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1 Title: Human NAIP/NLRC4 and NLRP3 inflammasomes detect *Salmonella* type III
2 secretion system activities to restrict intracellular bacterial replication
3
4 Short title: Human inflammasomes restrict *Salmonella* in macrophages
5
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21 Abstract

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

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43 and engagement of both the NAIP/NLRC4 and NLRP3 inflammasomes control

44 *Salmonella* infection in human macrophages.

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45 Author summary

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

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64 Introduction

65 *Salmonella enterica* serovar Typhimurium (referred to hereafter as *Salmonella*) is
66 a Gram-negative bacterial pathogen that causes self-limiting gastroenteritis in immune-
67 competent humans. Transmission of *Salmonella* typically occurs upon ingestion of
68 contaminated food or water. Once inside the host, *Salmonella* uses specialized
69 nanomachines known as type III secretion systems (T3SSs) to inject effectors into the
70 host cell cytosol [1]. Subsequently, these effectors remodel host cellular processes to
71 facilitate bacterial colonization. Thus, *Salmonella*'s T3SSs enable the enteric pathogen
72 to successfully colonize the intestinal tract and infect a variety of cell types, including
73 intestinal epithelial cells (IECs) and macrophages [1]. Specifically, *Salmonella* uses its
74 first T3SS, located on *Salmonella* Pathogenicity Island 1 (SPI-1), to invade host cells,
75 and its second T3SS, located on a second pathogenicity island, SPI-2, to persist and
76 replicate within host cells [2–8]. Numerous other Gram-negative bacterial pathogens
77 also use these evolutionarily conserved T3SSs to colonize the host [9]. While T3SSs
78 are required for these bacterial pathogens to cause disease, they also translocate
79 structural components of the T3SS or the flagellar apparatus into the cytosol, thus
80 enabling the host to detect the invading pathogen [10]. Unlike effectors, which display
81 significant diversity across bacterial species, structural components of the T3SS or the
82 flagellar apparatus retain significant structural homology across Gram-negative bacteria
83 [9,11]. Thus, these ligands serve as ideal targets of host immune sensors.

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

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87 [nucleotide-binding domain, leucine-rich repeat-containing] family, apoptosis inhibitory
88 proteins), recognize the structurally related SPI-1 T3SS needle protein, SPI-1 T3SS
89 inner rod protein, and flagellin, which are translocated into the host cell cytosol by the
90 SPI-1 T3SS [10,14,15]. Mice have multiple NAIPs, each specific to a particular ligand:
91 NAIP1 recognizes the T3SS needle protein, NAIP2 recognizes the T3SS inner rod
92 protein, and NAIP5 and NAIP6 both recognize flagellin [14–19]. Upon sensing a ligand,
93 NAIPs recruit the adaptor protein NLRC4 (nucleotide-binding domain, leucine-rich
94 repeat-containing family, CARD domain-containing protein 4) to form multimeric
95 signaling complexes called inflammasomes [20–22]. The NAIP/NLRC4 inflammasome
96 then recruits and activates the cysteine protease caspase-1 [23]. Active caspase-1
97 cleaves downstream substrates, including pro-IL-1 and pro-IL-18, as well as the pore-
98 forming protein gasdermin-D (GSDMD) [24–26]. Cleaved GSDMD creates pores in the
99 host plasma membrane, leading to the release of proinflammatory cytokines and an
100 inflammatory form of cell death known as pyroptosis, which effectively eliminates the
101 infected cell. The NAIP/NLRC4 inflammasome is critical for the control of *Salmonella*
102 infection in mice [27,28]. However, whether the NAIP/NLRC4 inflammasome recognizes
103 or controls *Salmonella* infection in humans has not been thoroughly investigated.
104 While mice express several different NAIPs that each respond to a particular
105 ligand, humans only express one functional NAIP [29,30]. In human macrophages, this
106 single NAIP is sufficient to respond to the cytosolic delivery of bacterial flagellin as well
107 as the SPI-1 T3SS inner rod (PrgJ) and needle (PrgI) proteins [31,32]. Interestingly, the
108 SPI-2 T3SS inner rod protein (SsaI) fails to induce inflammasome activation in both
109 murine and human macrophages [11,32], suggesting that the *Salmonella* SPI-2 T3SS

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

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

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128 Results

129

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

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

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

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

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

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174 the bacterial ligand alone released negligible levels of IL-1β, IL-18, and IL-1α and
175 exhibited minimal cell death (Fig. 1A, 1C, S3A-C, S4A-C). In agreement with previous
176 findings [32], WT THP-1s treated with both the PA and LFn subunits exhibited robust
177 inflammasome activation, and released substantial levels of IL-1β, IL-18, and IL-1α and
178 exhibited considerable cytotoxicity (Fig. 1A, 1C, S3A-C, S4A-C), indicating that robust
179 inflammasome activation requires cytosolic delivery of the ligands. In contrast, both
180 *NAIP*$^{-/-}$ THP-1s and *NLRC4*$^{-/-}$ THP-1s released negligible levels of inflammasome-
181 dependent cytokines and did not undergo cell death when treated with FlaTox, PrgJTox,
182 or YscFTox (Fig 1A, 1C, S3A-C, S4A-C). Importantly, the *NAIP*$^{-/-}$ and *NLRC4*$^{-/-}$ THP-1s
183 released IL-1β at levels comparable to those released by WT THP-1s in response to the
184 NLRP3 stimulus LPS + nigericin (Fig. 1B, 1D), indicating that CRISPR/Cas9 editing was
185 specific to the NAIP/NLRC4 inflammasome pathway [35]. In addition, release of the
186 inflammasome-independent cytokine TNF-α was unaffected in *NAIP*$^{-/-}$ or *NLRC4*$^{-/-}$ THP-
187 1s (Fig. S3D, S4D). Consistent with our prior results [32] and in agreement with recent
188 studies [36], these results collectively demonstrate that NAIP and NLRC4 are required
189 for inflammasome activation in response to the T3SS inner rod, T3SS needle, and
190 flagellin proteins in human macrophages.
191

