Broad detection of bacterial type III secretion system and flagellin proteins by the human NAIP/NLRC4 inflammasome

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Inflammasomes are cytosolic multiprotein complexes that initiate host defense against bacterial pathogens by activating caspase–1-dependent cytokine secretion and cell death. In mice, specific nucleotide-binding domain, leucine-rich repeat-containing family, apoptosis inhibitory proteins (NAIPs) activate the nucleotide-binding domain protein 4 (NLR4) inflammasome upon sensing components of the type III secretion system (T3SS) and flagellar apparatus. NAIP1 recognizes the T3SS needle protein, NAIP2 recognizes the T3SS inner rod protein, and NAIP5 and NAIP6 recognize flagellin. In contrast, humans encode a single functional NAIP, raising the question of whether human NAIP senses one or multiple bacterial ligands. Previous studies found that human NAIP detects both flagellin and the T3SS needle protein and suggested that the ability to detect both ligands was achieved by multiple isoforms encoded by the single human NAIP gene. Here, we show that human NAIP also senses Salmonella Typhimurium T3SS inner rod protein PrsJ and that T3SS inner rod proteins from multiple bacterial species are also detected. Furthermore, we show that a single human NAIP isoform is capable of sensing the T3SS inner rod, needle, and flagellin. Our findings indicate that, in contrast to murine NAIPs, promiscuous recognition of multiple bacterial ligands is conferred by a single human NAIP.

In response to pathogenic bacteria, the innate immune system is required for inflammatory responses that promote host defense. Host defense is initiated by the engagement of pattern recognition receptors (PRRs) by pathogen-associated molecular patterns (1). Cytosolic PRRs detect pathogens that introduce products into host cells as a consequence of bacterial virulence activities, such as specialized secretion systems. A subset of cytosolic PRRs, termed the nucleotide-binding domain, leucine-rich repeat-containing (NLR) family, is composed of 23 members in humans and 34 members in mice (2, 3). A subfamily of NLRs, known as nucleotide-binding domain, leucine-rich repeat-containing family, apoptosis inhibitory proteins (NAIPs), recognizes bacterial proteins that are translocated into the host cell by Gram-negative bacteria. One such pathogen is Salmonella, which uses a virulence-associated type III secretion system (T3SS) to inject effector proteins into the host cell cytosol that promote bacterial invasion and survival (4). These secretion systems also translocate structurally related components of the T3SS or closely related flagellar apparatus, enabling cytosolic detection of bacteria by NAIPs (5). In mice, ligands for four of seven distinct NAIPs are known: NAIP1 recognizes the T3SS needle protein, NAIP2 recognizes the T3SS inner rod protein, and both NAIP5 and NAIP6 recognize flagellin (6–11). Upon binding their cognate ligands, the NAIPs recruit the adaptor nucleotide-binding domain, leucine-rich repeat-containing family, CARD domain-containing protein 4 (NLR4) (12–14). The resulting NAIP/NLRC4 inflammasome then recruits and activates caspase-1 (15). Active caspase-1 mediates processing and secretion of IL-1 family cytokines and a proinflammatory cell death termed pyroptosis (16–18), which promotes antimicrobial functions critical for controlling bacterial infection (19–22). This inflammasome also plays a protective role in mouse models of colitis-associated colorectal cancer and may be a useful strategy in tumor immunotherapy (23, 24). However, the NLRC4 inflammasome can cause sepsis-like disease after antibiotic disruption of the microbiota, and activating NLRC4 mutations cause an autoinflammatory syndrome in humans (25–29). Defining the mechanisms of human NAIP sensing of bacterial ligands may, therefore, provide insight into therapeutic approaches for diverse infectious and autoinflammatory diseases.

Unlike in mice, the human NAIP locus has a number of pseudogenes and gene duplications and has retained a single functional copy of the full-length NAIP gene (30, 31). Initial studies with human monocytic cell lines suggested that human NAIP could only sense the T3SS needle protein (7–9). However, a recent study found that flagellin also triggers NAIP inflammasome activation in primary human macrophages and indicated that detection of flagellin was mediated by an alternate splice isoform of NAIP (32). These findings suggested that, in humans, specificity for different bacterial ligands is encoded by distinct splicing variants of the single NAIP gene.

Here, we show that, in addition to the T3SS needle protein and flagellin, primary human macrophages also mount NAIP inflammasome.

