absence of NLRP11 caused no significant additional decrease in cell death induced by LPS transfection (Fig. 2d and Extended Data Fig. 5a). Depletion of *CASP1* or *MyD88* was associated with no significant decrease in cell death in response to cLPS as compared to control depletion for either THP-1 macrophages containing NLRP11 or THP-1 macrophages lacking NLRP11 (Fig. 2d and Extended Data Fig. 5b). Despite testing several antibodies, we were unable to detect native cellular NLRP11 by western blot, presumably due to very low levels of native protein. These data indicate that NLRP11 is required for efficient activation of the non-canonical inflammasome, likely functioning in the caspase-4 pathway, and that NLRP11 function is independent of caspase-1 and extracellular Toll-like receptor signaling. Consistent with NLRP11 functioning specifically in the non-canonical inflammasome pathway, NLRP11 was not required for activation of the

canonical NLRP3 inflammasome, as cell death induced by the NLRP3 agonist nigericin was independent of NLRP11 (Extended Data Fig. 4b-d).

NLRP11 interacts with LPS and caspase-4

In contrast to NLRP3 and NLR family CARD domain containing 4 (NLRC4) inflammasomes, activation of the non-canonical pathway is independent of the adaptor protein ASC, as signaling in response to cytosolic LPS is normal in human or mouse-derived macrophages lacking ASC^{6,8} and NLRP11 does not co-localize with ASC specks^{20,27}. We tested whether NLRP11 interacts with other core components of the non-canonical pathway by ectopic expression of selected components in HEK293T cells, which lack most innate immune proteins, including ASC and inflammatory caspases. Biotin-conjugated LPS precipitated NLRP11 in the absence of caspase-4 (Fig. 3a), indicating that NLRP11 binds LPS independently of caspase-4. As previously described⁸, LPS also specifically precipitated caspase-4 and its catalytically inactive derivative (CASP4 C258A), including in the presence of NLRP11 (Fig. 3b), whereas the triacylated lipid Pam3CSK4 did not precipitate either NLRP11 or, as previously described⁸, caspase-4 (Fig. 3a). In addition to binding to LPS independent of caspase-4, NLRP11 specifically precipitated caspase-4 (Fig. 3c). To characterize the binding relationship of NLRP11 to caspase-4, we developed a plate binding assay using FLAG-NLRP11 in HEK293T cell lysates and purified caspase-4 (Methods and Extended Data Fig. 6a). In this assay, FLAG-NLRP11 binding depended on caspase-4, as binding of FLAG-NLRP11 in the absence of caspase-4 was at background levels (Fig. 3d, diamonds). Moreover, binding of NLRP11 to caspase-4 increased as a function of

increasing concentrations of both NLRP11 lysate and caspase-4, resembling the asymptotic phase of a sigmoidal curve, beginning to reach saturation at 8-10 µg/ml caspase-4 coating the wells (Fig. 3d).

NLRP11 displays caspase specificity

In addition to caspase-4, NLRP11 co-precipitated with caspase-1; of note, NLRP11 did not co-precipitate with caspase-5, a paralog of caspase-4 that binds LPS and is a component of at least some non-canonical inflammasomes^{8,14,28}, or the more divergent caspase-9 (Fig. 3e and Extended Data Fig. 7a). In these experiments the isoform of caspase-5 that was used was caspase-5/a, the most abundant isoform; although this isoform contains nearly all sequences present in other isoforms, it is theoretically plausible that another isoform(s) would interact with NLRP11. Although caspase-1 co-precipitates with NLRP11 (Fig. 3e), NLRP11 does not activate caspase-1 under conditions in which AIM2 did²⁰, and NLRP11 function in LPS-induced cell death of THP-1 macrophages is independent of caspase-1 (Fig. 2d), indicating that the observed interaction of caspase-1 with NLRP11 contributes minimally to activation of the non-canonical inflammasome.