192 **Fig 1. NAIP and NLRC4 are necessary for inflammasome responses to T3SS**
193 **ligands in human macrophages.** WT, *NAIP*$^{-/-}$ clone, or two independent clones of
194 *NLRC4*$^{-/-}$ THP-1 monocyte-derived macrophages were primed with 100 ng/mL
195 Pam3CSK4 for 16 hours. Cells were then treated with PBS (Mock), PA alone,
196 LFnFlaA$^{310–475}$ alone, LFnPrgJ alone, LFnYscF alone, PA+LFnFlaA$^{310–475}$ (FlaTox),

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197 PA+LFnPrgJ (PrgJTox), or PA+LFnYscF (YscFTox) for 6 hours (A, C). As a control,
198 cells were primed with 500 ng/mL LPS for 4 hours and treated with 10 μM nigericin for 6
199 hours (B, D). Release of IL-1β into the supernatant was measured by ELISA. ns – not
200 significant, **p* < 0.05, ***p* < 0.01, *****p* < 0.0001 by Šídák's multiple comparisons test
201 (A), or by unpaired t-test (B), or by Dunnett's multiple comparisons test (C, D). Data
202 shown are representative of at least three independent experiments.
203
204 NAIP and NLRC4 are partially required for inflammasome activation during *Salmonella*
205 infection of human macrophages
206 Human macrophages undergo SPI-1 T3SS-dependent inflammasome activation
207 during *Salmonella* infection [32]. To test whether this inflammasome activation requires
208 NAIP/NLRC4, we infected WT, *NAIP*$^{-/-}$, or *NLRC4*$^{-/-}$ THP-1 macrophages with WT
209 *Salmonella* (WT Stm) or *Salmonella* lacking its SPI-1 T3SS (Δ*sipB* Stm) and assayed
210 for subsequent inflammasome activation (Fig. 2, S5). WT THP-1s infected with WT Stm
211 released high levels of IL-1β, IL-18, and IL-1α and underwent cell death (Fig. 2, S5A-D).
212 This response was dependent on SPI-1 T3SS translocation into host cells, as cells
213 infected with Δ*sipB* Stm, which lack a component of the translocon, failed to undergo
214 robust inflammasome activation (Fig. 2, S5A-D). In *NAIP*$^{-/-}$ or *NLRC4*$^{-/-}$ THP-1
215 macrophages infected with WT Stm, we observed a significant decrease but not
216 complete abrogation of secreted IL-1β and IL-18 levels (Fig. 2), whereas levels of IL-1α
217 and cell death were largely unaffected (Fig. S5A–D). WT and *NAIP*$^{-/-}$ or *NLRC4*$^{-/-}$ THP-
218 1s released similar levels of the inflammasome-independent cytokine TNF-α (Fig. S5E,
219 S5F). Overall, these data indicate that NAIP and NLRC4 are partially required for

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220 inflammasome responses to *Salmonella* infection in human macrophages, in contrast to
221 what we observe with individual T3SS ligand delivery (Fig. 1, S3, S4), where
222 NAIP/NLRC4 is absolutely required for inflammasome activation. Thus, our data
223 indicate that in addition to the NAIP/NLRC4 inflammasome, *Salmonella* also induces a
224 NAIP/NLRC4-independent inflammasome response, in agreement with a recent study
225 [36].
226
227 **Fig 2. NAIP and NLRC4 are partially required for inflammasome activation during**
228 ***Salmonella* infection in human macrophages.** WT, *NAIP*$^{-/-}$ clone, or two independent
229 clones of *NLRC4*$^{-/-}$ THP-1 monocyte-derived macrophages were primed with 100 ng/mL
230 Pam3CSK4 for 16 hours. Cells were then infected with PBS (Mock), WT *S.*
231 Typhimurium, or Δ*sipB S*. Typhimurium for 6 hours. Release of IL-1β and IL-18 into the
232 supernatant were measured by ELISA. ns – not significant, ****p* < 0.001, *****p* < 0.0001
233 by Šídák's multiple comparisons test (A, C) or Dunnett's multiple comparisons test (B,
234 D). Data shown are representative of at least three independent experiments.
235
236 <u>*Salmonella* induces NAIP/NLRC4- and NLRP3-dependent inflammasome activation in</u>
237 <u>human macrophages</u>
238 In murine macrophages, *Salmonella* infection activates both the NAIP/NLRC4
239 and NLRP3 inflammasomes [37]. The NAIP/NLRC4 inflammasome is important for early
240 responses to *Salmonella* in the setting of SPI-1 activation, while the NLRP3
241 inflammasome is important at later timepoints following bacterial replication [38]. In
242 human THP-1s, *Salmonella* infection triggers recruitment of both NLRC4 and NLRP3 to