Significance

Inflammasomes are cytosolic multiprotein complexes that initiate innate immune responses to microbial infection. Inflammasome specificity is determined by cytosolic innate immune sensors, including nucleotide-binding domain, leucine-rich repeat-containing family, apoptosis inhibitory proteins (NAIPs). In mice, which encode seven different NAIPs, individual NAIPs recognize specific components of the structurally related bacterial type III secretion system (T3SS) and flagellar apparatus. Humans encode a single functional NAIP, raising the question of whether human NAIP recognizes the same repertoire of bacterial ligands. Here, we find that, in contrast to the ligand specificity exhibited by the murine NAIPs, the single human NAIP broadly detects multiple T3SS and flagellin proteins. These findings provide a basis for understanding the mechanisms underlying human-specific innate immune responses against bacterial infection.


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Results

Salmonella Typhimurium Induces Flagellin-Independent Inflammasome Responses in Primary Human Macrophages. In murine macrophages, the NAIPs induce inflammasome activation on direct recognition of proteins from the T3SS and the structurally related flagellar apparatus. The relative contribution of these components to the inflammasome response in human macrophages is still unclear. Thus, we examined cell death as well as secretion of IL-1α and IL-1β after infection of human monocyte-derived macrophages (hMDMs) with WT, SPI-1 T3SS-deficient (ΔsipB), or flagellin-deficient (ΔfliCB) Salmonella Typhimurium strains. Compared with WT Salmonella-infected macrophages, ΔfliCBΔsipB Salmonella-infected macrophages exhibited a slight but not statistically significant decrease in inflammasome activation as measured by IL-1α and IL-1β secretion, IL-1β processing, and cell death (Fig. 1). In contrast, inflammasome activation was abrogated in ΔsipB-infected macrophages (Fig. 1 A–C). Immunoblot analysis indicated no defect in pro–IL-1β production in ΔsipB-infected hMDMs, but inflammasome-mediated cleavage of pro–IL-1β into its active form was not observed (Fig. 1D). These results suggest that Salmonella infection of primary human macrophages induces robust flagellin-independent inflammasome activation that requires the SPI-1 T3SS.

Salmonella Typhimurium T3SS Inner Rod Protein PrgJ Activates the Inflammasome in Primary Human Macrophages. Previous studies using immortalized human monocyte cell lines found that the NAIP inflammasome could be activated by the T3SS needle protein but not flagellin or the T3SS inner rod (7–9). However, another study found that NAIP played a role in restricting the intracellular replication of flagellated bacteria (33). Recently, it was discovered that flagellin can activate the NAIP inflammasome in primary hMDMs (32). As our data suggested that there is a robust flagellin-independent, T3SS-dependent inflammasome response to Salmonella, we sought to determine whether, in addition to the T3SS needle protein PrgI, the T3SS inner rod protein PrgJ could induce inflammasome activation in primary hMDMs. We utilized the Gram-positive pathogen Listeria monocytogenes, which does not encode a T3SS apparatus, to directly deliver PrgJ or PrgI into host cells (34). After infection, Listeria uses the pore-forming toxin Listerialysin O (LLO) to escape into the cytosol, where it expresses the protein ActA on the bacterial surface to polymerize actin (35, 36). We utilized strains that ectopically express PrgJ or PrgI translationally fused to the N terminus of ActA and under control of the actA promoter. This approach of delivering flagellin into the host cell cytosol robustly activates the mouse NAIP5 inflammasome (34). Indeed, as expected, hMDMs infected with Listeria expressing PrgJ induced robust IL-1α and IL-1β secretion, IL-1β processing, and cell death above that of WT Listeria-infected cells (Fig. 2). Surprisingly, infection with PrgJ-expressing Listeria also induced robust IL-1α and IL-1β release, IL-1β processing, and cell death (Fig. 2). Importantly, cytosolic access was required for inflammasome activation, as PrgJ-expressing Listeria lacking llo, the gene encoding LLO, did not induce IL-1β secretion (Fig. S1).

