Title: Human NAIP/NLRC4 and NLRP3 inflammasomes detect *Salmonella* type III secretion system activities to restrict intracellular bacterial replication

Short title: Human inflammasomes restrict *Salmonella* in macrophages

Authors: Nawar Naseer^{1¶}, Marisa Egan^{1¶}, Valeria M. Reyes Ruiz^{1¶#a}, Igor E. Brodsky², and Sunny Shin^{1*}

Author affiliations:

¹Department of Microbiology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104

^{#a}Current address: Department of Pathology, Microbiology, and Immunology, Vanderbilt University Medical Center, Nashville, TN 37232

²Department of Pathobiology, University of Pennsylvania School of Veterinary Medicine, Philadelphia, PA 19104

* Corresponding author

E-mail: sunshin@pennmedicine.upenn.edu

[¶]These authors contributed equally to the work.

Abstract

Salmonella enterica serovar Typhimurium is a Gram-negative pathogen that uses two distinct type III secretion systems (T3SSs), termed *Salmonella* pathogenicity island (SPI)-1 and SPI-2, to deliver virulence factors into the host cell. The SPI-1 T3SS enables *Salmonella* to invade host cells, while the SPI-2 T3SS facilitates *Salmonella*'s intracellular survival. In mice, a family of cytosolic immune sensors, including NAIP1, NAIP2, and NAIP5/6, recognizes the SPI-1 T3SS needle, inner rod, and flagellin proteins, respectively. Ligand recognition triggers assembly of the NAIP/NLRC4 inflammasome, which mediates caspase-1 activation, IL-1 family cytokine secretion, and pyroptosis of infected cells. In contrast to mice, humans encode a single NAIP that broadly recognizes all three ligands. The role of NAIP/NLRC4 or other inflammasomes during *Salmonella* infection of human macrophages is unclear. We find that although the NAIP/NLRC4 inflammasome is essential for detecting SPI-1 T3SS ligands in human macrophages, it is partially required for responses to infection, as *Salmonella* also activated the NLRP3 and CASP4/5 inflammasomes. Importantly, we demonstrate that combinatorial NAIP/NLRC4 and NLRP3 inflammasome activation restricts *Salmonella* replication in human macrophages. In contrast to SPI-1, the SPI-2 T3SS inner rod is not sensed by human or murine NAIPs, which is thought to allow *Salmonella* to evade host recognition and replicate intracellularly. Intriguingly, we find that human NAIP detects the SPI-2 T3SS needle protein. Critically, in the absence of both flagellin and the SPI-1 T3SS, the NAIP/NLRC4 inflammasome still restricted intracellular *Salmonella* replication. These findings reveal that recognition of *Salmonella* SPI-1 and SPI-2 T3SSs

- 43 and engagement of both the NAIP/NLRC4 and NLRP3 inflammasomes control
- 44 *Salmonella* infection in human macrophages.

Author summary

Salmonella enterica serovar Typhimurium is a gastrointestinal bacterial pathogen that causes diarrheal disease and is a major cause of morbidity and mortality worldwide. *Salmonella* uses molecular syringe-like machines called type III secretion systems (T3SSs) to inject virulence factors into host cells. These T3SSs enable *Salmonella* to infect and survive within host cells such as macrophages. However, host cells contain a family of cytosolic immune receptors, termed NAIPs, that recognize T3SS and flagellin components. Upon detecting these components, NAIPs recruit the adaptor protein NLRC4 to form signaling complexes called inflammasomes. Inflammasomes activate host proteases called caspases that mount robust immune responses against the invading pathogen. While mice encode multiple NAIPs that have been extensively studied, much remains unknown about how the single human NAIP mediates inflammasome responses to *Salmonella* in macrophages. Our study reveals that while NAIP is necessary to detect individual T3SS ligands in human macrophages, it is only partially required for inflammasome responses to *Salmonella* infection. We found that the NLRP3 and CASP4/5 inflammasomes are also activated, and the combination of NAIP- and NLRP3-mediated recognition limits intracellular *Salmonella* replication in human macrophages. Our results demonstrate that human macrophages employ multiple inflammasomes to mount robust host defense against *Salmonella* infection.

Introduction

Salmonella enterica serovar Typhimurium (referred to hereafter as *Salmonella*) is a Gram-negative bacterial pathogen that causes self-limiting gastroenteritis in immune-competent humans. Transmission of *Salmonella* typically occurs upon ingestion of contaminated food or water. Once inside the host, *Salmonella* uses specialized nanomachines known as type III secretion systems (T3SSs) to inject effectors into the host cell cytosol [1]. Subsequently, these effectors remodel host cellular processes to facilitate bacterial colonization. Thus, *Salmonella*'s T3SSs enable the enteric pathogen to successfully colonize the intestinal tract and infect a variety of cell types, including intestinal epithelial cells (IECs) and macrophages [1]. Specifically, *Salmonella* uses its first T3SS, located on *Salmonella* Pathogenicity Island 1 (SPI-1), to invade host cells, and its second T3SS, located on a second pathogenicity island, SPI-2, to persist and replicate within host cells [2–8]. Numerous other Gram-negative bacterial pathogens also use these evolutionarily conserved T3SSs to colonize the host [9]. While T3SSs are required for these bacterial pathogens to cause disease, they also translocate structural components of the T3SS or the flagellar apparatus into the cytosol, thus enabling the host to detect the invading pathogen [10]. Unlike effectors, which display significant diversity across bacterial species, structural components of the T3SS or the flagellar apparatus retain significant structural homology across Gram-negative bacteria [9,11]. Thus, these ligands serve as ideal targets of host immune sensors.

The mammalian innate immune system is armed with pattern recognition receptors (PRRs) that detect pathogens by recognizing pathogen-associated molecular patterns (PAMPs) [12,13]. A subfamily of cytosolic PRRs, known as NAIPs (the NLR

[nucleotide-binding domain, leucine-rich repeat-containing] family, apoptosis inhibitory proteins), recognize the structurally related SPI-1 T3SS needle protein, SPI-1 T3SS inner rod protein, and flagellin, which are translocated into the host cell cytosol by the SPI-1 T3SS [10,14,15]. Mice have multiple NAIPs, each specific to a particular ligand: NAIP1 recognizes the T3SS needle protein, NAIP2 recognizes the T3SS inner rod protein, and NAIP5 and NAIP6 both recognize flagellin [14–19]. Upon sensing a ligand, NAIPs recruit the adaptor protein NLRC4 (nucleotide-binding domain, leucine-rich repeat-containing family, CARD domain-containing protein 4) to form multimeric signaling complexes called inflammasomes [20–22]. The NAIP/NLRC4 inflammasome then recruits and activates the cysteine protease caspase-1 [23]. Active caspase-1 cleaves downstream substrates, including pro-IL-1 and pro-IL-18, as well as the pore-forming protein gasdermin-D (GSDMD) [24–26]. Cleaved GSDMD creates pores in the host plasma membrane, leading to the release of proinflammatory cytokines and an inflammatory form of cell death known as pyroptosis, which effectively eliminates the infected cell. The NAIP/NLRC4 inflammasome is critical for the control of *Salmonella* infection in mice [27,28]. However, whether the NAIP/NLRC4 inflammasome recognizes or controls *Salmonella* infection in humans has not been thoroughly investigated.

While mice express several different NAIPs that each respond to a particular ligand, humans only express one functional NAIP [29,30]. In human macrophages, this single NAIP is sufficient to respond to the cytosolic delivery of bacterial flagellin as well as the SPI-1 T3SS inner rod (PrgJ) and needle (PrgI) proteins [31,32]. Interestingly, the SPI-2 T3SS inner rod protein (SsaI) fails to induce inflammasome activation in both murine and human macrophages [11,32], suggesting that the *Salmonella* SPI-2 T3SS

evades NAIP detection to enable *Salmonella* replication within macrophages. However, whether the SPI-2 T3SS needle protein (SsaG) is recognized by NAIP or whether NAIP contributes to the restriction of *Salmonella* replication within macrophages is unknown.

In this study, we found that while human macrophages require NAIP and NLRC4 for inflammasome responses to T3SS ligands, NAIP and NLRC4 are only partially required for the inflammasome response during *Salmonella* infection. Rather, we found that *Salmonella* infection of human macrophages also activates both the CASP4/5 inflammasome, which senses cytosolic LPS [33], and the NLRP3 inflammasome. Importantly, both the NAIP/NLRC4 and NLRP3 (NLR pyrin domain-containing protein 3) inflammasomes played a functional role in restricting *Salmonella*'s intracellular replication, indicating that they contribute to host defense in a cell-intrinsic manner, as well as via release of inflammatory mediators. Finally, we found that the NAIP/NLRC4 inflammasome recognizes the SPI-2 T3SS needle protein SsaG, and that SPI-1 T3SS and flagellin-independent, NAIP/NLRC4-dependent recognition of *Salmonella* mediates restriction of bacterial replication within human macrophages. Our findings highlight the multifaceted inflammasome response to *Salmonella* infection in human macrophages, and yield important insight into how human macrophages use inflammasomes to sense and respond to intracellular bacterial pathogens.

Results

NAIP and NLRC4 are necessary for inflammasome responses to T3SS ligands in human macrophages

In murine macrophages, multiple NAIPs are required for inflammasome responses to the *Salmonella* SPI-1 T3SS inner rod protein (PrgJ), the SPI-1 T3SS needle protein (PrgI), and flagellin [14–19]. In addition, the murine NAIPs and NLRC4 contribute to the inflammasome response during *in vivo* *Salmonella* infection [11,19]. In human macrophages, PrgJ, PrgI, and flagellin all activate the inflammasome, while the *Salmonella* SPI-2 inner rod protein (SsaI) does not [31,32]. Using siRNA-mediated silencing of *NAIP* in human macrophages, we have previously shown that human NAIP is important for maximal inflammasome responses to PrgJ and flagellin [32]. However, siRNA-mediated knockdown of *NAIP* did not completely abrogate inflammasome activation, either due to incomplete knockdown, or the potential contribution of other inflammasomes. Therefore, it remained unclear whether human NAIP or NLRC4 is absolutely required for inflammasome responses to these bacterial ligands or whether additional host sensors also mediate sensing of these ligands.

To test the requirement of the NAIP/NLRC4 inflammasome in human macrophages, we used the Clustered Regularly Interspersed Palindromic Repeat (CRISPR) system, in conjunction with the RNA-guided exonuclease Cas9, to disrupt the *NAIP* and *NLRC4* genes in the human monocytic cell line, THP-1 (Fig. S1A, S2A). We selected one independent single cell clone of *NAIP*^{-/-} THP-1s (*NAIP*^{-/-} Clone 12) that exhibited reduced *NAIP* mRNA expression by qRT-PCR compared to WT THP-1s (Fig.

S1C). Sequence validation confirmed that this clone contained a deletion of 1 or 2 nucleotides in both *NAIP* alleles, resulting in premature stop codons (Fig. S1B). We selected two independent single cell clones of *NLRC4*^{-/-} THP-1s (*NLRC4*^{-/-} Clone 4 and Clone 7), both of which showed complete loss of NLRC4 protein expression compared to WT THP-1s (Fig. S2D). Both clones were sequence-validated and both alleles of each clone contained mutations that resulted in premature stop codons (Fig. S2B, S2C). These sequence-validated *NAIP*^{-/-} and *NLRC4*^{-/-} THP-1 clones were used throughout this study.