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243 the same macromolecular complex [38]. The NLRP3 inflammasome can be activated by
244 diverse stimuli during bacterial infection, such as potassium efflux [39]. To determine if
245 the NAIP/NLRC4-independent inflammasome response we observed in our *Salmonella*-
246 infected human macrophages is NLRP3-dependent, we infected WT, *NAIP*$^{-/-}$, or *NLRC4*$^{-}$
247 $^{/-}$ THP-1s with *Salmonella* in the presence of MCC950, a potent chemical inhibitor of the
248 NLRP3 inflammasome [40], or the vehicle control DMSO. We subsequently assayed for
249 inflammasome activation by measuring IL-1α, IL-1β, and IL-18 secretion (Fig. 3, S6).
250 WT THP-1s treated with DMSO control released substantial amounts of IL-1α, IL-1β,
251 and IL-18 when infected with WT Stm. In contrast, infected WT THP-1s treated with
252 MCC950 secreted decreased levels of IL-1α, IL-1β, and IL-18, which are comparable to
253 levels observed in WT Stm-infected *NAIP*$^{-/-}$ or *NLRC4*$^{-/-}$ THP-1s. (Fig. 3, S6A-D).
254 Interestingly, WT Stm-infected *NAIP*$^{-/-}$ or *NLRC4*$^{-/-}$ THP-1s treated with MCC950 largely
255 had significantly decreased IL-1α, IL-1β, and IL-18 secretion compared to infected
256 *NAIP*$^{-/-}$ or *NLRC4*$^{-/-}$ THP-1s treated with DMSO or infected WT THP-1s treated with
257 MCC950 (Fig. 3, S6A-D). Furthermore, *NAIP*$^{-/-}$ or *NLRC4*$^{-/-}$ THP-1s treated with
258 MCC950 secreted negligible levels of IL-1α, IL-1β, and IL-18, similar to those observed
259 during Δ*sipB* Stm infection (Fig. 3, S6A-D). WT, *NAIP*$^{-/-}$, and *NLRC4*$^{-/-}$ THP-1s
260 demonstrated robust IL-1α, IL-1β, and IL-18 secretion in response to LPS + nigericin
261 that was significantly reduced by MCC950 treatment, indicating that this inhibitor
262 effectively blocked NLRP3 inflammasome activation, as expected (Fig. 3, S6A-D).
263 Release of the inflammasome-independent cytokine TNF-α was similar across the
264 various THP-1 genotypes and treatments following infection (Fig. S6E, S6F). Altogether,

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265 these data indicate that *Salmonella* infection induces both NAIP/NLRC4- and NLRP3-
266 dependent inflammasome activation in human macrophages.
267
268 **Fig 3. *Salmonella* induces NAIP/NLRC4- and NLRP3-dependent inflammasome**
269 **activation in human macrophages.** WT, *NAIP*$^{-/-}$, or *NLRC4*$^{-/-}$ THP-1 monocyte-derived
270 macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. One hour prior to
271 infection, cells were treated with 1 μM MCC950, a chemical inhibitor of the NLRP3
272 inflammasome or DMSO as a control. Cells were then infected with PBS (Mock), WT *S*.
273 Typhimurium, or Δ*sipB S*. Typhimurium for 6 hours. As a control, cells were primed with
274 500 ng/mL LPS for 4 hours and treated with 10 μM nigericin for 6 hours. Release of IL-
275 1β into the supernatant was measured by ELISA. ns – not significant, *$p < 0.05$, **$p <$
276 0.01, ***$p < 0.001$, ****$p < 0.0001$ by Tukey's multiple comparisons test (A, C) or by
277 Šídák's multiple comparisons test (B, D). Data shown are representative of at least
278 three independent experiments.
279
280 <u>*Salmonella* induces NAIP/NLRC4- and CASP4/5-dependent inflammasome activation in</u>
281 <u>human macrophages</u>
282 In mice, in addition to the NAIP/NLRC4 and NLRP3 inflammasomes, *Salmonella*
283 infection can also activate the caspase-11 inflammasome [41]. Caspase-11 detects
284 cytosolic LPS and forms the noncanonical inflammasome, which secondarily activates
285 the NLRP3 inflammasome [33,42]. Caspases-4 and 5 are human orthologs of murine
286 caspase-11 [33], and they can also sense cytosolic LPS to form the noncanonical
287 inflammasome in human cells. We have previously observed caspase-4-dependent

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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-1β secretion in
295 response to WT Stm. WT THP-1s treated with either *CASP4* or *CASP5* siRNAs
296 exhibited significantly decreased IL-1β 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-1β 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-1β 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-1β 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-1β secretion, as

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310 expected [45] (Fig. 4B). Taken together, these data suggest that the caspase-4/5
311 inflammasome is involved in the NAIP-independent response to *Salmonella*.
312
313 **Fig 4. *Salmonella* induces NAIP- and CASP4/5-dependent inflammasome**
314 **activation in human macrophages.** WT or *NAIP*$^{-/-}$ THP-1 monocyte-derived
315 macrophages were treated with siRNA targeting a control scrambled siRNA, siRNA
316 targeting *CASP4* or *CASP5*, or siRNA targeting both *CASP4* and *CASP5* for 48 hours.
317 Cells were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were then infected
318 with PBS (Mock) or WT *S*. Typhimurium for 6 hours. Release of IL-1β into the
319 supernatant were measured by ELISA. As a control, cells were transfected with LPS. ns
320 – not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ by Tukey's multiple
321 comparisons test. Data shown are representative of at least three independent
322 experiments.
323
324 <u>The NAIP/NLRC4 and NLRP3 inflammasomes restrict *Salmonella* replication within</u>
325 <u>human macrophages</u>
326 One of the mechanisms by which inflammasome activation leads to control of
327 bacterial infection is by restricting intracellular bacterial replication. In mice, the
328 NAIP/NLRC4 inflammasome is important for controlling *Salmonella* replication in the
329 intestine [28], whereas the NLRP3 inflammasome is dispensable for control of
330 *Salmonella* infection *in vivo* [46,47]. Caspases-1 and 11 restrict cytosolic *Salmonella*
331 replication within murine macrophages [48]. Whether inflammasome activation restricts
332 WT *Salmonella* replication in human macrophages is unknown. To test the hypothesis