To determine whether PrgJ alone could induce inflammasome activation independently of bacterial infection, we used an anthrax toxin-based delivery system (9, 11, 37). In this system, bacterial ligands are translationally fused to the N-terminal domain of Bacillus anthracis lethal factor (LFn). The LFn domain enables ligand translocation into the host cell cytosol through a membrane channel formed by the anthrax protective antigen (PA) protein. We used a translational fusion of LFn and PrgJ (LFn-PrgJ) as well as LFn fused to flagellin as a positive control for NAIP inflammasome activation. To avoid potential confounding effects of TLRS detection of flagellin, we used a truncated Legionella pneumophila flagellin that lacks the TLRS-activating region but retains the C-terminal 166 amino acids detected by murine NAIP5 (38, 39). In agreement with previous findings (32), hMDMs treated with PA+LFn-LFlaA100–475 (referred to as FlaTox) induced robust inflammasome activation as measured by significantly increased IL-1α and IL-1β cytokine release, IL-1β processing, and cell death (Fig. 3). Treatment with PA+LFn-PrgJ (referred to as PrgJTox) also induced robust IL-1α and IL-1β cytokine secretion, IL-1β processing, and cell death (Fig. 3). In contrast, treatment with PA, LFn-LFlaA100–475, or LFn-PrgJ alone did not activate, indicating that FlaA and PrgJ induce inflammasome activation only when delivered into the host cell cytosol via PA. Altogether, these results show that primary human macrophages undergo inflammasome activation on cytosolic sensing of the Salmonella Typhimurium T3SS inner rod protein.

Human NAIP is Required for Maximal Inflammasome Responses to the T3SS Inner Rod Protein PrgJ. Human NAIP is required for inflammasome responses to flagellin and the T3SS needle protein (7, 9, 32). To test whether NAIP is also necessary for detecting PrgJ, we used siRNAs to silence NAIp in primary hMDMs (Fig. S2). As expected (32), anti-NAIp siRNA treatment resulted in significantly decreased IL-1α and IL-1β secretion after FlaTox administration compared with control siRNA treatment. Anti-NAIP siRNA treatment also led to significantly decreased IL-1α and IL-1β secretion in response to PrgJTox administration relative to control siRNA-treated.

Reyes Ruiz et al.
cells, suggesting that NAIP is required for maximal inflammasome responses to PrgJ (Fig. 4A and B). Importantly, siRNA-mediated silencing of NAIP did not significantly affect inflammasome responses to LPS+Nigericin, which specifically activates the NLRP3 inflammasome and does not engage NAIP (40) (Fig. 4C and D). These results indicate that NAIP is required for maximal inflammasome responses to the T3SS inner rod.

**T3SS Inner Rod Proteins from Other Bacterial Species Induce Inflammasome Activation in Human Macrophages.** As T3SS inner rod proteins from multiple bacterial species activate the mouse NAIP2 inflammasome (41), we next examined whether other bacterial T3SS inner rod homologs similarly activate human cells. We engineered *Listeria* strains expressing the T3SS inner rod proteins from *Burkholderia thailandensis* (BsaK), *Shigella flexneri* (MxiI), and *Chromobacterium violaceum* (CprJ). In agreement with previous findings (41), mouse macrophages infected with *Listeria* expressing these inner rod homologs robustly secreted IL-1β (Fig. S3), hMDMs infected with *Listeria* expressing PrgJ, PrgJ, BsaK, and MxiI also resulted in robust IL-1β secretion and processing well above that of WT *Listeria*-infected cells (Fig. 5A and B). In contrast, CprJ-expressing *Listeria* induced relatively low levels of IL-1β secretion and processing. These findings show that human macrophages broadly detect and activate the inflammasome in response to T3SS inner rod proteins from multiple bacterial species.

*Salmonella Typhimurium* SPI-2 T3SS Inner Rod Protein SsaI Evades Immune Detection by Human Macrophages. *Salmonella Typhimurium* uses two different T3SSs, termed SPI-1 and SPI-2. The SPI-1 T3SS plays a role in bacterial invasion, whereas the SPI-2 T3SS is required for intracellular survival and replication (42–44), suggesting a need to evade host recognition of the SPI-2 T3SS. Indeed, while the SPI-1 T3SS inner rod protein, PrgJ, robustly activates the mouse NAIP2 inflammasome, the SPI-2 T3SS inner rod protein, SsaI, evades detection (41). We, therefore, asked whether SsaI also evades human NAIP by expressing SsaI in *Listeria*. Consistent with previous findings (41), mouse macrophages infected with *Listeria* expressing SsaI secreted negligible levels of IL-1β (Fig. S3). Infection of hMDMs with SsaI-expressing *Listeria* also resulted in negligible IL-1β secretion and cleavage compared with infection with *Listeria* expressing PrgJ or PrgI (Fig. 5C and D). These data suggest that the SPI-2 T3SS inner rod protein SsaI has evolved to evade NAIP recognition in both mice and humans.