To test if NAIP and NLRC4 are necessary for sensing and responding to bacterial T3SS ligands, we compared inflammasome responses in wild type (WT), *NAIP*^{-/-}, and *NLRC4*^{-/-} THP-1 macrophages to T3SS ligands delivered directly into the host cell cytosol. We used the *Bacillus anthracis* toxin system to deliver these bacterial ligands into the cytosol of THP-1s [34]. This system contains two subunits: a protective antigen (PA) that creates a pore in the host endosomal membrane and a truncated lethal factor (LFn) that is delivered through the PA pore into the cytosol. Our T3SS ligands of interest are fused to the N-terminal domain of the *B. anthracis* LFn. When the LFn is added to eukaryotic cells in conjunction with PA (collectively referred to as Tox), the bacterial ligand is delivered directly into the host cell cytosol. Using this system, we delivered a truncated version of *Legionella* flagellin (FlaTox), the *Salmonella* SPI-1 T3SS inner rod protein (PrgJTox), and the *Burkholderia* T3SS needle protein (YscFTox) into THP-1s. We then measured the release of the inflammasome-dependent IL-1 family cytokines IL-1α, IL-1β, and IL-18 and cell death as markers of inflammasome activation. Cells left untreated (Mock) or treated with the PA alone or the LFn fused to

the bacterial ligand alone released negligible levels of IL-1 β , IL-18, and IL-1 α and exhibited minimal cell death (Fig. 1A, 1C, S3A-C, S4A-C). In agreement with previous findings [32], WT THP-1s treated with both the PA and LFn subunits exhibited robust inflammasome activation, and released substantial levels of IL-1 β , IL-18, and IL-1 α and exhibited considerable cytotoxicity (Fig. 1A, 1C, S3A-C, S4A-C), indicating that robust inflammasome activation requires cytosolic delivery of the ligands. In contrast, both *NAIP*^{-/-} THP-1s and *NLRC4*^{-/-} THP-1s released negligible levels of inflammasome-dependent cytokines and did not undergo cell death when treated with FlaTox, PrgJTox, or YscFTox (Fig 1A, 1C, S3A-C, S4A-C). Importantly, the *NAIP*^{-/-} and *NLRC4*^{-/-} THP-1s released IL-1 β at levels comparable to those released by WT THP-1s in response to the NLRP3 stimulus LPS + nigericin (Fig. 1B, 1D), indicating that CRISPR/Cas9 editing was specific to the NAIP/NLRC4 inflammasome pathway [35]. In addition, release of the inflammasome-independent cytokine TNF- α was unaffected in *NAIP*^{-/-} or *NLRC4*^{-/-} THP-1s (Fig. S3D, S4D). Consistent with our prior results [32] and in agreement with recent studies [36], these results collectively demonstrate that NAIP and NLRC4 are required for inflammasome activation in response to the T3SS inner rod, T3SS needle, and flagellin proteins in human macrophages.

Fig 1. NAIP and NLRC4 are necessary for inflammasome responses to T3SS ligands in human macrophages. WT, *NAIP*^{-/-} clone, or two independent clones of *NLRC4*^{-/-} THP-1 monocyte-derived macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were then treated with PBS (Mock), PA alone, LFnFlaA³¹⁰⁻⁴⁷⁵ alone, LFnPrgJ alone, LFnYscF alone, PA+LFnFlaA³¹⁰⁻⁴⁷⁵ (FlaTox),

PA+LFnPrgJ (PrgJTox), or PA+LFnYscF (YscFTox) for 6 hours (A, C). As a control, cells were primed with 500 ng/mL LPS for 4 hours and treated with 10 μ M nigericin for 6 hours (B, D). Release of IL-1 β into the supernatant was measured by ELISA. ns – not significant, * p < 0.05, ** p < 0.01, **** p < 0.0001 by Šídák's multiple comparisons test (A), or by unpaired t-test (B), or by Dunnett's multiple comparisons test (C, D). Data shown are representative of at least three independent experiments.

NAIP and NLRC4 are partially required for inflammasome activation during *Salmonella* infection of human macrophages

Human macrophages undergo SPI-1 T3SS-dependent inflammasome activation during *Salmonella* infection [32]. To test whether this inflammasome activation requires NAIP/NLRC4, we infected WT, *NAIP*^{-/-}, or *NLRC4*^{-/-} THP-1 macrophages with WT *Salmonella* (WT Stm) or *Salmonella* lacking its SPI-1 T3SS ($\Delta sipB$ Stm) and assayed for subsequent inflammasome activation (Fig. 2, S5). WT THP-1s infected with WT Stm released high levels of IL-1 β , IL-18, and IL-1 α and underwent cell death (Fig. 2, S5A-D). This response was dependent on SPI-1 T3SS translocation into host cells, as cells infected with $\Delta sipB$ Stm, which lack a component of the translocon, failed to undergo robust inflammasome activation (Fig. 2, S5A-D). In *NAIP*^{-/-} or *NLRC4*^{-/-} THP-1 macrophages infected with WT Stm, we observed a significant decrease but not complete abrogation of secreted IL-1 β and IL-18 levels (Fig. 2), whereas levels of IL-1 α and cell death were largely unaffected (Fig. S5A–D). WT and *NAIP*^{-/-} or *NLRC4*^{-/-} THP-1s released similar levels of the inflammasome-independent cytokine TNF- α (Fig. S5E, S5F). Overall, these data indicate that NAIP and NLRC4 are partially required for

inflammasome responses to *Salmonella* infection in human macrophages, in contrast to what we observe with individual T3SS ligand delivery (Fig. 1, S3, S4), where NAIP/NLRC4 is absolutely required for inflammasome activation. Thus, our data indicate that in addition to the NAIP/NLRC4 inflammasome, *Salmonella* also induces a NAIP/NLRC4-independent inflammasome response, in agreement with a recent study [36].

Fig 2. NAIP and NLRC4 are partially required for inflammasome activation during *Salmonella* infection in human macrophages. WT, *NAIP*^{-/-} clone, or two independent clones of *NLRC4*^{-/-} THP-1 monocyte-derived macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were then infected with PBS (Mock), WT *S. Typhimurium*, or Δ *sipB* *S. Typhimurium* for 6 hours. Release of IL-1 β and IL-18 into the supernatant were measured by ELISA. ns – not significant, *** $p < 0.001$, **** $p < 0.0001$ by Šídák's multiple comparisons test (A, C) or Dunnett's multiple comparisons test (B, D). Data shown are representative of at least three independent experiments.

Salmonella induces NAIP/NLRC4- and NLRP3-dependent inflammasome activation in human macrophages

In murine macrophages, *Salmonella* infection activates both the NAIP/NLRC4 and NLRP3 inflammasomes [37]. The NAIP/NLRC4 inflammasome is important for early responses to *Salmonella* in the setting of SPI-1 activation, while the NLRP3 inflammasome is important at later timepoints following bacterial replication [38]. In human THP-1s, *Salmonella* infection triggers recruitment of both NLRC4 and NLRP3 to

the same macromolecular complex [38]. The NLRP3 inflammasome can be activated by diverse stimuli during bacterial infection, such as potassium efflux [39]. To determine if the NAIP/NLRC4-independent inflammasome response we observed in our *Salmonella*-infected human macrophages is NLRP3-dependent, we infected WT, *NAIP*^{-/-}, or *NLRC4*^{-/-} THP-1s with *Salmonella* in the presence of MCC950, a potent chemical inhibitor of the NLRP3 inflammasome [40], or the vehicle control DMSO. We subsequently assayed for inflammasome activation by measuring IL-1α, IL-1β, and IL-18 secretion (Fig. 3, S6). WT THP-1s treated with DMSO control released substantial amounts of IL-1α, IL-1β, and IL-18 when infected with WT Stm. In contrast, infected WT THP-1s treated with MCC950 secreted decreased levels of IL-1α, IL-1β, and IL-18, which are comparable to levels observed in WT Stm-infected *NAIP*^{-/-} or *NLRC4*^{-/-} THP-1s. (Fig. 3, S6A-D). Interestingly, WT Stm-infected *NAIP*^{-/-} or *NLRC4*^{-/-} THP-1s treated with MCC950 largely had significantly decreased IL-1α, IL-1β, and IL-18 secretion compared to infected *NAIP*^{-/-} or *NLRC4*^{-/-} THP-1s treated with DMSO or infected WT THP-1s treated with MCC950 (Fig. 3, S6A-D). Furthermore, *NAIP*^{-/-} or *NLRC4*^{-/-} THP-1s treated with MCC950 secreted negligible levels of IL-1α, IL-1β, and IL-18, similar to those observed during *ΔsipB* Stm infection (Fig. 3, S6A-D). WT, *NAIP*^{-/-}, and *NLRC4*^{-/-} THP-1s demonstrated robust IL-1α, IL-1β, and IL-18 secretion in response to LPS + nigericin that was significantly reduced by MCC950 treatment, indicating that this inhibitor effectively blocked NLRP3 inflammasome activation, as expected (Fig. 3, S6A-D). Release of the inflammasome-independent cytokine TNF-α was similar across the various THP-1 genotypes and treatments following infection (Fig. S6E, S6F). Altogether,

these data indicate that *Salmonella* infection induces both NAIP/NLRC4- and NLRP3-dependent inflammasome activation in human macrophages.

Fig 3. *Salmonella* induces NAIP/NLRC4- and NLRP3-dependent inflammasome activation in human macrophages. WT, *NAIP*^{-/-}, or *NLRC4*^{-/-} THP-1 monocyte-derived macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. One hour prior to infection, cells were treated with 1 μ M MCC950, a chemical inhibitor of the NLRP3 inflammasome or DMSO as a control. Cells were then infected with PBS (Mock), WT *S. Typhimurium*, or Δ *sipB* *S. Typhimurium* for 6 hours. As a control, cells were primed with 500 ng/mL LPS for 4 hours and treated with 10 μ M nigericin for 6 hours. Release of IL-1 β into the supernatant was measured by ELISA. ns – not significant, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 by Tukey's multiple comparisons test (A, C) or by Šídák's multiple comparisons test (B, D). Data shown are representative of at least three independent experiments.

Salmonella induces NAIP/NLRC4- and CASP4/5-dependent inflammasome activation in human macrophages

In mice, in addition to the NAIP/NLRC4 and NLRP3 inflammasomes, *Salmonella* infection can also activate the caspase-11 inflammasome [41]. Caspase-11 detects cytosolic LPS and forms the noncanonical inflammasome, which secondarily activates the NLRP3 inflammasome [33,42]. Caspases-4 and 5 are human orthologs of murine caspase-11 [33], and they can also sense cytosolic LPS to form the noncanonical inflammasome in human cells. We have previously observed caspase-4-dependent

288 inflammasome activation in response to *Salmonella* infection in primary human
 289 macrophages [43], and caspases-4 and 5 also contribute to inflammasome responses
 290 to *Salmonella* infection in THP-1s and human intestinal epithelial cells [44,45]. To test
 291 the relative contribution of both caspases-4 and 5 to NAIP-independent inflammasome
 292 responses during *Salmonella* infection of THP-1 macrophages, we treated WT or *NAIP*^{-/-}
 293 THP-1s with siRNAs targeting *CASP4*, *CASP5*, or both, achieving ~70-90% knockdown
 294 efficiency at the mRNA level (Fig. S7), and subsequently assayed for IL-1 β secretion in
 295 response to WT Stm. WT THP-1s treated with either *CASP4* or *CASP5* siRNAs
 296 exhibited significantly decreased IL-1 β secretion following WT Stm infection relative to
 297 WT THP-1s treated with control siRNA (Fig. 4A & B), in agreement with our previous
 298 observations in primary human macrophages [43]. *NAIP*^{-/-} THP-1s treated with *CASP5*
 299 siRNA showed a slight but significant decrease in IL-1 β secretion following *CASP5*
 300 siRNA treatment, but not *CASP4* siRNA treatment, compared to control siRNA-treated
 301 cells following WT Stm infection (Fig. 4A & B). WT and *NAIP*^{-/-} THP-1s treated with both
 302 *CASP4* and *CASP5* siRNAs displayed significantly reduced IL-1 β secretion relative to
 303 THP-1s treated with a scrambled control siRNA, although inflammasome activation was
 304 not completely abrogated when both *CASP4* and *CASP5* were knocked down in *NAIP*^{-/-}
 305 THP-1 cells (Fig. 4C). As a control, we assessed inflammasome activation in response
 306 to transfected *E. coli* LPS, which activates the caspase-4/5 inflammasome. Both WT
 307 and *NAIP*^{-/-} cells transfected with LPS displayed significantly decreased IL-1 β secretion
 308 when *CASP4* was silenced, either alone or in conjunction with *CASP5* (Fig. 4A & C),
 309 whereas knockdown of *CASP5* alone did not significantly affect IL-1 β secretion, as

expected [45] (Fig. 4B). Taken together, these data suggest that the caspase-4/5 inflammasome is involved in the NAIP-independent response to *Salmonella*.