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333 that inflammasome activation restricts *Salmonella* replication within human
334 macrophages, we infected WT or *NAIP*$^{-/-}$ THP-1 macrophages with WT Stm in the
335 presence or absence of the NLRP3 inhibitor MCC950 and determined the bacterial
336 colony forming units (CFU) at various timepoints post-infection to assay bacterial
337 replication. At 2 hours post-infection, we did not observe any differences in bacterial
338 uptake between the different conditions (Fig. S8A). At 6 or 24 hours post-infection, the
339 bacterial burden was the lowest in WT THP-1s, whereas *NAIP*$^{-/-}$ THP-1s harbored
340 significantly higher bacterial burdens (Fig. 5A, S8B). WT THP-1s treated with MCC950
341 also contained a significantly higher number of bacterial CFUs, comparable to those in
342 *NAIP*$^{-/-}$ THP-1s (Fig. 5A, S8B). *NAIP*$^{-/-}$ THP-1s treated with MCC950 had the highest
343 bacterial burdens, which were significantly higher than the bacterial burdens in DMSO
344 control-treated *NAIP*$^{-/-}$ THP-1s or WT THP-s treated with MCC950 (Fig. 5A). We then
345 examined the fold-change in bacterial replication at 6 and 24 hours relative to 2 hours
346 post-infection. The fold-change in bacterial replication was restricted the most effectively
347 in WT THP-1s, moderately restricted in *NAIP*$^{-/-}$ THP-1s or WT THP-1s treated with
348 MCC950, and the least restricted in *NAIP*$^{-/-}$ THP-1s treated with MCC950 (Fig. 5B).
349 Collectively, these data suggest that both the NAIP/NLRC4 and NLRP3 inflammasomes
350 restrict intracellular *Salmonella* replication within human macrophages at both early (6
351 hours post-infection) and late (24 hours post-infection) timepoints.
352
353 **Fig 5. The NAIP and NLRP3 inflammasomes restrict *Salmonella* replication within**
354 **human macrophages.** WT or *NAIP*$^{-/-}$ THP-1 monocyte-derived macrophages were
355 primed with 100 ng/mL Pam3CSK4 for 16 hours. One hour prior to infection, cells were

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356 treated with 1 μM MCC950, a chemical inhibitor of the NLRP3 inflammasome or DMSO
357 as a control. Cells were then infected with PBS (Mock) or WT *S*. Typhimurium. Cells
358 were lysed at the indicated time points and bacteria were plated to calculate CFU. (A)
359 CFU/well of bacteria at 6 hpi (B) Fold change in CFU/well of bacteria at indicated time
360 point, relative to 2 hpi CFU/well. ns – not significant, ***$p < 0.001$, ****$p < 0.0001$ by
361 Dunnett's multiple comparisons test (A) or Tukey's multiple comparisons test (B). Data
362 shown are representative of at least three independent experiments.
363

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

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

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379 infection, escapes from its vacuole into the cytosol where it expresses the protein ActA
380 on its surface. Fusing bacterial ligands of interest to the N-terminus of truncated ActA
381 allows these ligands to be delivered into the host cytosol, where they trigger
382 NAIP/NLRC4 inflammasome activation [32,53]. We infected hMDMs with WT *Listeria*
383 (Lm) or *Listeria* expressing PrgJ, SsaI, or SsaG and assayed for inflammasome
384 activation (Fig. 6A, S9). hMDMs infected with *Listeria* expressing the SPI-1 T3SS inner
385 rod protein PrgJ induced robust inflammasome activation, indicated by significantly
386 increased IL-18 secretion as well as robust IL-1α and IL-1β secretion compared to mock
387 infection or WT Lm infection alone (Fig. 6A, S9), in agreement with our previous findings
388 [32]. In contrast, and as we previously observed [32], *Listeria* expressing the SPI-2 inner
389 rod protein SsaI failed to induce IL-1β, IL-18, and IL-1α secretion or cell death in
390 hMDMs (Fig. 6A, S9). Intriguingly, we observed that *Listeria* expressing the SPI-2
391 needle protein SsaG induced significantly increased IL-18 and robust IL-1α and IL-1β
392 secretion compared to mock infection or WT Lm infection alone (Fig. 6A, S9).
393 To test whether NAIP or NLRC4 are required for inflammasome responses to
394 SsaG, we infected WT, *NAIP*$^{-/-}$, and *NLRC4*$^{-/-}$ THP-1s with WT *Listeria* (Lm) or *Listeria*
395 expressing PrgI or SsaG and assayed for subsequent inflammasome activation by
396 measuring levels of IL-1β, IL-18, and IL-1α secretion and cell death (Fig. 6B, S10).
397 Infection of WT THP-1s with *Listeria* expressing PrgI or SsaG led to robust release of
398 IL-1 cytokines and cytotoxicity. In contrast, *NAIP*$^{-/-}$ and *NLRC4*$^{-/-}$ THP-1s infected with
399 *Listeria* expressing PrgI or SsaG released significantly reduced levels of IL-1 cytokines
400 and cell death relative to WT THP-1s that were comparable to the background levels
401 secreted by THP-1s infected with WT Lm (Fig. 6B, S10). Altogether, these data

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402 demonstrate that the SPI-2 needle protein activates the human NAIP/NLRC4
403 inflammasome, providing evidence that human NAIP can sense and respond to the
404 *Salmonella* SPI-2 T3SS.
405
406 **Fig 6. *Salmonella* SPI-2 needle protein SsaG activates the NAIP/NLRC4**
407 **inflammasome in human macrophages.** (A) Primary hMDMs from four healthy human
408 donors were infected with PBS (Mock), WT *Listeria (*WT Lm*)*, *Listeria* expressing PrgJ
409 (Lm + PrgJ), SsaI (Lm + SsaI), or SsaG (Lm + SsaG) for 16 hours at MOI=5. Release of
410 IL-18 into the supernatant was measured by ELISA. Each dot represents the mean of
411 individual donors derived from triplicate wells. The grey bar represents the mean of all
412 donors. (B) WT or *NAIP*$^{-/-}$, *NLRC4*$^{-/-}$ THP-1 monocyte-derived macrophages were
413 primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were treated with PBS (Mock),
414 WT *Listeria (*WT Lm*)*, *Listeria* expressing PrgI (Lm + PrgI), or SsaG (Lm + SsaG) for 6
415 hours at MOI=20. Release of IL-1β into the supernatant was measured by ELISA. ns –
416 not significant, ***p < 0.001, ****p < 0.0001 paired t-test (A) or by Tukey's multiple
417 comparisons test (B). Data shown are representative of at least three independent
418 experiments.
419
420 <u>NAIP/NLRC4 inflammasome recognition of the SPI-2 T3SS restricts *Salmonella*</u>
421 <u>replication in human macrophages</u>
422 To determine if NAIP/NLRC4-mediated recognition of the SPI-2 T3SS needle
423 restricts *Salmonella* intracellular replication, we generated a *Salmonella* mutant strain
424 (Δ*prgIfliCfljB*) lacking flagellin and the SPI-1 T3SS needle protein, PrgI. This strain is

