# Supporting Information

## Reyes Ruiz et al. 10.1073/pnas.1710433114

### SI Materials and Methods

**hMDM Experiments.** In experiments where macrophages were primed with Pam3CSK4, cells were pretreated with 100 or 400 ng/mL Pam3CSK4 (Invivogen) for 3 h before bacterial infections or 4 h before anthrax toxin treatments, respectively. For experiments involving LPS, cells were pretreated with 500 ng/mL LPS (Sigma-Aldrich).

For infections with *Salmonella* Typhimurium, bacterial cultures were pelleted at 6,010 × *g* for 3 min and washed with PBS. Bacteria were then resuspended in PBS and added to the cells at a multiplicity of infection (MOI) of 20. The infected cells were then centrifuged at 290 × *g* for 10 min and incubated at 37 °C. After 1 h of infection, 100 μg/mL of gentamicin was added to each well to prevent extracellular growth. Infections proceeded at 37 °C for a total of 4 h. For infections with *Listeria monocytogenes*, bacterial cultures were back-diluted on the day of infection and grown until $OD_{600}$ = 0.8. Cultures were pelleted at 6,010 × *g* for 3 min and resuspended in PBS. Cells were infected with *L. monocytogenes* at an MOI of 5, 10, 20, or 75 and incubated at 37 °C. After 1 h of infection, 50 μg/mL of gentamicin was added to each well. Infections proceeded for a total of 16 h. For all experiments, control cells were mock-infected with PBS.

**Mouse Bone Marrow-Derived Macrophage Experiments.** All experiments performed with mouse bone marrow-derived macrophages were done so in accordance with the Animal Welfare Act and the recommendations in *Guide for the Care and Use of Laboratory Animals* of the NIH (1). The Institutional Animal Care and Use Committee of the University of Pennsylvania approved all procedures (protocol 804928).

Bone marrow was collected from the femurs and tibiae of C57BL/6J mice (Jackson Laboratory). Bone marrow cells were differentiated into macrophages by culturing the cells in RPMI containing 30% L929 cell supernatant, 20% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C. One day before infection, macrophages were replated in RPMI containing 15% L929 cell supernatant and 10% FBS at a concentration of 1.25 × $10^5$ cells per well in a 48-well plate. Cells were pretreated with 100 ng/mL Pam3CSK4 (Invivogen) for 16 h before infection and then either mock-infected with PBS or infected with *L. monocytogenes* at an MOI of 5. After 1 h of infection, 50 μg/mL of gentamicin was added to each well. Infections were continued for a total of 6 h.

**THP-1 Monocytic Cell Line Experiments.** THP-1 cells (TIB-202; American Type Culture Collection) were maintained in RPMI supplemented with 10% (vol/vol) heat-inactivated FBS, 0.05 mM β-mercaptoethanol, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified incubator. One day before infection, cells were replated in media lacking antibiotics at a concentration of 2.0 × $10^5$ cells per well in a 48-well plate. THP-1 cells were differentiated into macrophages with 200 nM phorbol 12-myristate 13-acetate for 24 h.

**Anthrax Toxin-Mediated Delivery of FlaA, PrgJ, and YscF.** Recombinant proteins (PA, LFn-FlaA, LFn-PrgJ, and LFn-YscF) were provided by Russell Vance, University of California, Berkeley, CA (2). In experiments with THP-1 and hMDMs, cells were plated in a 48-well plate at concentrations of 2.0 × $10^5$ and 1.0 × $10^5$ cells per well, respectively. PA and LFn doses for in vitro delivery were 1 μg/mL PA (for FlaTox), 4 μg/mL PA (for PrgJTox and YscFTox), 500 ng/mL LFn-$FlaA^{310–475}$ (truncated C terminus of *Legionella pneumophila* flagellin), 8 ng/mL LFn-PrgJ, 200 ng/mL LFn-YscF, and 2 μg/mL LFn-FlaA (full-length flagellin).