Fig 4. *Salmonella* induces NAIP- and CASP4/5-dependent inflammasome

activation in human macrophages. WT or *NAIP*^{-/-} THP-1 monocyte-derived macrophages were treated with siRNA targeting a control scrambled siRNA, siRNA targeting *CASP4* or *CASP5*, or siRNA targeting both *CASP4* and *CASP5* for 48 hours. Cells were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were then infected with PBS (Mock) or WT *S. Typhimurium* for 6 hours. Release of IL-1 β into the supernatant were measured by ELISA. As a control, cells were transfected with LPS. ns – not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by Tukey's multiple comparisons test. Data shown are representative of at least three independent experiments.

The NAIP/NLRC4 and NLRP3 inflammasomes restrict *Salmonella* replication within human macrophages

One of the mechanisms by which inflammasome activation leads to control of bacterial infection is by restricting intracellular bacterial replication. In mice, the NAIP/NLRC4 inflammasome is important for controlling *Salmonella* replication in the intestine [28], whereas the NLRP3 inflammasome is dispensable for control of *Salmonella* infection *in vivo* [46,47]. Caspases-1 and 11 restrict cytosolic *Salmonella* replication within murine macrophages [48]. Whether inflammasome activation restricts WT *Salmonella* replication in human macrophages is unknown. To test the hypothesis

that inflammasome activation restricts *Salmonella* replication within human macrophages, we infected WT or *NAIP*^{-/-} THP-1 macrophages with WT Stm in the presence or absence of the NLRP3 inhibitor MCC950 and determined the bacterial colony forming units (CFU) at various timepoints post-infection to assay bacterial replication. At 2 hours post-infection, we did not observe any differences in bacterial uptake between the different conditions (Fig. S8A). At 6 or 24 hours post-infection, the bacterial burden was the lowest in WT THP-1s, whereas *NAIP*^{-/-} THP-1s harbored significantly higher bacterial burdens (Fig. 5A, S8B). WT THP-1s treated with MCC950 also contained a significantly higher number of bacterial CFUs, comparable to those in *NAIP*^{-/-} THP-1s (Fig. 5A, S8B). *NAIP*^{-/-} THP-1s treated with MCC950 had the highest bacterial burdens, which were significantly higher than the bacterial burdens in DMSO control-treated *NAIP*^{-/-} THP-1s or WT THP-1s treated with MCC950 (Fig. 5A). We then examined the fold-change in bacterial replication at 6 and 24 hours relative to 2 hours post-infection. The fold-change in bacterial replication was restricted the most effectively in WT THP-1s, moderately restricted in *NAIP*^{-/-} THP-1s or WT THP-1s treated with MCC950, and the least restricted in *NAIP*^{-/-} THP-1s treated with MCC950 (Fig. 5B). Collectively, these data suggest that both the NAIP/NLRC4 and NLRP3 inflammasomes restrict intracellular *Salmonella* replication within human macrophages at both early (6 hours post-infection) and late (24 hours post-infection) timepoints.

Fig 5. The NAIP and NLRP3 inflammasomes restrict *Salmonella* replication within human macrophages. WT or *NAIP*^{-/-} THP-1 monocyte-derived macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. One hour prior to infection, cells were

treated with 1 μ M MCC950, a chemical inhibitor of the NLRP3 inflammasome or DMSO as a control. Cells were then infected with PBS (Mock) or WT *S. Typhimurium*. Cells were lysed at the indicated time points and bacteria were plated to calculate CFU. (A) CFU/well of bacteria at 6 hpi (B) Fold change in CFU/well of bacteria at indicated time point, relative to 2 hpi CFU/well. ns – not significant, *** $p < 0.001$, **** $p < 0.0001$ by Dunnett's multiple comparisons test (A) or Tukey's multiple comparisons test (B). Data shown are representative of at least three independent experiments.

Salmonella SPI-2 needle protein SsaG activates the NAIP/NLRC4 inflammasome in human macrophages

The *Salmonella* flagellin, SPI-1 T3SS inner rod (PrgJ), and needle (PrgI) proteins all activate NAIP in primary human macrophages, whereas the *Salmonella* SPI-2 T3SS inner rod protein (SsaI) is not sensed by NAIP [31,32]. Similarly in mice, SsaI is not sensed by NAIP2 [11]. These findings have led to models proposing that the SPI-2 T3SS evades inflammasome detection to allow *Salmonella* to replicate or persist in both murine and human cells [11,32]. However, our data indicate that the NAIP/NLRC4 inflammasome restricts *Salmonella* replication within macrophages even at late timepoints, when the SPI-1 T3SS and flagellin are thought to be downregulated [49–51]. As *Salmonella* utilizes the SPI-2 T3SS to replicate within macrophages [52], we asked whether the human NAIP/NLRC4 inflammasome detects the SPI-2 T3SS needle SsaG. To address this question, we delivered bacterial ligands into the cytosol of primary human monocyte-derived macrophages (hMDMs) derived from anonymous healthy human donors using the Gram-positive bacterium *Listeria monocytogenes*, which, upon

infection, escapes from its vacuole into the cytosol where it expresses the protein ActA on its surface. Fusing bacterial ligands of interest to the N-terminus of truncated ActA allows these ligands to be delivered into the host cytosol, where they trigger NAIP/NLRC4 inflammasome activation [32,53]. We infected hMDMs with WT *Listeria* (Lm) or *Listeria* expressing PrgJ, SsaI, or SsaG and assayed for inflammasome activation (Fig. 6A, S9). hMDMs infected with *Listeria* expressing the SPI-1 T3SS inner rod protein PrgJ induced robust inflammasome activation, indicated by significantly increased IL-18 secretion as well as robust IL-1 α and IL-1 β secretion compared to mock infection or WT Lm infection alone (Fig. 6A, S9), in agreement with our previous findings [32]. In contrast, and as we previously observed [32], *Listeria* expressing the SPI-2 inner rod protein SsaI failed to induce IL-1 β , IL-18, and IL-1 α secretion or cell death in hMDMs (Fig. 6A, S9). Intriguingly, we observed that *Listeria* expressing the SPI-2 needle protein SsaG induced significantly increased IL-18 and robust IL-1 α and IL-1 β secretion compared to mock infection or WT Lm infection alone (Fig. 6A, S9).

To test whether NAIP or NLRC4 are required for inflammasome responses to SsaG, we infected WT, *NAIP*^{-/-}, and *NLRC4*^{-/-} THP-1s with WT *Listeria* (Lm) or *Listeria* expressing PrgI or SsaG and assayed for subsequent inflammasome activation by measuring levels of IL-1 β , IL-18, and IL-1 α secretion and cell death (Fig. 6B, S10). Infection of WT THP-1s with *Listeria* expressing PrgI or SsaG led to robust release of IL-1 cytokines and cytotoxicity. In contrast, *NAIP*^{-/-} and *NLRC4*^{-/-} THP-1s infected with *Listeria* expressing PrgI or SsaG released significantly reduced levels of IL-1 cytokines and cell death relative to WT THP-1s that were comparable to the background levels secreted by THP-1s infected with WT Lm (Fig. 6B, S10). Altogether, these data

demonstrate that the SPI-2 needle protein activates the human NAIP/NLRC4 inflammasome, providing evidence that human NAIP can sense and respond to the *Salmonella* SPI-2 T3SS.

Fig 6. *Salmonella* SPI-2 needle protein SsaG activates the NAIP/NLRC4

inflammasome in human macrophages. (A) Primary hMDMs from four healthy human donors were infected with PBS (Mock), WT *Listeria* (WT Lm), *Listeria* expressing PrgJ (Lm + PrgJ), Ssal (Lm + Ssal), or SsaG (Lm + SsaG) for 16 hours at MOI=5. Release of IL-18 into the supernatant was measured by ELISA. Each dot represents the mean of individual donors derived from triplicate wells. The grey bar represents the mean of all donors. (B) WT or *NAIP*^{-/-}, *NLRC4*^{-/-} THP-1 monocyte-derived macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were treated with PBS (Mock), WT *Listeria* (WT Lm), *Listeria* expressing PrgI (Lm + PrgI), or SsaG (Lm + SsaG) for 6 hours at MOI=20. Release of IL-1β into the supernatant was measured by ELISA. ns – not significant, ****p* < 0.001, *****p* < 0.0001 paired t-test (A) or by Tukey's multiple comparisons test (B). Data shown are representative of at least three independent experiments.

NAIP/NLRC4 inflammasome recognition of the SPI-2 T3SS restricts *Salmonella* replication in human macrophages

To determine if NAIP/NLRC4-mediated recognition of the SPI-2 T3SS needle restricts *Salmonella* intracellular replication, we generated a *Salmonella* mutant strain (Δ *prgIfljCfljB*) lacking flagellin and the SPI-1 T3SS needle protein, PrgI. This strain is

therefore unable to assemble a functional SPI-1 T3SS, but still expresses a functional SPI-2 T3SS. We infected WT or *NAIP*^{-/-} THP-1 macrophages with *ΔprgIfljCfljB* and determined the CFUs at various timepoints to assay bacterial replication (Fig. 7, S11). Bacterial replication of *ΔprgIfljCfljB* over a 24-hour post-infection time course was restricted the most effectively in WT THP-1s and was significantly less restricted in *NAIP*^{-/-} THP-1s (Fig. 7, S11). Collectively, our data suggest that there is SPI-1 T3SS/flagellin-independent, NAIP/NLRC4 inflammasome-dependent control of *Salmonella* replication in human macrophages, and that NAIP/NLRC4 recognition of the SPI-2 T3SS needle SsaG mediates such restriction of *Salmonella* replication in human macrophages.

Fig 7. NAIP/NLRC4 inflammasome recognition of the SPI-2 T3SS restricts

Salmonella* replication in human macrophages.** WT or *NAIP*^{-/-} THP-1 monocyte-derived macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were then infected with PBS (Mock) or *ΔprgIfljCfljB* *S. Typhimurium*. Cells were lysed at the indicated time points and bacteria were plated to calculate CFU. Fold change in CFU/well of bacteria at indicated time point, relative to 2 hpi CFU/well. ns – not significant, *p* < 0.001, *****p* < 0.0001 by Tukey's multiple comparisons test.