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425 therefore unable to assemble a functional SPI-1 T3SS, but still expresses a functional
426 SPI-2 T3SS. We infected WT or *NAIP*$^{-/-}$ THP-1 macrophages with Δ*prgIfliCfljB* and
427 determined the CFUs at various timepoints to assay bacterial replication (Fig. 7, S11).
428 Bacterial replication of Δ*prgIfliCfljB* over a 24-hour post-infection time course was
429 restricted the most effectively in WT THP-1s and was significantly less restricted in
430 *NAIP*$^{-/-}$ THP-1s (Fig. 7, S11). Collectively, our data suggest that there is SPI-1
431 T3SS/flagellin-independent, NAIP/NLRC4 inflammasome-dependent control of
432 *Salmonella* replication in human macrophages, and that NAIP/NLRC4 recognition of the
433 SPI-2 T3SS needle SsaG mediates such restriction of *Salmonella* replication in human
434 macrophages.
435

436 **Fig 7. NAIP/NLRC4 inflammasome recognition of the SPI-2 T3SS restricts**
437 ***Salmonella* replication in human macrophages.** WT or *NAIP*$^{-/-}$ THP-1 monocyte-
438 derived macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were
439 then infected with PBS (Mock) or Δ*prgIfliCfljB S*. Typhimurium. Cells were lysed at the
440 indicated time points and bacteria were plated to calculate CFU. Fold change in
441 CFU/well of bacteria at indicated time point, relative to 2 hpi CFU/well. ns – not
442 significant, ***$p < 0.001$, ****$p < 0.0001$ by Tukey's multiple comparisons test.

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443 ## Discussion

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

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

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

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

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

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

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

530 The NLRP3 inflammasome can be activated by a variety of different stimuli,
531 including potassium efflux. It can also be activated downstream of the CASP4/5
532 inflammasome [42,45], leading to non-canonical NLRP3 inflammasome activation.
533 Given that we observed only partial loss of inflammasome activation in the *NAIP*$^{-/-}$ THP-
534 1s treated with siRNA targeting *CASP4* and *CASP5*, we hypothesize that at least part of

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535 the NLRP3-dependent response is due to canonical activation (Fig. 4), although this
536 partial loss may also be due to incomplete knockdown of *CASP4* and *CASP5*. A recent
537 study also found that *Salmonella* infection induces NLRC4- and NLRP3-dependent
538 inflammasome activation in human macrophages, and observed that full-length
539 *Salmonella* flagellin can activate the NLRP3 inflammasome [36]. In contrast, we found
540 the response to flagellin to be entirely dependent on the NAIP/NLRC4 inflammasome
541 (Fig. 1, S3, S4). The reason for this apparent discrepancy is unclear, but in our studies,
542 we used a truncated flagellin that only contains the C-terminal D0 domain and thus does
543 not stimulate TLR5 signaling [56,68]. It is possible that full-length flagellin, in addition to
544 activating the NAIP/NLRC4 inflammasome, also stimulates TLR5 signaling, perhaps
545 potentiating NLRP3-dependent responses.
546 We observed NAIP/NLRC4- and NLRP3-dependent restriction of *Salmonella*
547 (Fig. 5, S8), but the mechanism by which inflammasome activation promotes bacterial
548 restriction is unclear. Inflammasome activation often triggers host cell death, thereby
549 eliminating the pathogen's intracellular replicative niche. *In vivo*, pyroptosis can trigger
550 formation of pore-induced intracellular traps (PITs). These PITs can trap intracellular
551 bacteria that can subsequently be efferocytosed by neutrophils [69]. However, in murine
552 macrophages, inhibition of *Salmonella* replication by caspase-1 and caspase-11 occurs
553 prior to host cell death, indicating that caspase-1 and caspase-11 restrict *Salmonella*
554 through a mechanism distinct from cell death [48]. Another mechanism of
555 inflammasome-dependent restriction may be through promoting phagolysomal
556 maturation. In murine macrophages infected with *Legionella*, NAIP5 activation results in
557 increased colocalization of *Legionella*-containing vacuoles with the lysosomal markers

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558 cathepsin-D and Lamp-1 [70,71]. Perhaps a similar process occurs during *Salmonella*
559 infection of human macrophages.
560 The current model is that the SPI-2 T3SS subverts inflammasome activation to
561 facilitate *Salmonella*'s intracellular survival, based on the observation that the SPI-2
562 T3SS inner rod SsaI is not detected in murine or human macrophages [11,32].
563 Moreover, the SPI-2 T3SS effectors are critical for biogenesis and maintenance of the
564 SCV [52]. Thus, evasion of inflammasome activation by the SPI-2 T3SS was thought to
565 confer an advantage to the pathogen. However, our findings indicate that the
566 NAIP/NLRC4 inflammasome detects the *Salmonella* SPI-2 T3SS needle protein SsaG.
567 Furthermore, we find that SPI-1-independent, flagellin-independent, NAIP-dependent
568 detection of *Salmonella* mediates restriction of intracellular bacterial replication in
569 human macrophages. Perhaps the NAIP/NLRC4-mediated detection of SsaG is a
570 consequence of a functional constraint placed upon SsaG's role as a T3SS needle
571 protein. It is thus possible that SsaG is unable to evade immune detection due to such
572 functional constraints.
573 While we focused here primarily on inflammasome responses in human
574 macrophages, *Salmonella*'s first cellular encounters are with intestinal epithelial cells. In
575 mice, NAIP/NLRC4 inflammasome activation in intestinal epithelial cells results in
576 extrusion of infected cells from the epithelial layer [27,28]. It has been proposed that this
577 mechanism eliminates *Salmonella* from the host and helps control bacterial burdens.
578 Whether similar NAIP/NLRC4-dependent mechanisms are engaged in human intestinal
579 epithelial cells remains to be elucidated.