**The THP-1 Monocytic Cell Line Undergoes Inflammasome Activation in Response to T3SS Inner Rod and Flagellin Proteins.** Previous studies using the U937 and THP-1 monocytic cell lines found that anthrax toxin-mediated delivery of flagellin or inner rod proteins did not induce inflammasome activation (7, 9). Transfection of purified PrgJ protein into these cells also failed to activate the inflammasome (8). In contrast, recent findings and the data presented here show that hMDMs mount robust inflammasome responses to flagellin (32) and the T3SS inner rod. A previously proposed explanation for these discrepant findings is that distinct NAIP splicing isoforms possess differing ligand specificities and that primary human macrophages and immortalized cells express differing levels of particular isoforms (32). Alternatively, human NAIP may recognize all three bacterial ligands regardless of isoform type. As THP-1 cells express lower levels of NAIP and NLRC4 than primary macrophages (32), the method of ligand delivery or specific bacterial proteins previously used may not have been sufficient for inflammasome activation in this cell type. Previous studies utilized the C. violaceum inner rod protein CprJ (7, 9), which we found to be a poor inflammasome activator in hMDMs relative to other T3SS inner rod homologs (Fig. 5A and B). Another study used transfection-based delivery of PrgJ protein (8), which is likely not as efficient at delivering proteins into host cells as the anthrax toxin system.

Thus, we next tested whether THP-1 cells activate inflammasome responses to PrgJ delivered via *Listeria* or the anthrax toxin.
system. Although PrgJ-expressing _Listeria_ induced IL-1α and IL-1β secretion in THP-1 cells, PrgJ-expressing _Listeria_ failed to do so (Fig. S4), despite robustly activating hMDMs (Fig. 2). In contrast, THP-1 cells treated with PrgJTox robustly secreted IL-1α and IL-1β (Fig. 6A and B). Consistent with previous findings (7, 9), anthrax toxin-mediated delivery of full-length flagellin failed to activate THP-1 cells (Fig. S5). Intriguingly, anthrax toxin-mediated delivery of a truncated version of flagellin robustly triggered IL-1α and IL-1β secretion (Fig. 6A and B), likely because of more efficient delivery of truncated flagellin. These data show that THP-1 cells are capable of detecting the T3SS needle, inner rod, and flagellin but are less responsive than hMDMs, as the type of bacterial ligand and route of delivery influence the extent of inflammasome activation.

**A Single NAIP Isoform Mediates Inflammasome Responses to T3SS Needle, Inner Rod, and Flagellin Proteins.** Our data show that both THP-1 cells and hMDMs recognize T3SS needle, inner rod, and flagellin and that NAIP contributes to ligand detection. We next sought to understand how a single human NAIP gene could confer recognition of all three ligands in contrast to mice, which utilize distinct NAIPs to recognize each ligand. Interestingly, studies in which chimeric mouse NAIPs were generated to define the ligand specificity domain identified a chimeric mouse NAIP capable of recognizing multiple ligands (45), suggesting the possibility that human NAIP might function as a broad receptor. Human monocytic cell lines express lower levels than hMDMs of a particular full-length NAIP splicing isoform (termed NAIP*) that enables sensing of flagellin (32). We, therefore, sought to test whether a single NAIP isoform possesses specificity for a given bacterial ligand or is capable of detecting all three bacterial ligands.

We ectopically expressed the NAIP* isoform previously shown to recognize flagellin (32) along with other NLRC4 inflammasome components in HEK293 cells and then used the anthrax toxin system to deliver bacterial ligands into these cells. As expected, HEK293 cells expressing the NAIP* isoform robustly processed IL-1β in response to flagellin (Fig. 6C). Unexpectedly, however, delivery of PrgJ or the _Bordetella_ T3SS needle protein (YscF) also induced robust IL-1β processing (Fig. 6C). Critically, inflammasome activation by FlaA, PrgJ, and YscF required NAIP, as delivery of bacterial ligands into cells only expressing NLRC4, caspase-1, and IL-1β did not result in IL-1β processing. Inflammasome activation also required delivery of the bacterial ligands, as untreated cells or PA treatment alone did not process IL-1β. Altogether, these data indicate that a single human NAIP isoform is sufficient to mediate inflammasome responses to the T3SS needle, inner rod, and flagellin proteins.