Discussion

Our data show that human macrophages engage multiple inflammasome pathways to sense and respond to *Salmonella* infection. Using *NAIP^{-/-}* and *NLRC4^{-/-}* THP-1s (Fig. S1, S2), we found inflammasome activation in response to individual SPI-1 T3SS ligands to be entirely dependent on the NAIP/NLRC4 inflammasome in human macrophages (Fig. 1, S3, S4). In contrast, *Salmonella* infection induced activation of inflammasome responses that depended partially on NAIP/NLRC4, NLRP3, and CASP4/5 (Fig. 2-4, S5-7). Our findings are in agreement with a recent study demonstrating that both NLRC4 and NLRP3 are required for inflammasome responses to *Salmonella* in human macrophages [36]. Importantly, our data also reveal that both the NAIP/NLRC4 and NLRP3 inflammasomes contribute to restriction of *Salmonella* replication in human macrophages (Fig. 5, S8). Furthermore, contrary to the prevailing model that the SPI-2 T3SS evades NAIP detection, we show that the NAIP/NLRC4 inflammasome can recognize the *Salmonella* SPI-2 T3SS needle SsaG (Fig. 6, S9, S10), and that NAIP/NLRC4-dependent recognition of the SPI-2 T3SS restricts bacterial replication within human macrophages (Fig. 6, S11).

Many Gram-negative bacteria use evolutionarily conserved T3SSs to deliver virulence factors, or effectors, into host cells. We have previously shown that T3SS inner rod proteins from various Gram-negative bacteria activate the inflammasome in human macrophages [32]. In this study, we used T3SS inner rod, needle, or flagellin proteins from three different Gram-negative bacteria, *Salmonella*, *Burkholderia*, and *Legionella*, and observed that inflammasome activation in response to an isolated ligand is entirely dependent on NAIP/NLRC4 (Fig. 1, S3, S4, 6B). How the single human

NAIP senses and responds to these diverse bacterial structures remains an open question. The *Salmonella* SPI-1 T3SS inner rod (PrgJ), SPI-1 T3SS needle (PrgI), and flagellin proteins exhibit low total sequence conservation, but they all retain several conserved hydrophobic amino acid residues within their structurally homologous C-terminal helices [11,54]. In particular, both PrgJ and flagellin contain C-terminal leucine residues, specifically in their LLR motifs, which are critical for recognition by mNAIP2 and mNAIP5, respectively [55–59]. Instead of the LLR motif, PrgI has other hydrophobic amino acids, including valine and isoleucine residues, within its C-terminal helical domain. These terminal hydrophobic residues within PrgI are important for mediating inflammasome activation in human macrophages [17]. Interestingly, an alignment of the amino acid sequences of the SPI-2 T3SS needle protein (SsaG), PrgJ, and PrgI using Clustal Omega revealed that SsaG also contains conserved hydrophobic amino acid residues in its C-terminus (Fig S12A). Specifically, SsaG has C-terminal isoleucine residues like PrgI. To further compare these ligands at the structural level, we examined published three-dimensional structures of PrgJ and PrgI and used PHYRE2 Protein Fold Recognition Server to predict the structure of SsaG. Similar to PrgJ and PrgI, SsaG also displays an alpha-helical structure at its C-terminus (Fig S12B). Thus, SsaG displays secondary structural and sequence motifs similar to those retained by the other T3SS ligands recognized by human NAIP. Unlike these T3SS ligands, the *Salmonella* SPI-2 inner rod protein, SsaI, does not retain such conserved C-terminal residues. Perhaps this is why SsaI is not detected by human NAIP. Still, the specific ligand residues recognized by human NAIP remain unknown. Murine NAIPs use their nucleotide-binding domain (NBD)-associated domains to detect these conserved

residues of their respective cognate bacterial ligand [55–60]. It remains to be determined if the single human NAIP uses a similar mechanism to broadly detect its bacterial ligands.

Human NAIP is a generalist, as it detects multiple bacterial ligands, while the murine NAIPs are specialists, as they each recognize a particular ligand. The functional consequences of being a generalist NAIP is unclear. It is possible that recognizing a broad array of structures diminishes the affinity with which human NAIP binds its ligands. Alternatively, human NAIP may recognize its bacterial ligands with varying affinities. Furthermore, under physiological conditions, all bacterial ligands may not be delivered to the cytosol to the same extent or recognized with the same sensitivity. Varying levels of inflammasome activation with the different ligands may have distinct downstream consequences. It would be interesting to determine whether restriction of bacterial replication varies depending on which bacterial ligand is sensed.

Salmonella infection induces NAIP/NLRC4-, CASP4/5-, and NLRP3-dependent inflammasome activation in human macrophages (Fig. 2-4, S5-7). This suggests that there is redundancy in the inflammasome pathways when sensing and responding to *Salmonella* infection, such that loss of just one inflammasome does not result in severe loss of inflammasome activation in human macrophages. Given our observations with individual ligand delivery (Fig. 1, S3, S4), it is likely that the NAIP/NLRC4 inflammasome is sensing the *Salmonella* SPI-1 T3SS inner rod, SPI-1 and SPI-2 needle, and flagellin proteins during infection. However, it remains unknown how NLRP3 and CASP4/5 inflammasomes are activated in human macrophages during *Salmonella* infection. CASP4/5 detects intracellular LPS [33], but given that *Salmonella* is normally a vacuolar

pathogen in macrophages, it is unclear how CASP4/5 may be accessing LPS. In murine macrophages, a small percentage of *Salmonella*-containing vacuoles rupture, allowing bacteria to escape into the host cell cytosol [61]. In human intestinal epithelial cells, a subpopulation of *Salmonella* that escape the vacuole and replicates in the cytosol activates the CASP4/5 inflammasome at late timepoints of infection [44]. Moreover, other host immune factors can potentiate inflammasome signaling by promoting the release of PAMPs, such as LPS, into the host cell cytosol. For example, a family of host immune factors called guanylate binding proteins (GBPs) can localize to pathogen-containing vacuoles [62]. Murine GBPs promote rupture of the *Salmonella*-containing vacuole (SCV) [61]. Human GBP-1 can localize to the SCV in macrophages [63], and in human epithelial cells, GBP1 binds to bacterial LPS on the surface of cytosolic *Salmonella* and promotes the recruitment and activation of caspase-4 [64,65]. Another mechanism by which LPS can access the cytosol is through bacterial outer membrane vesicles (OMVs), and this mechanism has been shown to activate the caspase-11 inflammasome in murine models [66,67]. Future studies will explore if the CASP4/5-dependent inflammasome activation we have observed in human macrophages is facilitated by *Salmonella* escape into the host cell cytosol, GBP1 activity, OMVs, or other mechanisms.

The NLRP3 inflammasome can be activated by a variety of different stimuli, including potassium efflux. It can also be activated downstream of the CASP4/5 inflammasome [42,45], leading to non-canonical NLRP3 inflammasome activation. Given that we observed only partial loss of inflammasome activation in the *NAIP*^{-/-} THP-1s treated with siRNA targeting *CASP4* and *CASP5*, we hypothesize that at least part of

the NLRP3-dependent response is due to canonical activation (Fig. 4), although this partial loss may also be due to incomplete knockdown of *CASP4* and *CASP5*. A recent study also found that *Salmonella* infection induces NLRC4- and NLRP3-dependent inflammasome activation in human macrophages, and observed that full-length *Salmonella* flagellin can activate the NLRP3 inflammasome [36]. In contrast, we found the response to flagellin to be entirely dependent on the NAIP/NLRC4 inflammasome (Fig. 1, S3, S4). The reason for this apparent discrepancy is unclear, but in our studies, we used a truncated flagellin that only contains the C-terminal D0 domain and thus does not stimulate TLR5 signaling [56,68]. It is possible that full-length flagellin, in addition to activating the NAIP/NLRC4 inflammasome, also stimulates TLR5 signaling, perhaps potentiating NLRP3-dependent responses.

We observed NAIP/NLRC4- and NLRP3-dependent restriction of *Salmonella* (Fig. 5, S8), but the mechanism by which inflammasome activation promotes bacterial restriction is unclear. Inflammasome activation often triggers host cell death, thereby eliminating the pathogen's intracellular replicative niche. *In vivo*, pyroptosis can trigger formation of pore-induced intracellular traps (PITs). These PITs can trap intracellular bacteria that can subsequently be efferocytosed by neutrophils [69]. However, in murine macrophages, inhibition of *Salmonella* replication by caspase-1 and caspase-11 occurs prior to host cell death, indicating that caspase-1 and caspase-11 restrict *Salmonella* through a mechanism distinct from cell death [48]. Another mechanism of inflammasome-dependent restriction may be through promoting phagolysosomal maturation. In murine macrophages infected with *Legionella*, NAIP5 activation results in increased colocalization of *Legionella*-containing vacuoles with the lysosomal markers

cathepsin-D and Lamp-1 [70,71]. Perhaps a similar process occurs during *Salmonella* infection of human macrophages.

The current model is that the SPI-2 T3SS subverts inflammasome activation to facilitate *Salmonella*'s intracellular survival, based on the observation that the SPI-2 T3SS inner rod SsaI is not detected in murine or human macrophages [11,32]. Moreover, the SPI-2 T3SS effectors are critical for biogenesis and maintenance of the SCV [52]. Thus, evasion of inflammasome activation by the SPI-2 T3SS was thought to confer an advantage to the pathogen. However, our findings indicate that the NAIP/NLRC4 inflammasome detects the *Salmonella* SPI-2 T3SS needle protein SsaG. Furthermore, we find that SPI-1-independent, flagellin-independent, NAIP-dependent detection of *Salmonella* mediates restriction of intracellular bacterial replication in human macrophages. Perhaps the NAIP/NLRC4-mediated detection of SsaG is a consequence of a functional constraint placed upon SsaG's role as a T3SS needle protein. It is thus possible that SsaG is unable to evade immune detection due to such functional constraints.

While we focused here primarily on inflammasome responses in human macrophages, *Salmonella*'s first cellular encounters are with intestinal epithelial cells. In mice, NAIP/NLRC4 inflammasome activation in intestinal epithelial cells results in extrusion of infected cells from the epithelial layer [27,28]. It has been proposed that this mechanism eliminates *Salmonella* from the host and helps control bacterial burdens. Whether similar NAIP/NLRC4-dependent mechanisms are engaged in human intestinal epithelial cells remains to be elucidated.

580 Overall, these data indicate that *Salmonella* infection of human macrophages
581 triggers activation of multiple inflammasomes, and at least two of these inflammasomes,
582 the NAIP/NLRC4, and the NLRP3 inflammasomes, appear to be essential for controlling
583 bacterial replication within macrophages. Furthermore, our data indicate that the human
584 NAIP/NLRC4 inflammasome detects the SPI-2 needle protein SsaG, and that
585 NAIP/NLRC4-mediated detection of the SPI-2 T3SS restricts *Salmonella* replication
586 within macrophages. Collectively, our findings provide fundamental insight into how
587 *Salmonella* is sensed and restricted by human macrophages. Moreover, these results
588 offer a foundation for further understanding of how each of these pathways is activated
589 and how these inflammasomes interact to mediate downstream responses that promote
590 control of *Salmonella* infection in human macrophages.

Materials and Methods

Ethics statement

All studies involving primary human monocyte-derived macrophages (hMDMs) were performed in compliance with the requirements of the US Department of Health and Human Services and the principles expressed in the Declaration of Helsinki. hMDMs were derived from samples obtained from the University of Pennsylvania Human Immunology Core. These samples 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.