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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.

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591 ## Materials and Methods

592

593 ### Ethics statement

594 All studies involving primary human monocyte-derived macrophages (hMDMs)
595 were performed in compliance with the requirements of the US Department of Health
596 and Human Services and the principles expressed in the Declaration of Helsinki.
597 hMDMs were derived from samples obtained from the University of Pennsylvania
598 Human Immunology Core. These samples are considered to be a secondary use of
599 deidentified human specimens and are exempt via Title 55 Part 46, Subpart A of 46.101
600 (b) of the Code of Federal Regulations.

601

602 ### Bacterial strains and growth conditions

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

608 WT, Δ*sipB* [74], and Δ*prgIfliCfljB* isogenic strains were routinely grown overnight
609 in Luria-Bertani (LB) broth with streptomycin (100 μg/ml) at 37°C. For infection of
610 cultured cells, overnight cultures were diluted in LB containing 300 mM NaCl and grown
611 standing for 3 hours at 37°C to induce SPI-1 expression [75].

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

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614 heterologous bacterial ligand *S.* Typhimurium PrgJ translationally fused to the truncated
615 N-terminus of ActA and under the control of the *actA* promoter was used [53]. The
616 *Listeria* strains expressing *S.* Typhimurium SsaI and SsaG were constructed using
617 codon-optimized gene fragments (IDT) cloned into the pPL2 vector and introduced into
618 *Listeria* as previously described [53,76].
619
620 Cell culture of THP-1s
621 THP-1 cells (TIB-202; American Type Culture Collection) were maintained in
622 RPMI supplemented with 10% (vol/vol) heat-inactivated FBS, 0.05 nM β-
623 mercaptoethanol, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C in a
624 humidified incubator. Two days before experimentation, the cells were replated in media
625 without antibiotics in a 48-well plate at a concentration of 2 × 10^5 cells/well and
626 incubated with phorbol 12-myristate 13-acetate (PMA) for 24 hours to allow
627 differentiation into macrophages. Macrophages were primed with 100 ng/mL
628 Pam3CSK4 (Invivogen) for 16 hours prior to bacterial infections or anthrax toxin
629 treatments. For experiments involving LPS, cells were pretreated with 500 ng/mL LPS
630 (Sigma-Aldrich) for 3 hours. For experiments involving Nigericin, cells were treated with
631 10 µM Nigericin (EMD Millipore) for 6 hours. For experiments involving MCC950, cells
632 were treated with 1 µM MCC950 (Sigma Aldrich) 1 hour prior to infection.
633
634 Cell culture of primary human monocyte-derived macrophages (hMDMs)
635 Purified human monocytes from de-identified healthy human donors were
636 obtained from the University of Pennsylvania Human Immunology Core. Monocytes

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637 were cultured in RPMI supplemented with 10% (vol/vol) heat-inactivated FBS, 2 mM L-
638 glutamine, 100 IU/mL penicillin, 100 µg/ml streptomycin, and 50 ng/ml recombinant
639 human M-CSF (Gemini Bio-Products) for 6 days to promote differentiation into hMDMs.
640 One day prior to infection, adherent hMDMs were replated in media with 25 ng/ml
641 human M-CSF lacking antibiotics at 1.0×10^5 cells/well in a 48-well plate.
642

643 Bacterial infections

644 Overnight cultures of *Salmonella* were diluted into LB broth containing 300 mM
645 NaCl and grown for 3 hours standing at 37°C to induce SPI-1 expression [75]. Overnight
646 cultures of *L. monocytogenes* were diluted and grown for 3 hours in BHI. All cultures
647 were pelleted at 6,010 × *g* for 3 minutes, washed once with PBS, and resuspended in
648 PBS. THP-1 cells were infected with *S.* Typhimurium or *L. monocytogenes* at a
649 multiplicity of infection (MOI) of 20. hMDMs were infected with *L. monocytogenes* at an
650 MOI of 5. Infected cells were centrifuged at 290 × *g* for 10 min and incubated at 37°C. 1
651 hour post-infection, cells were treated with 100 ng/mL or 50 ng/mL of gentamicin to kill
652 any extracellular *S.* Typhimurium or *L. monocytogenes* respectively. *Salmonella* and
653 *Listeria* infections in THP-1s proceeded at 37°C for 6 hours. *Listeria* infection of hMDMs
654 proceeded at 37°C for 16 hours. For all experiments, control cells were mock-infected
655 with PBS.
656

657 Anthrax toxin-mediated delivery of bacterial ligands

658 Recombinant proteins (PA, LFn-FlaA$^{310\text{-}475}$, LFn-PrgJ, and LFn-YscF) were kindly
659 provided by Russell Vance [18]. PA and LFn doses for *in vitro* delivery were: 1 µg/ml PA

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660 for FlaTox; 4 μg/ml PA for PrgJTox and YscFTox; 500 ng/ml LFn-FlaA$^{310-475}$; 8 ng/ml
661 LFn-PrgJ; and 200 ng/mL LFn-YscF.
662
663 siRNA-mediated knockdown of genes
664 All Silencer Select siRNA oligos were purchased from Ambion (Life
665 Technologies). For *CASP4*, siRNA ID# s2412 was used. For *CASP5*, siRNA ID# s2417
666 was used. The two Silencer Select negative control siRNAs (Silencer Select Negative
667 Control No. 1 siRNA and Silencer Select Negative Control No. 2 siRNA) were used as a
668 control. Two days before infection, 30 nM of siRNA was transfected into macrophages
669 using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) following
670 the manufacturer's protocol. 16 hours before infection, the media was replaced with
671 fresh antibiotic-free media containing 100 ng/ml Pam3CSK4. In parallel, siRNA-
672 transfected cells were also transfected with 2 μg/ml of *E. coli* LPS strain W3110 (kindly
673 provided by Robert Ernst) using FuGENE HD transfection reagent (Promega) for 6
674 hours.
675
676 Bacterial intracellular replication assay
677 Cells were infected with WT or Δ*prgIfliCfljB S.* Typhimurium as usual at an MOI
678 of 20. 1 hour post-infection, cells were treated with 100 μg/ml of gentamicin to kill any
679 extracellular bacteria. 2 hours post-infection, the media was replaced with fresh media
680 containing 10 μg/ml of gentamicin. At the indicated time points, cells were lysed with
681 PBS containing 0.5% Triton to collect all intracellular bacteria. Harvested bacteria were
682 serially diluted in PBS and plated on LB agar with streptomycin (100 μg/ml) plates to