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

Host GBPs bind intracellular LPS, promote non-canonical inflammasome activation²⁹⁻³³, and are encoded by genes that are strongly induced by interferon-gamma (IFN γ)³⁴. Human GBP1 binds LPS on the *S. flexneri* surface, whereupon it enhances caspase-4

recruitment³⁵. We tested whether IFN γ stimulation altered the role of NLRP11 in activation of the non-canonical inflammasome. IFN γ pre-treatment of THP-1 macrophages induced GBP1, as expected, yet NLRP11 was still required for efficient activation of *S. flexneri*-induced cell death (Extended Data Fig. 8a-b). Moreover, during the timeframe of this infection, GBP1 was not induced by *S. flexneri* infection alone, depending exclusively on pre-treatment with IFN γ (Extended Data Fig. 8c), indicating that is not required for non-canonical inflammasome induced pyroptosis or NLRP11 function in non-canonical inflammasome induced pyroptosis. Moreover, IFN γ and GBP1 do not bypass the requirement for NLRP11 in this pathway.

Requirement for NLRP11 in primary human macrophages

Following treatment of primary human monocyte-derived macrophages with siRNA to NLRP11, although overall levels of cell death are lower than for immortalized THP-1 cells, cell death induced by cLPS was significantly diminished (Fig. 2e). As in THP-1 macrophages, in primary macrophages, the defect in cLPS-induced cell death depended on caspase-4, but not MyD88 (Figs. 2f-g and Extended Data Fig. 5c-d), indicating that in these assays, the trigger was cLPS rather than extracellular LPS, and demonstrating that the requirement for NLRP11 in the non-canonical inflammasome pathway extends to primary human cells.

In summary, the data reported here identify NLRP11 as a primate-specific cytosolic sensor of bacterial LPS in the activation of the caspase-4 dependent inflammasome and caspase-4 dependent pyroptosis. These functions imply that NLRP11 is a key component of this inflammasome. Galectin-3, one among a family of β -galactoside–

194 binding proteins, senses cytosolic pathogens, promotes delivery of GBPs to ruptured
 195 pathogen-containing vacuoles, and enhances assembly of LPS-triggered caspase-4/11
 196 oligomerization and activation³⁶⁻³⁸. Whether galectin-3 activity synergizes with the
 197 interaction of cLPS and NLRP11 is uncertain; however, the independence of NLRP11
 198 function from GBP1 (Extended Data Fig. 8) suggests that any contribution of galectin-3
 199 to this process would be minor. Additionally, it remains uncertain whether the unique
 200 presence of NLRP11 in primates and the absence of it or identifiable homologs in mice
 201 contributes to the observed $\sim 10^4$ -fold increased susceptibility to gram-negative bacterial
 202 sepsis and LPS of humans as compared to mice^{39,40}; additional insights into these
 203 relationships would potentially enable much-needed improved mouse models for the
 204 study of bacterial sepsis.

Methods

Bacterial strains, plasmids, antibodies, and reagents. *Shigella flexneri* serotype 2a wildtype strain 2457T and the *S. flexneri* mutant strains *virB::Tn5*, BS103, and $\Delta ospC3$, which are isogenic to 2457T, have been described previously⁴¹⁻⁴⁴. *Salmonella enterica* serovar Typhimurium and the isogenic $\Delta sifA$ mutant²⁶ were gifts from L. Knodler. *Burkholderia thailandensis*⁴⁵ was a gift from P. Cotter. *Listeria monocytogenes* 10403s and 10403 LLO⁴⁶ were gifts from M. Kielian and J. Miller. *S. flexneri* was grown overnight in tryptic soy broth at 37°C with aeration. *L. monocytogenes* was grown overnight in brain heart infusion broth at 30°C at a stationary slant. *B. thailandensis* was grown overnight in low salt Luria broth (LB) at 37°C with aeration. *S. enterica* Typhimurium was grown in LB broth at 37°C with aeration.

Caspase-1 (Abcam, ab207802) rabbit monoclonal antibody was used at 0.5 µg/mL (1:1000). Caspase-4 (Santa Cruz, sc-56056), and gasdermin D (Santa Cruz, sc-81868) mouse monoclonal antibodies were used at 0.5 µg/mL (1:200). FLAG M2 mouse monoclonal antibody (Sigma, F3165) was used at 76 ng/mL (1:5000). Myc rabbit monoclonal antibody (Cell Signaling, 2278) was used at 1:1000. GBP1 mouse monoclonal antibody (Abcam, ab119236) was used at 1:1000. For western blots of immunoprecipitations, the secondary antibody used was TrueBlot horseradish peroxidase-conjugated anti-mouse Ig (Rockland, 18-8817-31). For detecting β-actin, horseradish peroxidase-conjugated anti-β-actin antibody (Sigma, A3854) was used. For all other western blots, the secondary antibody used was horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG antibodies (Jackson

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

Strain and plasmid construction. The *S. flexneri* *ospl* deletion mutant was generated in the 2457T strain background using the lambda red method by introducing a chloramphenicol acetyltransferase (*cat*) cassette into the *orf169b* gene (which encodes Ospl), as described⁴⁷.