Bacterial strains and growth conditions

Targeted deletion strains used in this study were made on the *Salmonella enterica* serovar Typhimurium SL1344 strain background. The $\Delta prgI fliCfljB$ strain was engineered using the $\Delta fliCfljB$ background [72], in which the SPI-1 T3SS needle, *prgI*, was deleted through a chloramphenicol resistance cassette insertion into *prgI* (*fliCfljBprgI::CmR*) using standard methods [73].

WT, $\Delta sipB$ [74], and $\Delta prgI fliCfljB$ isogenic strains were routinely grown overnight in Luria-Bertani (LB) broth with streptomycin (100 μ g/ml) at 37°C. For infection of cultured cells, overnight cultures were diluted in LB containing 300 mM NaCl and grown standing for 3 hours at 37°C to induce SPI-1 expression [75].

Listeria monocytogenes WT and isogenic strains on the 10403S background were cultured in brain heart infusion (BHI) medium [53]. The *Listeria* strain encoding the

heterologous bacterial ligand *S. Typhimurium* PrgJ translationally fused to the truncated N-terminus of ActA and under the control of the *actA* promoter was used [53]. The *Listeria* strains expressing *S. Typhimurium* SsaI and SsaG were constructed using codon-optimized gene fragments (IDT) cloned into the pPL2 vector and introduced into *Listeria* as previously described [53,76].

Cell culture of THP-1s

THP-1 cells (TIB-202; American Type Culture Collection) were maintained in RPMI supplemented with 10% (vol/vol) heat-inactivated FBS, 0.05 nM β -mercaptoethanol, 100 IU/mL penicillin, and 100 μ g/mL streptomycin at 37°C in a humidified incubator. Two days before experimentation, the cells were replated in media without antibiotics in a 48-well plate at a concentration of 2×10^5 cells/well and incubated with phorbol 12-myristate 13-acetate (PMA) for 24 hours to allow differentiation into macrophages. Macrophages were primed with 100 ng/mL Pam3CSK4 (Invivogen) for 16 hours prior to bacterial infections or anthrax toxin treatments. For experiments involving LPS, cells were pretreated with 500 ng/mL LPS (Sigma-Aldrich) for 3 hours. For experiments involving Nigericin, cells were treated with 10 μ M Nigericin (EMD Millipore) for 6 hours. For experiments involving MCC950, cells were treated with 1 μ M MCC950 (Sigma Aldrich) 1 hour prior to infection.

Cell culture of primary human monocyte-derived macrophages (hMDMs)

Purified human monocytes from de-identified 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 days to promote differentiation into hMDMs. One day prior to infection, adherent hMDMs were replated in media with 25 ng/ml human M-CSF lacking antibiotics at 1.0×10^5 cells/well in a 48-well plate.

Bacterial infections

Overnight cultures of *Salmonella* were diluted into LB broth containing 300 mM NaCl and grown for 3 hours standing at 37°C to induce SPI-1 expression [75]. Overnight cultures of *L. monocytogenes* were diluted and grown for 3 hours in BHI. All cultures were pelleted at $6,010 \times g$ for 3 minutes, washed once with PBS, and resuspended in PBS. THP-1 cells were infected with *S. Typhimurium* or *L. monocytogenes* at a multiplicity of infection (MOI) of 20. hMDMs were infected with *L. monocytogenes* at an MOI of 5. Infected cells were centrifuged at $290 \times g$ for 10 min and incubated at 37°C. 1 hour post-infection, cells were treated with 100 ng/mL or 50 ng/mL of gentamicin to kill any extracellular *S. Typhimurium* or *L. monocytogenes* respectively. *Salmonella* and *Listeria* infections in THP-1s proceeded at 37°C for 6 hours. *Listeria* infection of hMDMs proceeded at 37°C for 16 hours. For all experiments, control cells were mock-infected with PBS.

Anthrax toxin-mediated delivery of bacterial ligands

Recombinant proteins (PA, LFn-FlaA³¹⁰⁻⁴⁷⁵, LFn-PrgJ, and LFn-YscF) were kindly provided by Russell Vance [18]. 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-FlaA³¹⁰⁻⁴⁷⁵; 8 ng/ml LFn-PrgJ; and 200 ng/mL LFn-YscF.

siRNA-mediated knockdown of genes

All Silencer Select siRNA oligos were purchased from Ambion (Life Technologies). For *CASP4*, siRNA ID# s2412 was used. For *CASP5*, siRNA ID# s2417 was used. The two Silencer Select negative control siRNAs (Silencer Select Negative Control No. 1 siRNA and Silencer Select Negative Control No. 2 siRNA) were used as a control. Two days before infection, 30 nM of siRNA was transfected into macrophages using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) following the manufacturer's protocol. 16 hours before infection, the media was replaced with fresh antibiotic-free media containing 100 ng/ml Pam3CSK4. In parallel, siRNA-transfected cells were also transfected with 2 µg/ml of *E. coli* LPS strain W3110 (kindly provided by Robert Ernst) using FuGENE HD transfection reagent (Promega) for 6 hours.

Bacterial intracellular replication assay

Cells were infected with WT or $\Delta prgIfljCfljB$ *S. Typhimurium* as usual at an MOI of 20. 1 hour post-infection, cells were treated with 100 µg/ml of gentamicin to kill any extracellular bacteria. 2 hours post-infection, the media was replaced with fresh media containing 10 µg/ml of gentamicin. At the indicated time points, cells were lysed with PBS containing 0.5% Triton to collect all intracellular bacteria. Harvested bacteria were serially diluted in PBS and plated on LB agar with streptomycin (100 µg/ml) plates to

enumerate colony forming units (CFUs). Plates were incubated at 37°C overnight and then CFUs were counted.

ELISAs

Harvested supernatants from infected cells were assayed using ELISA kits for human IL-1α (R&D Systems), IL-18 (R&D Systems), IL-1β (BD Biosciences), and TNF-α (R&D Systems).

LDH cytotoxicity assays

Harvested supernatants from infected cells were assayed for cytotoxicity by measuring loss of cellular membrane integrity via lactate dehydrogenase (LDH) activity. LDH release was quantified using an LDH Cytotoxicity Detection Kit (Clontech) according to the manufacturer's instructions and normalized to mock-infected cells.

Quantitative RT-PCR analysis

RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) following the manufacturer's instructions. Cells were lysed in 350 µL RLT buffer with β-mercaptoethanol and centrifuged through a QIAshredder spin column (Qiagen). cDNA was synthesized from isolated RNA using SuperScript II Reverse Transcriptase (Invitrogen) following the manufacturer's protocol. Quantitative PCR was conducted with the CFX96 real-time system from Bio-Rad using the SsoFast EvaGreen Supermix with Low ROX (Bio-Rad). For analysis, mRNA levels of siRNA-treated cells were normalized to housekeeping gene *HPRT* and control siRNA-treated cells using the $2^{-\Delta\Delta CT}$ (cycle

threshold) method [77] to calculate knockdown efficiency. The following primers from PrimerBank were used. The PrimerBank identifications are *CASP4* (73622124c1), and *CASP5* (209870072c2), and *HPRT* (164518913c1); all 5'–3':

CASP4 forward: CAAGAGAAGCAACGTATGGCA

CASP4 reverse: AGGCAGATGGTCAAACCTCTGTA

CASP5 forward: TTCAACACCACATAACGTGTCC

CASP5 reverse: GTCAAGGTTGCTCGTTCTATGG

HPRT forward: CCTGGCGTCGTGATTAGTGAT

HPRT reverse: AGACGTTTCAGTCCTGTCCATAA

Statistical analysis

Prism 9.1.1 (GraphPad Software) was utilized for the graphing of data and all statistical analyses. Statistical significance for experiments with THP-1 cells was determined using the appropriate test and are indicated in each figure legend. Differences were considered statistically significant if the *p* value was <0.05.

721 Acknowledgements

722 We thank members of Igor Brodsky's and Sunny Shin's laboratories for scientific
 723 discussion. We thank Meghan Wynosky-Dolfi for technical advice. We thank Russell
 724 Vance, Randilea Nichols, Isabella Rauch, and Jeannette Tenthorey for providing
 725 anthrax toxin-based reagents, JD Sauer for providing the *Listeria* strains and constructs
 726 for generating ActA fusion proteins, and Robert Ernst for providing *E. coli* LPS. We
 727 thank the Human Immunology Core of the Penn Center for AIDS Research and
 728 Abramson Cancer Center for providing purified primary human monocytes.

References

1. Crowley SM, Knodler LA, Vallance BA. Salmonella and the inflammasome: battle for intracellular dominance. *Inflammasome signaling and bacterial infections*. 2016; 43–67.
2. Mills DM, Bajaj V, Lee CA. A 40 kb chromosomal fragment encoding Salmonella typhimurium invasion genes is absent from the corresponding region of the Escherichia coli K-12 chromosome. *Molecular Microbiology*. 2006;15: 749–759. doi:10.1111/j.1365-2958.1995.tb02382.x
3. Shea JE, Hensel M, Gleeson C, Holden DW. Identification of a virulence locus encoding a second type III secretion system in Salmonella typhimurium. *Proceedings of the National Academy of Sciences*. 1996;93: 2593–2597. doi:10.1073/pnas.93.6.2593
4. Hensel M, Shea JE, Waterman SR, Mundy R, Nikolaus T, Banks G, et al. Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. *Molecular Microbiology*. 1998;30: 163–174. doi:10.1046/j.1365-2958.1998.01047.x
5. Galan JE, Zhou D. Striking a balance: modulation of the actin cytoskeleton by Salmonella. *Proc Natl Acad Sci U S A*. 2000;97: 8754–8761. doi:10.1073/pnas.97.16.8754
6. Galán JE. Interaction of Salmonella with host cells through the centisome 63 type III secretion system. *Curr Opin Microbiol*. 1999;2: 46–50. doi:10.1016/s1369-5274(99)80008-3
7. Galán JE, Collmer A. Type III secretion machines: bacterial devices for protein delivery into host cells. *Science*. 1999;284: 1322–1328. doi:10.1126/science.284.5418.1322
8. Galán JE, Curtiss R. Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells. *Proc Natl Acad Sci U S A*. 1989;86: 6383–6387. doi:10.1073/pnas.86.16.6383
9. Zhao Y, Shao F. The NAIP-NLRC4 inflammasome in innate immune detection of bacterial flagellin and type III secretion apparatus. *Immunol Rev*. 2015;265: 85–102. doi:10.1111/imr.12293
10. Sun Y-H, Rolán HG, Tsolis RM. Injection of flagellin into the host cell cytosol by Salmonella enterica serotype Typhimurium. *Journal of Biological Chemistry*. 2007;282: 33897–33901.
11. Miao EA, Mao DP, Yudkovsky N, Bonneau R, Lorang CG, Warren SE, et al. Innate immune detection of the type III secretion apparatus through the NLRC4 inflammasome. *Proceedings of the National Academy of Sciences*. 2010;107: 3076–3080.
12. Medzhitov R, Janeway CA. Decoding the patterns of self and nonself by the innate immune system. *Science*. 2002;296: 298–300.