**Expression Plasmids Encoding Human Inflammasome Components.** pCMV6-XL5 plasmids encoding NAIP (NM_004536), IL-1β (NM_000576), or empty vector were purchased from Origene. The pCI plasmid encoding human caspase-1 (NM_033292.3) was a gift from Kate Fitzgerald, University of Massachusetts Medical School, Worcester, MA (plasmid 41552; Addgene) (3). The NLRC4 (NM_021209) ORF was amplified from an expression vector (GeneCopoeia) between flanking BamHI and NotI sites, and a Kozak sequence (GCCACC) was engineered to precede the start codon. The following primers were used (5′–3′): NLRC4 forward: AAAAGGATCCGCCACCATGAATTTCATAAAGGACAATAGCC and NLRC4 reverse: TTTTTGCGGCCGCTTAAGCAGTTACTAGTTTAAAATCACC. The digested NLRC4 PCR product was cloned into a BglII/NotI-digested MSCV2.2 vector, which was a gift from Russell Vance (plasmid 60206; Addgene) (4). Plasmids were prepared with the Qiagen EndoFree Plasmid Maxi Kit.

**Reconstitution of the NAIP/NLRC4 Inflammasome in HEK293 cells.** HEK293 cells were maintained in DMEM supplemented with 10% (vol/vol) heat-inactivated FBS, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C. Cells were replated at 7 × $10^4$ cells per well in 500 μL replating media (DMEM + 10% FBS + 2 mM L-glutamine) in a 24-well plate. Transfection of expression plasmids (described above) was performed using Lipofectamine 2000 (Thermo Fisher Scientific). The amounts of plasmids used were 20 ng of NAIP, 20 ng of NLRC4, 10 ng of caspase-1, and 400 ng of pro–IL-1β. Eighteen hours later, cells were treated with anthrax toxin components for cytosolic delivery of FlaA, PrgJ, or YscF. Cells were harvested 9 h later and subjected to immunoblot analysis.

**siRNA Knockdown Experiments.** All Silencer Select siRNA oligos were purchased from Ambion (Life Technologies). For NAIP, the siRNAs used were siRNA identifications s9262, s9263, and s9264. To knockdown NAIP, 10 nM each of the three oligos was used per well. As a control, Silencer Select negative control siRNAs (Silencer Select Negative Control No. 1 siRNA 4390843 and Silencer Select Negative Control No. 2 siRNA 4390846) were used at 15 nM each per well. Transfection of the pooled siRNAs into macrophages was performed using HiPerfect transfection reagent (Qiagen) following the manufacturer's protocol for "Transfection of Differentiated Macrophage Cell Lines, Including THP-1." Treatment with appropriate siRNAs was performed for 48 h. After 24 h, fresh media lacking antibiotics were added to each well. After a total of 48 h, treatment with anthrax toxin components was performed as described above. In parallel, siRNA-transfected hMDMs were treated with LPS + Nigericin (500 ng/mL and 10 μM, respectively).

**qRT-PCR Analysis.** Cells were lysed, and RNA was isolated using the RNeasy Plus Kit (Qiagen). Synthesis of the first strand cDNA was performed using SuperScript II reverse transcriptase and oligo (dT) primer (Invitrogen). qPCR was performed with the CFX96 real-time system (Bio-Rad) using the SsoFast EvaGreen Supermix with the LOW ROX kit (Bio-Rad). The following primers from PrimerBank (5–7) were used. The PrimerBank identifications are *NAIP* (119393877c3) and *HPRT* (164518913c1; all 5′–3′):

*NAIP* forward: CCCATTAGACGATCACACCAGA;

*NAIP* reverse: GGAGTCACTTCCGCAGAGG;

*HPRT* forward: CCTGGCGTCGTGATTAGTGAT; and

*HPRT* reverse: AGACGTTCAGTCCTGTCCATAA.

For analysis, mRNA levels of siRNA-treated cells were normalized to control siRNA-treated cells using the $2^{-\Delta\Delta CT}$ (cycle threshold) (8) method to calculate fold induction.

**Cytotoxicity Assays.** Cells were infected as described above and were assayed for cell death as determined by measuring loss of cellular membrane integrity via lactate dehydrogenase (LDH) activity in the supernatant. LDH release was quantified using an LDH Cytotoxicity Detection Kit (Clontech) and normalized to mock infected cells.

**ELISA.** Harvested supernatants from infected cells were assayed using ELISA kits for human IL-1α (R&D Systems) and IL-1β (BD Biosciences).