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683 enumerate colony forming units (CFUs). Plates were incubated at 37°C overnight and
684 then CFUs were counted.
685
686 ELISAs
687 Harvested supernatants from infected cells were assayed using ELISA kits for
688 human IL-1α (R&D Systems), IL-18 (R&D Systems), IL-1β (BD Biosciences), and TNF-α
689 (R&D Systems).
690
691 LDH cytotoxicity assays
692 Harvested supernatants from infected cells were assayed for cytotoxicity by
693 measuring loss of cellular membrane integrity via lactate dehydrogenase (LDH) activity.
694 LDH release was quantified using an LDH Cytotoxicity Detection Kit (Clontech)
695 according to the manufacturer's instructions and normalized to mock-infected cells.
696
697 Quantitative RT-PCR analysis
698 RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) following the
699 manufacturer's instructions. Cells were lysed in 350 μL RLT buffer with β-
700 mercaptoethanol and centrifuged through a QIAshredder spin column (Qiagen). cDNA
701 was synthesized from isolated RNA using SuperScript II Reverse Transcriptase
702 (Invitrogen) following the manufacturer's protocol. Quantitative PCR was conducted with
703 the CFX96 real-time system from Bio-Rad using the SsoFast EvaGreen Supermix with
704 Low ROX (Bio-Rad). For analysis, mRNA levels of siRNA-treated cells were normalized
705 to housekeeping gene *HPRT* and control siRNA-treated cells using the $2^{-\Delta\Delta CT}$ (cycle

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706 threshold) method [77] to calculate knockdown efficiency. The following primers from
707 PrimerBank were used. The PrimerBank identifications are *CASP4* (73622124c1), and
708 *CASP5* (209870072c2), and *HPRT* (164518913c1); all 5′–3′:
709 *CASP4* forward: CAAGAGAAGCAACGTATGGCA
710 *CASP4* reverse: AGGCAGATGGTCAAACTCTGTA
711 *CASP5* forward: TTCAACACCACATAACGTGTCC
712 *CASP5* reverse: GTCAAGGTTGCTCGTTCTATGG
713 *HPRT* forward: CCTGGCGTCGTGATTAGTGAT
714 *HPRT* reverse: AGACGTTCAGTCCTGTCCATAA
715
716 Statistical analysis
717 Prism 9.1.1 (GraphPad Software) was utilized for the graphing of data and all statistical
718 analyses. Statistical significance for experiments with THP-1 cells was determined using
719 the appropriate test and are indicated in each figure legend. Differences were
720 considered statistically significant if the p value was <0.05.

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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.

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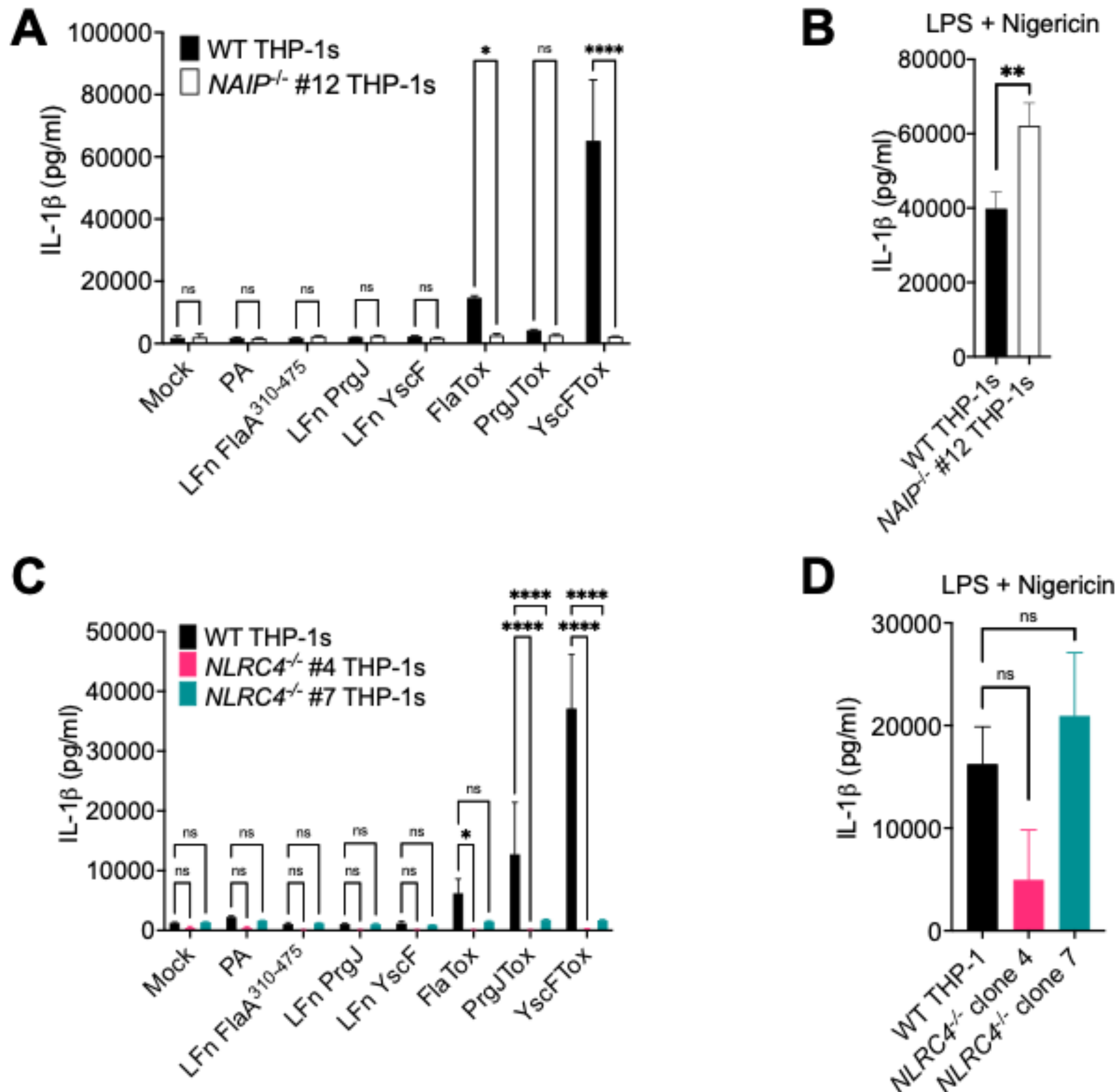
955 Main Body Figures and Figure Legends