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

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

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

Generation of *TRAF6* depletion cell lines. pLKO.1-shTRAF6 (⁴⁸, gift of A. Goldfeld), which encodes shRNA to *TRAF6*, was packaged into lentivirus in HEK293T cells. Lentivirus were introduced into NLRP11+/+ and NLRP11-/- THP-1 cell lines and cells containing pLKO.1-shTRAF6 were selected, expanded by maintenance in 0.8 μ g/mL puromycin, as described⁴⁸, and stable clones were isolated by limiting dilution.

Mammalian cell culture. All cell lines were obtained from ATCC. HEK293T cells were maintained in Dulbecco's Modified Eagle's medium (Gibco, 11965118). THP1 cells were maintained in RPMI-1640 (Gibco, 21870092). All media was supplemented with 10% (vol/vol) fetal bovine serum (heat inactivated, sterile filtered, R&D Systems). For THP-1 cells, RPMI-1640 media was also supplemented with 10 mM HEPES (Gibco, 15630080) and 2 mM L-glutamine (Gibco, 25030081). All cells were grown at 37°C in 5% CO₂. To differentiate monocyte cell lines into macrophage-like cells, 50 ng/mL phorbol-12-myristate-13-acetate (PMA) was added 48 h before experiments. Where indicated, cells were pretreated with 10 ng/mL IFN γ 16-20 h prior to infection.

Precipitation experiments. HEK293T cells were transiently transfected using Fugene6 (Promega). A stock solution of 10x Buffer A (100 mM HEPES, 1500 mM NaCl, 10 mM EGTA, 1 mM MgCl₂, pH 7.4) was prepared. The cell culture medium was removed 24-48 h after transfection. Cells were washed with cold PBS and lysed in 1x Buffer A, 0.5%

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

Streptavidin pulldown was largely as described⁸, with minor modifications. Cell lysates were diluted to 4 mg/mL before a 2 h incubation with beads. For co-precipitation experiments, separate cells were transfected with each DNA construct, and pre-specified amounts of lysates were combined with each other or with untransfected lysate, such that the total amount of lysate were similar in each experimental condition; these lysates were then incubated at 37°C overnight. To reduce non-specific binding, lysates were pre-cleared with streptavidin beads and then were incubated for 2 h at 30°C with 4 µg biotinylated *E. coli* O111:B4 LPS loaded onto 8 µg of Streptavidin Mag sepharose (GE, 28985738, or Pierce, 88816).

Plate binding assays. Clear flat bottom Nunc Maxisorp ELISA plates (Thermo Fisher, 442404) were coated with various concentrations of purified recombinant caspase-4 (Origene, TP760359) overnight at 4°C. The plates were washed 3x with PBS then blocked for 2 h at RT with 1% bovine serum albumin (Fisher, BP9700100) in PBS. Excess blocking solution was removed. HEK 293T cells transiently expressing FLAG-NLRP11 and control untransfected cells were lysed into 50 mM Tris-HCl pH 7.4, 150

mM NaCl, 5 mM MgCl₂, 100 µg/ml digitonin (Thermo Fisher Scientific, BN2006) and complete Mini EDTA-free protease inhibitor (Roche), as described³². Total protein concentration of lysates was normalized to 2 mg/mL. Serial dilutions were in untransfected HEK293T cell lysate. Varying concentrations of cell lysates were incubated with pre-blocked ELISA plates for 1 h at RT. Excess lysate was removed by washing 3 times with PBS. For detection of FLAG-NLRP11, wells were incubated with 0.8 µg/mL anti-FLAG rabbit (Sigma, F7425-.2MG) was incubated for 1 h at RT, and after washing with PBS, 0.16 µg/mL goat-anti-rabbit conjugated to horseradish peroxidase (HRP) (Jackson, 111-035-144). To detect caspase-4 bound to the plate, wells were incubated with 0.5 µg/mL anti-caspase-4 (Santa Cruz, sc-56056) and, after washing with PBS, 0.16 µg/mL goat-anti-mouse conjugated to HRP (Jackson, 115-035-003), each for 1 hr. After 6 washes with PBS, 50 µL of One Step Ultra-TMB (Fisher Scientific, PI34029) was added to each well and incubated for 5 min before stopping with 4 N sulfuric acid and reading in a BioTek plate reader.