**Immunoblotting.** Infected or treated cells were lysed directly with 1× SDS/PAGE sample buffer, and low-volume (90 μL per well of a 48-well plate) supernatants were mixed 1:1 with 2× SDS/PAGE buffer containing Complete Mini EDTA-Free Protease Inhibitor Mixture (Roche). Protein samples were boiled for 5 min, separated by SDS/PAGE, and transferred to PVDF Immobilon-P membranes (Millipore). Samples were then probed with antibodies specific for IL-1β (8516; R&D Systems), NAIP (ab25968; Abcam), NLRC4 (12421S; Cell Signaling), and caspase-1 (2225S; Cell Signaling). As a loading control, all blots were probed with anti–β-actin (4967L; Cell Signaling). Detection was performed with HRP-conjugated anti-mouse IgG (F00011; Cell Signaling) or anti-rabbit IgG (7074S; Cell Signaling).

**Statistical Analysis.** Prism 6.0 (GraphPad Software) was utilized for the graphing of data and all statistical analyses. Statistical significance for hMDMs was determined using the paired two-way *t* test in experiments delivering bacterial ligands via anthrax toxin and infections with *Salmonella* Typhimurium and the paired Wilcoxon signed rank test in experiments delivering bacterial ligands via engineered *L. monocytogenes*. All hMDM data are graphed such that each data point represents the mean of triplicate infected wells for a given donor. Individual experiments in figures were performed using primary hMDMs from at least four different donors. Statistical significance for experiments with THP-1 cells was determined using the unpaired two-way *t* test. Statistical analyses for experiments with mouse bone marrow-derived macrophages were determined using the one-way ANOVA test and Tukey's multiple comparisons test. Differences were considered statistically significant if the *P* value was <0.05.


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3. Hornung V, et al. (2009) AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* 458:514–518.
4. Kofoed EM, Vance RE (2011) Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity. *Nature* 477:592–595.
5. Wang X, Seed B (2003) A PCR primer bank for quantitative gene expression analysis. *Nucleic Acids Res* 31:e154.
6. Spandidos A, et al. (2008) A comprehensive collection of experimentally validated primers for polymerase chain reaction quantitation of murine transcript abundance. *BMC Genomics* 9:633.
7. Spandidos A, Wang X, Wang H, Seed B (2010) PrimerBank: A resource of human and mouse PCR primer pairs for gene expression detection and quantification. *Nucleic Acids Res* 38:D792–D799.
8. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta C(T)) Method. *Methods* 25:402–408.


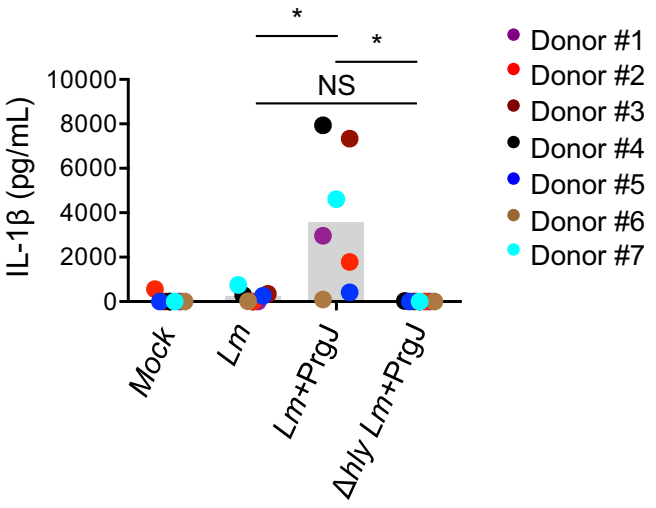


**Fig. S1.** *L. monocytogenes*-mediated delivery of the T3SS inner rod protein PrgJ requires LLO for inflammasome activation in primary human macrophages. hMDMs were primed with Pam3CSK4 for 3 h and infected with PBS (mock), WT *Listeria* (*Lm*) expressing PrgJ, or Δ*hly Lm* expressing PrgJ at an MOI of five for 16 h. IL-1β supernatant levels were measured by ELISA. Each data point represents the mean of triplicate infected wells for each of seven different human donors. Shaded bars represent the overall mean of the donors. NS, not significant. *$P < 0.05$ by paired Wilcoxon signed rank test.

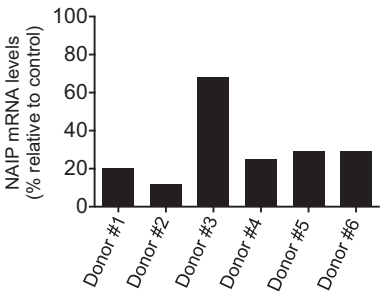


**Fig. S2.** *NAIP* siRNA knockdown efficiency in primary human macrophages. qRT-PCR was performed to quantitate *NAIP* mRNA levels in hMDMs treated with either control siRNA or *NAIP* siRNA. For the *NAIP* siRNA-treated cells from each donor, *NAIP* mRNA levels were normalized to human HPRT mRNA levels, and each sample was normalized to control siRNA-treated cells from the same donor.