- 763 13. Janeway CA. Approaching the asymptote? Evolution and revolution in immunology. Cold
764 Spring Harb Symp Quant Biol. 1989;54 Pt 1: 1–13.
- 765 14. Kofoed EM, Vance RE. Innate immune recognition of bacterial ligands by NAIPs determines
766 inflammasome specificity. Nature. 2011;477: 592–595.
- 767 15. Zhao Y, Yang J, Shi J, Gong Y-N, Lu Q, Xu H, et al. The NLRC4 inflammasome receptors for
768 bacterial flagellin and type III secretion apparatus. Nature. 2011;477: 596–600.
- 769 16. Rayamajhi M, Zak DE, Chavarria-Smith J, Vance RE, Miao EA. Cutting edge: Mouse NAIP1
770 detects the type III secretion system needle protein. The Journal of Immunology.
771 2013;191: 3986–3989.
- 772 17. Yang J, Zhao Y, Shi J, Shao F. Human NAIP and mouse NAIP1 recognize bacterial type III
773 secretion needle protein for inflammasome activation. Proceedings of the National
774 Academy of Sciences. 2013;110: 14408–14413.
- 775 18. Rauch I, Tenthorey JL, Nichols RD, Al Moussawi K, Kang JJ, Kang C, et al. NAIP proteins are
776 required for cytosolic detection of specific bacterial ligands in vivo. Journal of
777 Experimental Medicine. 2016;213: 657–665.
- 778 19. Zhao Y, Shi J, Shi X, Wang Y, Wang F, Shao F. Genetic functions of the NAIP family of
779 inflammasome receptors for bacterial ligands in mice. Journal of Experimental Medicine.
780 2016;213: 647–656.
- 781 20. Diebold CA, Halff EF, Koster AJ, Huizinga EG, Koning RI. Cryoelectron tomography of the
782 NAIP5/NLRC4 inflammasome: implications for NLR activation. Structure. 2015;23: 2349–
783 2357.
- 784 21. Hu Z, Zhou Q, Zhang C, Fan S, Cheng W, Zhao Y, et al. Structural and biochemical basis for
785 induced self-propagation of NLRC4. Science. 2015;350: 399–404.
- 786 22. Zhang L, Chen S, Ruan J, Wu J, Tong AB, Yin Q, et al. Cryo-EM structure of the activated
787 NAIP2-NLRC4 inflammasome reveals nucleated polymerization. Science. 2015;350: 404–
788 409.
- 789 23. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering
790 activation of inflammatory caspases and processing of proIL- β . Molecular cell. 2002;10:
791 417–426.
- 792 24. Kuida K, Lippke JA, Ku G, Harding MW, Livingston DJ, Su M, et al. Altered cytokine export
793 and apoptosis in mice deficient in interleukin-1 beta converting enzyme. science.
794 1995;267: 2000–2003.

- 795 25. Li P, Allen H, Banerjee S, Franklin S, Herzog L, Johnston C, et al. Mice deficient in IL-1 β -
796 converting enzyme are defective in production of mature IL-1 β and resistant to endotoxic
797 shock. *Cell*. 1995;80: 401–411.
- 798 26. Shi J, Zhao Y, Wang K, Shi X, Wang Y, Huang H, et al. Cleavage of GSDMD by inflammatory
799 caspases determines pyroptotic cell death. *Nature*. 2015/09/16 ed. 2015;526: 660–5.
800 doi:10.1038/nature15514
- 801 27. Sellin ME, Müller AA, Felmy B, Dolowschik T, Diard M, Tardivel A, et al. Epithelium-
802 intrinsic NAIP/NLRC4 inflammasome drives infected enterocyte expulsion to restrict
803 *Salmonella* replication in the intestinal mucosa. *Cell host & microbe*. 2014;16: 237–248.
- 804 28. Rauch I, Deets KA, Ji DX, von Moltke J, Tenthorey JL, Lee AY, et al. NAIP-NLRC4
805 inflammasomes coordinate intestinal epithelial cell expulsion with eicosanoid and IL-18
806 release via activation of caspase-1 and -8. *Immunity*. 2017;46: 649–659.
- 807 29. Romanish MT, Lock WM, Van De Lagemaat LN, Dunn CA, Mager DL. Repeated recruitment
808 of LTR retrotransposons as promoters by the anti-apoptotic locus NAIP during mammalian
809 evolution. *PLoS Genet*. 2007;3: e10.
- 810 30. Romanish MT, Nakamura H, Lai CB, Wang Y, Mager DL. A novel protein isoform of the
811 multicopy human NAIP gene derives from intragenic Alu SINE promoters. *PloS one*.
812 2009;4: e5761.
- 813 31. Kortmann J, Brubaker SW, Monack DM. Cutting edge: inflammasome activation in primary
814 human macrophages is dependent on flagellin. *The Journal of Immunology*. 2015;195:
815 815–819.
- 816 32. Reyes Ruiz VM, Ramirez J, Naseer N, Palacio NM, Siddharthan IJ, Yan BM, et al. Broad
817 detection of bacterial type III secretion system and flagellin proteins by the human
818 NAIP/NLRC4 inflammasome. *Proceedings of the National Academy of Sciences*. 2017;114:
819 13242–13247.
- 820 33. Shi J, Zhao Y, Wang Y, Gao W, Ding J, Li P, et al. Inflammatory caspases are innate immune
821 receptors for intracellular LPS. *Nature*. 2014;514: 187–192.
- 822 34. von Moltke J, Trinidad NJ, Moayeri M, Kintzer AF, Wang SB, van Rooijen N, et al. Rapid
823 induction of inflammatory lipid mediators by the inflammasome in vivo. *Nature*. 2012;490:
824 107–111.
- 825 35. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, et al.
826 Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature*. 2006;440:
827 228–232.

- 828 36. Gram AM, Wright JA, Pickering RJ, Lam NL, Booty LM, Webster SJ, et al. Salmonella
829 Flagellin Activates NAIP/NLRC4 and Canonical NLRP3 Inflammasomes in Human
830 Macrophages. *The Journal of Immunology*. 2021;206: 631–640.
- 831 37. Broz P, Newton K, Lamkanfi M, Mariathasan S, Dixit VM, Monack DM. Redundant roles for
832 inflammasome receptors NLRP3 and NLRC4 in host defense against Salmonella. *Journal of*
833 *Experimental Medicine*. 2010;207: 1745–1755.
- 834 38. Man SM, Hopkins LJ, Nugent E, Cox S, Glück IM, Turlomousis P, et al. Inflammasome
835 activation causes dual recruitment of NLRC4 and NLRP3 to the same macromolecular
836 complex. *Proceedings of the National Academy of Sciences*. 2014;111: 7403–7408.
- 837 39. Muñoz-Planillo R, Kuffa P, Martínez-Colón G, Smith BL, Rajendiran TM, Núñez G. K⁺ efflux
838 is the common trigger of NLRP3 inflammasome activation by bacterial toxins and
839 particulate matter. *Immunity*. 2013;38: 1142–53. doi:10.1016/j.immuni.2013.05.016
- 840 40. Coll RC, Robertson AA, Chae JJ, Higgins SC, Muñoz-Planillo R, Inserra MC, et al. A small-
841 molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory
842 diseases. *Nature medicine*. 2015;21: 248–255.
- 843 41. Broz P, Ruby T, Belhocine K, Bouley DM, Kayagaki N, Dixit VM, et al. Caspase-11 increases
844 susceptibility to Salmonella infection in the absence of caspase-1. *Nature*. 2012;490: 288–
845 291. doi:10.1038/nature11419
- 846 42. Schmid-Burgk JL, Gaidt MM, Schmidt T, Ebert TS, Bartok E, Hornung V. Caspase-4 mediates
847 non-canonical activation of the NLRP3 inflammasome in human myeloid cells. *European*
848 *journal of immunology*. 2015;45: 2911–2917.
- 849 43. Casson CN, Yu J, Reyes VM, Taschuk FO, Yadav A, Copenhaver AM, et al. Human caspase-4
850 mediates noncanonical inflammasome activation against gram-negative bacterial
851 pathogens. *Proceedings of the National Academy of Sciences*. 2015;112: 6688–6693.
- 852 44. Knodler LA, Crowley SM, Sham HP, Yang H, Wrande M, Ma C, et al. Noncanonical
853 inflammasome activation of caspase-4/caspase-11 mediates epithelial defenses against
854 enteric bacterial pathogens. *Cell host & microbe*. 2014;16: 249–256.
- 855 45. Baker PJ, Boucher D, Bierschenk D, Tebartz C, Whitney PG, D'Silva DB, et al. NLRP3
856 inflammasome activation downstream of cytoplasmic LPS recognition by both caspase-4
857 and caspase-5. *Eur J Immunol*. 2015;45: 2918–2926. doi:10.1002/eji.201545655
- 858 46. Hausmann A, Böck D, Geiser P, Berthold DL, Fattinger SA, Furter M, et al. Intestinal
859 epithelial NAIP/NLRC4 restricts systemic dissemination of the adapted pathogen
860 Salmonella Typhimurium due to site-specific bacterial PAMP expression. *Mucosal*
861 *immunology*. 2020;13: 530–544.

- 862 47. De Jong HK, Koh GC, van Lieshout MH, Roelofs JJ, van Dissel JT, van der Poll T, et al.
863 Limited role for ASC and NLRP3 during in vivo Salmonella Typhimurium infection. BMC
864 immunology. 2014;15: 1–11.
- 865 48. Thurston TLM, Matthews SA, Jennings E, Alix E, Shao F, Shenoy AR, et al. Growth inhibition
866 of cytosolic Salmonella by caspase-1 and caspase-11 precedes host cell death. Nat
867 Commun. 2016;7: 13292. doi:10.1038/ncomms13292
- 868 49. Adams P, Fowler R, Kinsella N, Howell G, Farris M, Coote P, et al. Proteomic detection of
869 PhoPQ- and acid-mediated repression of Salmonella motility. Proteomics. 2001;1: 597–
870 607. doi:10.1002/1615-9861(200104)1:4<597::AID-PROT597>3.0.CO;2-P
- 871 50. Lou L, Zhang P, Piao R, Wang Y. Salmonella Pathogenicity Island 1 (SPI-1) and Its Complex
872 Regulatory Network. Front Cell Infect Microbiol. 2019;9: 270.
873 doi:10.3389/fcimb.2019.00270
- 874 51. Boddicker JD, Jones BD. Lon Protease Activity Causes Down-Regulation of *Salmonella*
875 Pathogenicity Island 1 Invasion Gene Expression after Infection of Epithelial Cells. Infect
876 Immun. 2004;72: 2002–2013. doi:10.1128/IAI.72.4.2002-2013.2004
- 877 52. Haraga A, Ohlson MB, Miller SI. Salmonellae interplay with host cells. Nat Rev Microbiol.
878 2008;6: 53–66. doi:10.1038/nrmicro1788
- 879 53. Sauer J-D, Pereyre S, Archer KA, Burke TP, Hanson B, Lauer P, et al. *Listeria monocytogenes*
880 engineered to activate the Nlrp4 inflammasome are severely attenuated and are poor
881 inducers of protective immunity. Proceedings of the National Academy of Sciences.
882 2011;108: 12419–12424.
- 883 54. Kubori T, Sukhan A, Aizawa S-I, Galán JE. Molecular characterization and assembly of the
884 needle complex of the Salmonella typhimurium type III protein secretion system.
885 Proceedings of the National Academy of Sciences. 2000;97: 10225–10230.
- 886 55. Tenthorey JL, Haloupek N, López-Blanco JR, Grob P, Adamson E, Hartenian E, et al. The
887 structural basis of flagellin detection by NAIP5: A strategy to limit pathogen immune
888 evasion. Science. 2017;358: 888–893.
- 889 56. Lightfield KL, Persson J, Brubaker SW, Witte CE, Von Moltke J, Dunipace EA, et al. Critical
890 function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of
891 flagellin. Nature immunology. 2008;9: 1171–1178.
- 892 57. Halff EF, Diebolder CA, Versteeg M, Schouten A, Brondijk THC, Huizinga EG. Formation and
893 structure of a NAIP5-NLRC4 inflammasome induced by direct interactions with conserved
894 N-and C-terminal regions of flagellin. Journal of Biological Chemistry. 2012;287: 38460–
895 38472.