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

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

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

Isolation of primary human peripheral blood mononuclear cells (PBMCs) and CD14 monocytes. Cells were isolated from leukopaks from individual healthy donors (Crimson Core Laboratories, Brigham and Women's Hospital) using density-gradient

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

LPS transfection. Electroporation of cells with LPS was performed using the Neon Transfection System (Invitrogen) according to the manufacturer's instructions using S. Minnesota LPS at indicated concentrations. THP-1 cells were seeded in low-attachment 10 cm dishes with 50 ng/mL PMA in RPMI. After 48 h, the cells were lifted in PBS (without calcium or magnesium). For electroporation, 1.5×10^6 THP-1 cells were pelleted, resuspended in Resuspension Buffer R with 2 $\mu\text{g/mL}$ of LPS or water control. Using a 10 μL Neon tip, cells were pulsed once at 1400 V and 10 ms pulse width. For primary human macrophage cells, 7.5×10^5 CD14 macrophages were pelleted, then resuspended in Resuspension Buffer T with 2 $\mu\text{g/mL}$ of LPS or water control. Using a 10 μL Neon tip, cells were pulsed 2 times at 2250 V and 20 ms pulse width. Electroporated cells were immediately resuspended in 2 mL of RPMI and placed into

wells of a 24 well plate. Cells were incubated for 2.5 h at 37°C before measuring LDH release.

siRNA transient knockdowns. siRNAs used for NLRP11 knockdowns were Hs_NALP11_1 FlexiTube siRNA and Hs_NALP11_5 FlexiTube siRNA at 10 nM total (5 nM each siRNA); for caspase-4 knockdowns were 3941, 4036 and 4130 at 30 nM total (10 nM each siRNA) (ThermoFisher); for Caspase-1 Hs_CASP1_14 FlexiTube siRNA and Hs_CASP1_13 FlexiTube siRNA at 30 nM total (15 nM each siRNA); and for MyD88 Hs_MYD88_5 FlexiTube siRNA and Hs_MYD88_11 FlexiTube siRNA at 30 nM total (15 nM each siRNA) (Qiagen). AllStars Negative Control siRNAs (Qiagen) were used as negative controls at 10 nM or 30 nM, accordingly. siRNAs were transfected using the HiPerfect reagent (Qiagen) into primary human monocyte-derived macrophages 72 h before or into THP-1 cells 48 h before experimental assays, following the manufacturer's protocol for "Transfection of Differentiated Macrophage Cell Lines." After transfection, cells were maintained at 37°C in 5% CO₂. Degree of knock-down was assessed by western blot of lysates of equivalent numbers of cells for each condition.

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

LDH assay. LDH was measured using an LDH Cytotoxicity Kit (Pierce, 88953) or Cytotoxicity Detection Kit (Sigma, 11644793001) per the manufacturer's instructions. Measurements at 490 nm and 680 nm were taken immediately after addition of Stop

Solution using a BioTek Plate Reader. To determine LDH activity, the 680 nm absorbance value (background) was subtracted from the 490-nm absorbance value, and cytotoxicity was calculated for each sample as LDH activity as a fraction of the total LDH activity for cells treated with the Triton X-100 lysis buffer provided by the manufacturer. For experiments involving primary cells, a different donor was used for each biological replicate; due to inherent variability from donor to donor in the baseline levels of death in cell death assays, within-donor data were normalized to the negative control condition before compilation of the biological replicates.

Statistics and reproducibility. Unless otherwise indicated, all results are data from at least 3 independent biological replicates. On bar graphs, each individual symbol is data from an independent biological replicate. Statistical analysis was performed with Prism version 9.