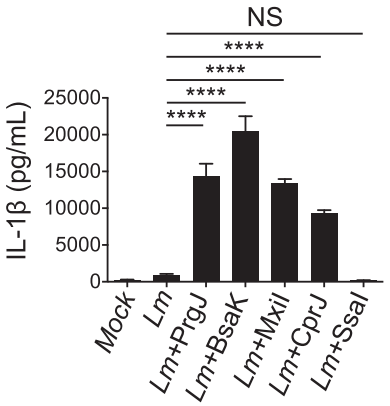


**Fig. S3.** *L. monocytogenes* strains ectopically expressing T3SS inner rod homologs induce inflammasome activation in mouse macrophages. Bone marrow-derived macrophages were primed with Pam3CSK4 for 16 h and infected with WT *Listeria* (*Lm*) or strains ectopically expressing PrgJ, BsaK MxiI, CprJ, or SsaI at an MOI of five for 6 h. Cells were treated with PBS for the mock control. IL-1β levels in the supernatants was measured by ELISA. Bar graphs display the mean ± SD of triplicate wells. Representative of two independent experiments. NS, not significant. ****$P < 0.0001$ by Tukey's multiple comparisons test.

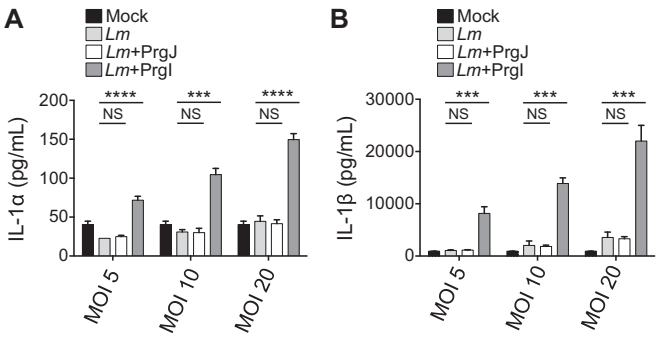


**Fig. S4.** *L. monocytogenes*-mediated delivery of PrgJ does not induce inflammasome activation in THP-1 cells. THP-1 cells were primed with Pam3CSK4 for 16 h and infected with WT *Listeria* (*Lm*) or strains ectopically expressing PrgJ or PrgI at an MOI of 5, 10, or 20 for 6 h. Cells were treated with PBS for the mock control. (*A* and *B*) IL-1α and IL-1β supernatant levels were measured by ELISA. Bar graphs display the mean ± SD of triplicate wells. Representative of three independent experiments. NS, not significant. ***$P < 0.001$ by unpaired *t* test; ****$P < 0.0001$ by unpaired *t* test.

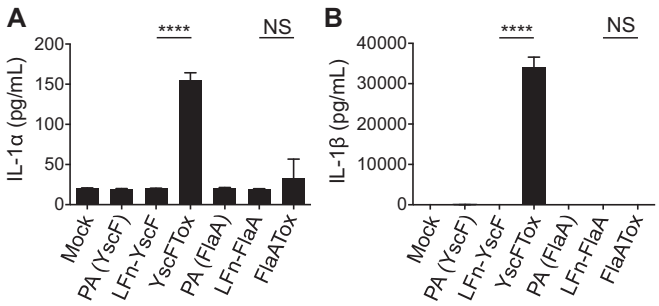


**Fig. S5.** Anthrax toxin-mediated delivery of full-length flagellin fails to induce inflammasome activation in THP-1 cells. (*A* and *B*) THP-1 cells were primed with Pam3CSK4 for 4 h and treated with PA alone, LFn-FlaA (full-length FlaA) alone, LFn-YscF alone, PA+LFn-FlaA (FlaTox; full-length FlaA), or PA+LFn-YscF (YscFTox) for 16 h. IL-1α and IL-1β supernatant levels were measured by ELISA. Bar graphs display the mean ± SD of triplicate wells. Representative of two independent experiments. NS, not significant. ****$P < 0.0001$ by unpaired *t* test.