**Discussion**

Our data show that, like murine cells, human macrophages sense multiple bacterial ligands from the T3SS and flagellar apparatus. In addition to the T3SS needle and flagellin, T3SS inner rod proteins from multiple bacterial species activate the human NAIP inflammasome. Furthermore, a single human NAIP isoform can mediate inflammasome responses to all three bacterial proteins in contrast to mouse NAIPs, which are highly selective for recognition of individual flagellin or T3SS proteins (6–11). Consistent with our findings, a recent study found that the _Pseudomonas aeruginosa_ T3SS inner rod also activates the human NAIP inflammasome (46). The region of murine NAIPs that confers ligand specificity has been mapped to an internal region composed of several nucleotide-binding domain (NBD)-associated α-helical domains (45). This region has evolved under positive selection in both rodents and primates (45), suggesting that this domain mediates ligand detection in human NAIP as well. How NAIP achieves broad recognition of multiple ligands and whether NAIP binds these ligands with similar or differing affinities or binding kinetics are unclear. The T3SS inner rod, needle, and flagellin proteins exhibit low sequence conservation but have some structural conservation, as the T3SS is thought to...
After 18 h, cells were treated with PA ELISA. Bar graphs display the mean ± SD of triplicate wells. Representative of three independent experiments. 

Fig. 6. A single NAIP isoform is sufficient for inflammasome responses to flagellin, the T3SS inner rod protein, and the T3SS needle protein. (A and B) THP-1 cells were primed with Pam3CSK4 and treated with PA alone, LFn-FlaAalone, LFn-PrgJ alone, PA+LFn-FlaAalone (FlaTox), or PA+LFn-PrgJ (PrgTox) for 5 h. IL-1α and IL-1β supernatant levels were measured by ELISA. Bar graphs display the mean ± 5D of triplicate wells. Representative of three independent experiments. **P < 0.001 by unpaired t test; ****P < 0.0001 by unpaired t test. (C) HEK293 cells were transfected with expression vectors encoding NLRC4, caspase-1, and IL-1β. Where indicated, cells were also transfected with vectors encoding NAIP* (+) or empty vector control (−). After 18 h, cells were treated with PA+LFn-PrgJ, PA+LFn-FlaA, PA+ LFn-YscF, or PA alone for 9 h. Immunoblot analysis was performed on cell lysates for mature and pro-IL-1β, NAIP*, NLRC4, caspase-1, and β-actin as a loading control. Representative of three independent experiments.

have evolved from the flagellar apparatus (47). Thus, human NAIP may recognize structural elements common to all three ligands. It will be of interest to determine whether NAIP detection of these three ligands is functionally redundant or distinct in the initiation of antimicrobial activities.

Our study raises intriguing questions about the evolution of the NAIP/NLRC4 inflammasome. It is likely that a single NAIP progenitor was present in the last common ancestor of primates and rodents (48). In mice, there has been an expansion of NAIP genes as a consequence of several gene duplication events (49); interestingly, the murine NAIPs are specialists, as they each recognize only one of three bacterial proteins derived from the evolutionarily related T3SS and flagellar apparatus. In contrast, the single human NAIP is a generalist, as it is capable of functionally detecting all three bacterial proteins. The promiscuity displayed by human NAIP may provide a selective advantage, as it may be more difficult for pathogens to simultaneously evade recognition of all three ligands by human NAIP.

Promiscuous ligand recognition may be a general strategy used by the innate immune system to diversify protein functionality as a means of promoting responses against different pathogenic stimuli. For example, the natural killer (NK) activating receptor NKG2D broadly recognizes several MHC class I-related proteins in contrast to other NK receptors, which typically recognize a single ligand. The ability of NKG2D to recognize a broad array of stress-inducible host ligands may provide an evolutionary advantage against viruses that use mechanisms to down-regulate NKG2D ligands as well as rapidly evolving cancers (50). Furthermore, the TLR adaptor TIRAP-promiscuously detects multiple ligands, which diversify subcellular signals of TLR signaling and thus, enable responses to both extracellular and endosomal pathogens (51). However, one possible tradeoff with a more promiscuous mode of sensing is that human NAIP may possess weaker affinities or altered binding kinetics for its bacterial ligands and hence, decreased signaling potency. In contrast, a given mouse NAIP may possess higher affinity or half-life in binding its particular ligand and thus, confer heightened immune responses. Indeed, compared with mouse macrophages, human macrophages do not seem to be as responsive to cytosolic flagellin, as they are more permissive for intracellular replication of flagellated bacteria (33). While the precise basis for this difference is unknown, one possibility is that human NAIP detects flagellin with lower affinity or altered binding kinetics than mouse NAIP5.