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

395 **Supplementary information**

396 The online version contains supplementary material.

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405 **Author contributions**

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

410 **Competing interests**

411 The authors declare no competing interests.

412 **Materials and correspondence**

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Figures and figure legends

Fig. 1. In the absence of *NLRP11*, cell death of human-derived macrophage-like cells induced by intracellular gram-negative bacteria is defective.

(a) Current (blue/black arrows) and proposed (pink/black arrows) paradigms of non-canonical recognition of cLPS; with high cLPS concentration, both pathways are proposed to occur. CASP4, caspase-4; GSDMD, gasdermin D; GSDMD-N, processed N-terminal domain of GSDMD. (b-c) Death of THP-1 macrophages upon infection with the cytosolic gram-negative pathogens *S. flexneri* (b), and *B. thailandensis* (c). Cell death in response to *S. flexneri* infection in each of two independent *NLRP11*^{-/-} THP-1 cell lines (b). *n* = 5 (b: WT, KO #1) or 3 experiments (b: KO #2, c). (d) *NLRP11* dependence of efficient THP-1 macrophage death induced by *S. Typhimurium*. *sifA*

571 mutant, which is largely cytosolic, and WT *S. Typhimurium*, which is largely vacuolar. *n*
572 = 3 experiments. **(e-f)** *NLRP11* dependence of efficient caspase-4 **(e)** and gasdermin D
573 **(f)** processing during *S. flexneri* infection of THP-1 macrophages; western blot of culture
574 supernatants. In the absence of OspC3, processing was similar to induced by WT *S.*
575 *flexneri*; in mouse bone marrow-derived macrophages and human epithelial cell lines,
576 OspC3 arginine ADP riboxanates caspase-4, thereby inhibiting processing of gasdermin
577 D^{49,50}. *n* = 3 experiments. Cell death is by LDH release, reported as percent of total
578 Triton X-100 cell lysis. Mean ± S.E.M. Two-way ANOVA with Dunnett *post hoc* test **(b,**
579 **d)** or with Sidak *post hoc* test **(e-f)**, or student T-test **(c)**. *, *p*<0.05; **, *p*<0.01; ***,
580 *p*<0.001; ****, *p*<0.0001; 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. *n* = 4 experiments. (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. *n* = 3 (b) or 4 (c) experiments. (d) *NLRP11*-dependent cell death of THP-1 macrophage-like cells depends on caspase-4, but not caspase-1 or MyD88. Cell death following siRNA depletion of *CASP4*, *CASP1*, or *MyD88*. *n* = 5 experiments. (e-g) *NLRP11* is required for efficient cell death of primary human monocyte-derived

594 macrophages in response to cLPS. Cell death in isolated CD14⁺ cells differentiated to
 595 macrophages, treated with siRNA to *NLRP11* (**e**), *caspase-4* (**f**), or *MyD88* (**g**), and
 596 subsequently electroporated with LPS. Each experiment performed with cells from a
 597 different donor. Cell death measured as LDH release relative to complete lysis control;
 598 (**e-g**), normalized to mock transfection following scRNA treatment. *n* = 4 (**e-f**) or 3 (**g**)
 599 experiments. Tfxn, transfection; mock, carrier alone; scRNA, scrambled siRNA; CASP4,
 600 caspase-4, CASP1, caspase-1. Two-way ANOVA with Sidak *post hoc* test (**a**) or Tukey
 601 *post hoc* test (**b-d**); mixed-effects analyses (**e-g**). Mean ± S.E.M. **, *p*<0.01; ***,
 602 *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. $n = 3$ experiments. (c) NLRP11 precipitates wildtype CASP4. $n = 3$ experiments (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 (untxd) lysate. $n = 3$ experiments. Two-way ANOVA with Tukey *post hoc* test. a.u., arbitrary units. (e) Specificity of NLRP11 for caspase-4 and caspase-1. FLAG-tagged catalytically inactive

615 caspases (CASP1[C285A]-FLAG, CASP5a[C315A]-FLAG, CASP9[C287S]-FLAG)
616 precipitation of Myc-NLRP11. CASP*, catalytically inactive caspase. IP: FLAG, pull
617 down with beads coated with FLAG antibody. Detection by western blot with anti-FLAG
618 or anti-Myc antibody. *n* = 3 experiments.