- 896 58. Yang J, Zhao Y, Li P, Yang Y, Zhang E, Zhong M, et al. Sequence determinants of specific
897 pattern-recognition of bacterial ligands by the NAIP–NLRC4 inflammasome. *Cell discovery*.
898 2018;4: 1–12.
- 899 59. Yang X, Yang F, Wang W, Lin G, Hu Z, Han Z, et al. Structural basis for specific flagellin
900 recognition by the NLR protein NAIP5. *Cell research*. 2018;28: 35–47.
- 901 60. Tenthorey JL, Kofoed EM, Daugherty MD, Malik HS, Vance RE. Molecular basis for specific
902 recognition of bacterial ligands by NAIP/NLRC4 inflammasomes. *Molecular cell*. 2014;54:
903 17–29.
- 904 61. Meunier E, Dick MS, Dreier RF, Schürmann N, Kenzelmann Broz D, Warming S, et al.
905 Caspase-11 activation requires lysis of pathogen-containing vacuoles by IFN-induced
906 GTPases. *Nature*. 2014/04/16 ed. 2014;509: 366–70. doi:10.1038/nature13157
- 907 62. Kim B-H, Shenoy AR, Kumar P, Das R, Tiwari S, MacMicking JD. A family of IFN- γ -inducible
908 65-kD GTPases protects against bacterial infection. *Science*. 2011;332: 717–721.
- 909 63. Fisch D, Bando H, Clough B, Hornung V, Yamamoto M, Shenoy AR, et al. Human GBP 1 is a
910 microbe-specific gatekeeper of macrophage apoptosis and pyroptosis. *The EMBO journal*.
911 2019;38: e100926.
- 912 64. Santos JC, Boucher D, Schneider LK, Demarco B, Dilucca M, Shkarina K, et al. Human GBP1
913 binds LPS to initiate assembly of a caspase-4 activating platform on cytosolic bacteria. *Nat*
914 *Commun*. 2020;11: 3276. doi:10.1038/s41467-020-16889-z
- 915 65. Wandel MP, Kim B-H, Park E-S, Boyle KB, Nayak K, Lagrange B, et al. Guanylate-binding
916 proteins convert cytosolic bacteria into caspase-4 signaling platforms. *Nat Immunol*. 2020
917 [cited 29 May 2021]. doi:10.1038/s41590-020-0697-2
- 918 66. Vanaja SK, Russo AJ, Behl B, Banerjee I, Yankova M, Deshmukh SD, et al. Bacterial Outer
919 Membrane Vesicles Mediate Cytosolic Localization of LPS and Caspase-11 Activation. *Cell*.
920 2016/05/05 ed. 2016;165: 1106–1119. doi:10.1016/j.cell.2016.04.015
- 921 67. Santos JC, Dick MS, Lagrange B, Degrandi D, Pfeffer K, Yamamoto M, et al. LPS targets host
922 guanylate-binding proteins to the bacterial outer membrane for non-canonical
923 inflammasome activation. *EMBO J*. 2018;37. doi:10.15252/embj.201798089
- 924 68. Lightfield KL, Persson J, Trinidad NJ, Brubaker SW, Kofoed EM, Sauer J-D, et al. Differential
925 Requirements for NAIP5 in Activation of the NLRC4 Inflammasome. McCormick BA, editor.
926 *Infect Immun*. 2011;79: 1606–1614. doi:10.1128/IAI.01187-10
- 927 69. Jorgensen I, Zhang Y, Krantz BA, Miao EA. Pyroptosis triggers pore-induced intracellular
928 traps (PITs) that capture bacteria and lead to their clearance by efferocytosis. *Journal of*
929 *Experimental Medicine*. 2016;213: 2113–2128.

930 70. Amer A, Franchi L, Kanneganti T-D, Body-Malapel M, Özören N, Brady G, et al. Regulation
931 of Legionella phagosome maturation and infection through flagellin and host Ipaf. Journal
932 of Biological Chemistry. 2006;281: 35217–35223.

933 71. Fortier A, De Chastellier C, Balor S, Gros P. Birc1e/Naip5 rapidly antagonizes modulation of
934 phagosome maturation by Legionella pneumophila. Cellular microbiology. 2007;9: 910–
935 923.

936 72. Wynosky-Dolfi MA, Snyder AG, Philip NH, Doonan PJ, Poffenberger MC, Avizonis D, et al.
937 Oxidative metabolism enables Salmonella evasion of the NLRP3 inflammasome. Journal of
938 Experimental Medicine. 2014;211: 653–668.

939 73. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in Escherichia coli
940 K-12 using PCR products. Proceedings of the National Academy of Sciences. 2000;97:
941 6640–6645. doi:10.1073/pnas.120163297

942 74. Lawley TD, Chan K, Thompson LJ, Kim CC, Govoni GR, Monack DM. Genome-wide screen
943 for Salmonella genes required for long-term systemic infection of the mouse. PLoS Pathog.
944 2006;2: e11.

945 75. Lee CA, Falkow S. The ability of Salmonella to enter mammalian cells is affected by
946 bacterial growth state. Proceedings of the national academy of sciences. 1990;87: 4304–
947 4308.

948 76. Lauer P, Chow MYN, Loessner MJ, Portnoy DA, Calendar R. Construction, characterization,
949 and use of two Listeria monocytogenes site-specific phage integration vectors. Journal of
950 bacteriology. 2002;184: 4177–4186.

951 77. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time
952 Quantitative PCR and the 2- $\Delta\Delta$ CT Method. Methods. 2001;25: 402–408.
953 doi:10.1006/meth.2001.1262

954

Main Body Figures and Figure Legends

Fig 1. NAIP and NLRC4 are necessary for inflammasome responses to T3SS ligands in human macrophages. WT, NAIP^{-/-} clone, or two independent clones of NLRC4^{-/-} THP-1 monocyte-derived macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were then treated with PBS (Mock), PA alone, LFnFlaA³¹⁰⁻⁴⁷⁵ alone, LFnPrgJ alone, LFnYscF alone, PA+LFnFlaA³¹⁰⁻⁴⁷⁵ (FlATox), PA+LFnPrgJ (PrgJTox), or PA+LFnYscF (YscFTox) for 6 hours (A, C). As a control, cells were primed with 500 ng/mL LPS for 4 hours and treated with 10 μM nigericin for 6

964 hours (B, D). Release of IL-1 β into the supernatant was measured by ELISA. ns – not
 965 significant, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ by Šídák's multiple comparisons test
 966 (A), or by unpaired t-test (B), or by Dunnett's multiple comparisons test (C, D). Data
 967 shown are representative of at least three independent experiments.

Fig 2. NAIP and NLRC4 are partially required for inflammasome activation during

***Salmonella* infection in human macrophages.** WT, NAIP^{-/-} clone, or two independent

clones of NLRC4^{-/-} THP-1 monocyte-derived macrophages were primed with 100 ng/mL

Pam3CSK4 for 16 hours. Cells were then infected with PBS (Mock), WT *S.*

Typhimurium, or ΔsipB *S. Typhimurium* for 6 hours. Release of IL-1β and IL-18 into the

supernatant were measured by ELISA. ns – not significant, ****p* < 0.001, *****p* < 0.0001

by Šídák's multiple comparisons test (A, C) or Dunnett's multiple comparisons test (B,

D). Data shown are representative of at least three independent experiments.

Fig 3. *Salmonella* induces NAIP/NLRC4- and NLRP3-dependent inflammasome activation in human macrophages. WT, *NAIP*^{-/-}, or *NLRC4*^{-/-} THP-1 monocyte-derived

980 macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. One hour prior to
 981 infection, cells were treated with 1 μ M MCC950, a chemical inhibitor of the NLRP3
 982 inflammasome or DMSO as a control. Cells were then infected with PBS (Mock), WT *S.*
 983 Typhimurium, or $\Delta sipB$ *S. Typhimurium* for 6 hours. As a control, cells were primed with
 984 500 ng/mL LPS for 4 hours and treated with 10 μ M nigericin for 6 hours. Release of IL-
 985 1 β into the supernatant was measured by ELISA. ns – not significant, * $p < 0.05$, ** $p <$
 986 0.01, *** $p < 0.001$, **** $p < 0.0001$ by Tukey's multiple comparisons test (A, C) or by
 987 Šídák's multiple comparisons test (B, D). Data shown are representative of at least
 988 three independent experiments.

Fig 4. *Salmonella* induces NAIP- and CASP4/5-dependent inflammasome

activation in human macrophages. WT or *NAIP*^{-/-} THP-1 monocyte-derived macrophages were treated with siRNA targeting a control scrambled siRNA, siRNA targeting *CASP4* or *CASP5*, or siRNA targeting both *CASP4* and *CASP5* for 48 hours. Cells were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were then infected with PBS (Mock) or WT *S. Typhimurium* for 6 hours. Release of IL-1β into the

996 supernatant were measured by ELISA. As a control, cells were transfected with LPS. ns
 997 – not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by Tukey's multiple
 998 comparisons test. Data shown are representative of at least three independent
 999 experiments.

Fig 5. The NAIP and NLRP3 inflammasomes restrict *Salmonella* replication within human macrophages. WT or *NAIP*^{-/-} THP-1 monocyte-derived macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. One hour prior to infection, cells were treated with 1 μ M MCC950, a chemical inhibitor of the NLRP3 inflammasome or DMSO as a control. Cells were then infected with PBS (Mock) or WT *S. Typhimurium*. Cells were lysed at the indicated time points and bacteria were plated to calculate CFU. (A) CFU/well of bacteria at 6 hpi (B) Fold change in CFU/well of bacteria at indicated time point, relative to 2 hpi CFU/well. ns – not significant, *** p < 0.001, **** p < 0.0001 by

1009 Dunnett's multiple comparisons test (A) or Tukey's multiple comparisons test (B). Data
1010 shown are representative of at least three independent experiments.

Fig 6. *Salmonella* SPI-2 needle protein SsaG activates the NAIP/NLRC4

inflammasome in human macrophages. (A) Primary hMDMs from four healthy human donors were infected with PBS (Mock), WT *Listeria* (WT Lm), *Listeria* expressing PrgJ (Lm + PrgJ), SsaI (Lm + SsaI), or SsaG (Lm + SsaG) for 16 hours at MOI=5. Release of IL-18 into the supernatant was measured by ELISA. Each dot represents the mean of individual donors derived from triplicate wells. The grey bar represents the mean of all donors. (B) WT or NAIP^{-/-}, NLRC4^{-/-} THP-1 monocyte-derived macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were treated with PBS (Mock), WT *Listeria* (WT Lm), *Listeria* expressing PrgI (Lm + PrgI), or SsaG (Lm + SsaG) for 6 hours at MOI=20. Release of IL-1β into the supernatant was measured by ELISA. ns – not significant, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ paired t-test (A) or by Dunnett's multiple comparisons test (B). Data shown are representative of at least three independent experiments.

Fig 7. NAIP/NLRC4 inflammasome recognition of the SPI-2 T3SS restricts

***Salmonella* replication in human macrophages.** WT or *NAIP*^{-/-} THP-1 monocyte-

derived macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were

then infected with PBS (Mock) or *ΔprgIfljCfljB* *S. Typhimurium*. Cells were lysed at the

indicated time points and bacteria were plated to calculate CFU. Fold change in

CFU/well of bacteria at indicated time point, relative to 2 hpi CFU/well. ns – not

significant, ****p* < 0.001, *****p* < 0.0001 by Tukey's multiple comparisons test.