It will be of interest to examine how coevolution with Gram-negative bacteria shaped the NAIP genes in humans and other mammals and whether pathogens have evolved strategies for evading human NAIP. Functional NAIP copy number varies among human populations, and increased copy number has been postulated to confer a selective advantage in antibacterial defense (52). Studies in mice have shown that inappropriate activation of the NAIP/NLRC4 inflammasome can lead to lethal systemic inflammation resembling sepsis (29, 37). Moreover, gain-of-function mutations in human NLRC4 result in pathologic enterocolitis and autoinflammation (25–28). Perhaps gain-of-function mutations in human NAIP confer similar pathological outcomes.

Our results provide insight into human NAIP detection of bacterial proteins from the T3SS and flagellar apparatus. The data presented here provide an important basis for elucidating the mechanisms underlying human NAIP inflammasome responses to bacterial infection, which could prove crucial to understanding how the NAIP/NLRC4 inflammasome contributes to human health and disease.

Materials and Methods

Ethics Statement. All studies involving hMDMs were performed in compliance with the requirements of the US Department of Health and Human Services and the principles in the Declaration of Helsinki. Samples obtained from the University of Pennsylvania Human Immunology Core are considered to be a secondary use of deidentified human specimens and are exempt via Title 55 Part 46, Subpart A of 46.101 (b) of the Code of Federal Regulations. 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 (53). The Institutional Animal Care and Use Committee of the University of Pennsylvania approved all procedures (protocol 804928).

Bacterial Strains and Growth Conditions. Salmonella enterica serovar Typhimurium WT, ΔsipB (54), and ΔlfiCfliB (55) isogenic strains on the SL1344 background were used. Three hours before infection, Salmonella was diluted into Luria–Bertani broth containing 300 mM NaCl and grown for 3 h standing at 37 °C to induce SPI-1 expression (50). L. monocytogenes WT and isogenic strains on the 10403S background were cultured in brain heart infusion medium (34). Listeria strains encoding heterologous bacterial ligands (L. pneumophila FlaA, Salmonella Typhimurium PrgL, and Salmonella Typhimurium PrgJ) translationally fused to the truncated N terminus of ActA and under the control of the actA promoter were used (34). The pPL2 vector encoding PrgJ was introduced into Δply Listeria as previously described (34, 57). Listeria strains expressing Salmonella Typhimurium SsaI, B. thailandensis BsaK, S. flexneri MxiI, and C. violaceum Cps were constructed using codon-optimized gene fragments (IDT) cloned into the pPL2 vector and introduced into Listeria as previously described (34, 57).

Cellular Assays. Purified human monocytes from deidentified healthy human donors were obtained from the University of Pennsylvania Human Immunology Core. Monocytes were cultured in RPMI supplemented with 10% (vol/vol) heat-inactivated FBS, 2 mM l-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 50 ng/mL recombinant human M-CSF (Gemini Bio-Products) for 6 d to promote differentiation into hMDMs. One day before infection, adherent
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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 OD600 = 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 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 10% FBS, 100 μM 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 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-FlaA160–67 (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_033292.2) was a gift from Kate Fitzgerald, University of Massachusetts Medical School, Worcester, MA (plasmid 41522; 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: AAAGAGATCCGCCCATGAAATCTCAAAAGGACATAGCC and NLRC4 reverse: TTGTGCGGCGTAAAGCAGTTACTAGTTTAAACACCC. The digested NLRC4 PCR product was cloned into a BgII/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 replying 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 PCR system using the SsoFast EvaGreen Supermix with the LOW ROX kit (Bio-Rad). The following primers from PrimerBank (5′-3′) were used. The PrimerBank identifications are NAIP (119393877c3) and HPRT (164518913c1; all 5′-3′):

NAIP forward: CCCATAGACGATCACACCCAGA;
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-1\( \alpha \) (R&D Systems) and IL-1\( \beta \) (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-1\( \beta \) (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.


**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 Δhly *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.