1033 Supporting Information

A

B

C

1034

1035 **S1 Fig. Validation of *NAIP* mutant THP-1 single cell clones generated with**

1036 **CRISPR/Cas9 genome editing.** (A) Schematic representation of the *NAIP* gene with

1037 exons (filled boxes) and introns (filled lines). gRNA target sequence is highlighted in

1038 red. (B) Sequence alignments of WT THP-1 and *NAIP*^{-/-} clone 12 are shown for both
 1039 alleles. Red boxes represent the mutated region. Purple text represents the predicted
 1040 impact of the mutation on the amino acid sequence. (C) qRT-PCR was performed to
 1041 quantitate *NAIP* mRNA levels in WT THP-1 and *NAIP*^{-/-} THP-1 cells. For the *NAIP*^{-/-}
 1042 THP-1 cells, *NAIP* mRNA levels were normalized to human HPRT mRNA levels and
 1043 WT THP-1 cells.

S2 Fig. Validation of *NLRC4* mutant THP-1 single cell clones generated with CRISPR/Cas9-mediated genome editing. (A) Schematic representation of the *NLRC4* gene with exons (filled boxes) and introns (lines). gRNA target sequence is highlighted in red. (B-C) Sequence alignments of WT THP-1 and *NLRC4*^{-/-} clones are shown for both alleles per clone. Red boxes highlight the mutated region. Purple text represents

1050 the predicted impact of the mutation on the amino acid sequence. (D) Immunoblot
1051 analysis was performed on cell lysates for human NLRC4, and β -actin as a loading
1052 control.

S3 Fig. (related to Figure 1) NAIP is necessary for inflammasome responses to T3SS ligands in human macrophages. WT or NAIP^{-/-} THP-1 monocyte-derived macrophages were primed with 100 ng/ml Pam3CSK4 for 16 hours. Cells were then treated with PBS (Mock), PA alone, LFn FlaA³¹⁰⁻⁴⁷⁵ alone, LFn PrgJ alone, LF YscF alone, PA+LFn FlaA³¹⁰⁻⁴⁷⁵ (FlaTox), PA+LFn PrgJ (PrgJTox), or PA+LFn YscF (YscFTox) for 6 hours. (A, B, D) Release of cytokines IL-18, IL-1α, and TNF-α into the supernatant were measured by ELISA. (C) Cell death (percentage cytotoxicity) was measured by lactate dehydrogenase release assay and normalized to Mock-treated

1062 cells. ns – not significant, $**p < 0.01$, $****p < 0.0001$ by Šídák's multiple comparisons
 1063 test. Data shown are representative of at least three independent experiments.

S4 Fig. (related to Figure 1) NLRC4 is necessary for inflammasome responses to T3SS ligands in human macrophages. WT or two independent clones of *NLRC4*^{-/-} THP-1 monocyte-derived macrophages were primed with 100 ng/ml Pam3CSK4 for 16 hours. Cells were then treated with PBS (Mock), PA alone, LFn FlaA³¹⁰⁻⁴⁷⁵ alone, LFn PrgJ alone, LFn YscF alone, PA+LFn FlaA³¹⁰⁻⁴⁷⁵ (FlaTox), PA+LFn PrgJ (PrgJTox), or PA+LFn YscF (YscFTox) for 6 hours. (A, B, D) Release of cytokines IL-18, IL-1α, and

1071 TNF- α into the supernatant were measured by ELISA. (C) Cell death (percentage
 1072 cytotoxicity) was measured by lactate dehydrogenase release assay and normalized to
 1073 Mock-treated cells. ns – not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p <$
 1074 0.0001 by Dunnett's multiple comparisons test (A-C). Data shown are representative of
 1075 at least three independent experiments.

S5 Fig. (related to Figure 2) NAIP and NLRC4 are partially required for inflammasome activation during *Salmonella* infection in human macrophages. WT, *NAIP*^{-/-}, or two independent clones of *NLRC4*^{-/-} THP-1 monocyte-derived macrophages were primed with 100 ng/uL Pam3CSK4 for 16 hours. Cells were then

1081 infected with PBS (Mock), WT *S. Typhimurium*, or $\Delta sipB$ *S. Typhimurium* for 6 hours. As
 1082 a control, cells were primed with 500 ng/mL LPS for 4 hours and treated with 10 μ M
 1083 nigericin for 6 hours. (A, C, E, F) Release of cytokines IL-1 α and TNF- α into the
 1084 supernatant were measured by ELISA. (B, D) Cell death (percentage cytotoxicity) was
 1085 measured by lactate dehydrogenase release assay and normalized to Mock-treated
 1086 cells. ns – not significant, * $p < 0.05$, *** $p < 0.001$ by Šídák's multiple comparisons test
 1087 (A, B, E) or by Dunnett's multiple comparisons test (C, D, F). Data shown are
 1088 representative of at least three independent experiments.

S6 Fig. (related to Figure 3) *Salmonella* induces NAIP/NLRC4- and NLRP3-dependent inflammasome activation in human macrophages. WT, *NAIP*^{-/-}, or *NLRC4*^{-/-} THP-1 monocyte-derived macrophages were primed with 100 ng/uL

1093 Pam3CSK4 for 16 hours. One hour prior to infection, cells were treated with 1 μ M
 1094 MCC950, a chemical inhibitor of the NLRP3 inflammasome. Cells were then infected
 1095 with PBS (Mock), WT *S. Typhimurium*, or $\Delta sipB$ *S. Typhimurium* for 6 hours. (B) As a
 1096 control, cells were primed with 500 ng/mL LPS for 4 hours and treated with 10 μ M
 1097 nigericin for 6 hours. (A-F) Release of cytokines IL-18, IL-1 α , TNF- α into the
 1098 supernatant were measured by ELISA. ns – not significant, * $p < 0.05$, ** $p < 0.01$, *** $p <$
 1099 0.001, **** $p < 0.0001$ by Tukey's multiple comparisons test.

A Average knockdown efficiency

THP-1s	<i>CASP4</i>	<i>CASP5</i>
WT	82.5%	89.5%
<i>NAIP</i> ^{-/-} #12	79.5%	79.5%

B Average knockdown efficiency

THP-1s	<i>CASP4</i>	<i>CASP5</i>
WT	81%	74.5%
<i>NAIP</i> ^{-/-} #12	73.5%	76.5%

1100

1101 **S7 Fig. (related to Figure 4) Knockdown efficiencies of siRNA-mediated silencing**
 1102 **of *CASP4* and *CASP5* in human macrophages.** Knockdown efficiencies following
 1103 siRNA treatment were measured by qRT-PCR and normalized to housekeeping gene
 1104 *HPRT*, and calculated relative to control-siRNA-treated cells. (A) siRNA targeting
 1105 *CASP4* or *CASP5*. (B) siRNA targeting *CASP4* and *CASP5*. Data shown are averages
 1106 of at least three independent experiments.

S8 Fig. (related to Figure 5) NAIP and NLRP3 restrict replication of *Salmonella* in human macrophages. WT or NAIP^{-/-} THP-1 monocyte-derived macrophages were primed with 100 ng/ml Pam3CSK4 for 16 hours. One hour prior to infection, cells were treated with 1 μ M MCC950 or DMSO as a control. Cells were then infected with WT *S. Typhimurium*. Cells were lysed at the indicated time points and bacterial were plated to calculate CFU. (A) CFU/well of bacteria at 2 hpi (B) CFU/well of bacteria at 24 hpi. **p* <

1114 0.05, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ by Tukey's multiple comparisons test. Data
1115 shown are representative of at least three independent experiments.

S9 Fig. (related to Figure 6A) *Salmonella* SPI-2 needle protein SsaG activates the

inflammasome in human macrophages. Primary hMDMs from four healthy human

donors was infected with PBS (Mock), WT *Listeria* (WT Lm), *Listeria* expressing PrgJ

(Lm + PrgJ), Ssal (Lm + Ssal), or SsaG (Lm + SsaG) for 16 hours at MOI=5. Each dot

represents the triplicate mean of one donor. The grey bar represents the mean of all

donors. Release of cytokines IL-1 β and IL-1 α , was measured by ELISA. p values based

on paired t-tests.

S10 Fig. (related to Figure 6B) NAIP/NLRC4 are necessary for inflammasome responses to the *Salmonella* SPI-2 needle protein SsaG in human macrophages.

WT, *NAIP*^{-/-}, or *NLRC4*^{-/-} THP-1 monocyte-derived macrophages were primed with 100 ng/ml Pam3CSK4 for 16 hours. Cells were then treated with PBS (Mock), WT *Listeria* (WT Lm), *Listeria* expressing PrgI (Lm + PrgJ) or SsaG (Lm + SsaG) for 6 hours at MOI=20. (A, B) Release of cytokines IL-18, and IL-1α was measured by ELISA. (C) Cell death was measured by lactate dehydrogenase (LDH) release. ns – not significant, **p* <

1132 0.05, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by Dunnett's multiple comparisons test.

1133 Data shown are representative of at least three independent experiments.

S11 Fig. (related to Figure 6C) NAIP/NLRC4 inflammasome recognition of the SPI-2 T3SS restricts *Salmonella* replication in human macrophages. WT or *NAIP*^{-/-} THP-1 monocyte-derived macrophages were primed with 100 ng/ml Pam3CSK4 for 16

1138 hours. Cells were then infected with a SPI-1 T3SS/flagellin-deficient strain of S.
 1139 Typhimurium, $\Delta prgI fliC fljB$. (A) CFU/well of bacteria at 2 hpi (B) CFU/well of bacteria at
 1140 6 hpi. (C) CFU/well of bacteria at 24 hpi. $**p < 0.01$, $***p < 0.001$, by unpaired t-test.
 1141 Data shown are representative of at least three independent experiments.

A

PrgJ	MSIATIVPENAVIGQAVNIRSMETDIVSLDDRLLQAFSGSAIATAVDKQITNRIEDPNL	60
SsaG	-----MDIAQLVD-MLSHMAH-QA-----GQAINDKM-NGND	29
PrgI	-----MATPWSGYLDDVSAKFDTGVNDLQT-QV-----TEALD-KL-A-AK	37
	*: * :. :	::: ::
PrgJ	VTDPKELAISQEMISDYNLYVSMVSTLTKGVGAVETLLRS--	101
SsaG	LLNPESMIKAQFALQQYSTFINYESLIKMIKMLSGIIAKI-	71
PrgI	PSDPALLAAYQSKLSEYNLYRNAQSNTVKVFKDIDAAIQNFR	80
	::* : * :.:*.: . *. : . ::.	

B

S12 Fig. Sequence alignment and three-dimensional structural prediction of

SsaG. (A) The primary sequences of PrgJ, PrgI, and SsaG were aligned using Multiple Sequence Alignment by Clustal Omega. * indicates single, *fully conserved* residue, : indicates conservation between groups of *strongly* similar properties, and . indicates conservation between groups of *weakly* similar properties. Small, hydrophobic residues are indicated in red (AVFPMILW). Acidic residues are indicated in blue (DE). Basic residues are indicated in magenta (RK). The remaining residues are indicated in green (STYHCNGQ). (B) The three-dimensional structure of SsaG was predicted with high confidence and high coverage using the PHYRE2 server. The structure is colored from N to C terminus using the colors of the rainbow (red, orange, yellow, green, and blue).