956

957 **Fig 1. NAIP and NLRC4 are necessary for inflammasome responses to T3SS**

958 **ligands in human macrophages.** WT, *NAIP*$^{-/-}$ clone, or two independent clones of

959 *NLRC4*$^{-/-}$ THP-1 monocyte-derived macrophages were primed with 100 ng/mL

960 Pam3CSK4 for 16 hours. Cells were then treated with PBS (Mock), PA alone,

961 LFnFlaA$^{310–475}$ alone, LFnPrgJ alone, LFnYscF alone, PA+LFnFlaA$^{310–475}$ (FlaTox),

962 PA+LFnPrgJ (PrgJTox), or PA+LFnYscF (YscFTox) for 6 hours (A, C). As a control,

963 cells were primed with 500 ng/mL LPS for 4 hours and treated with 10 μM nigericin for 6

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964 hours (B, D). Release of IL-1β 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.

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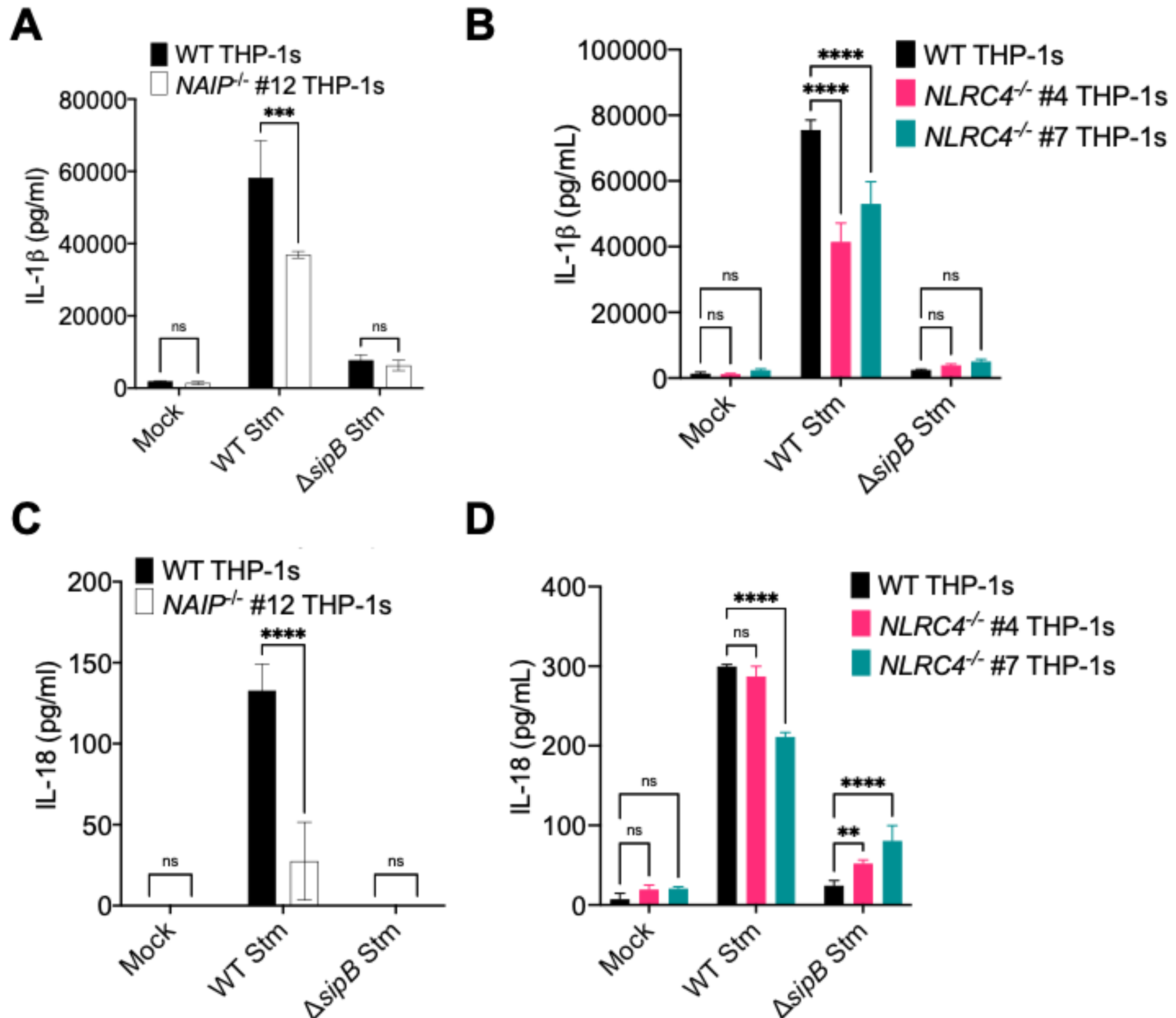

968

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

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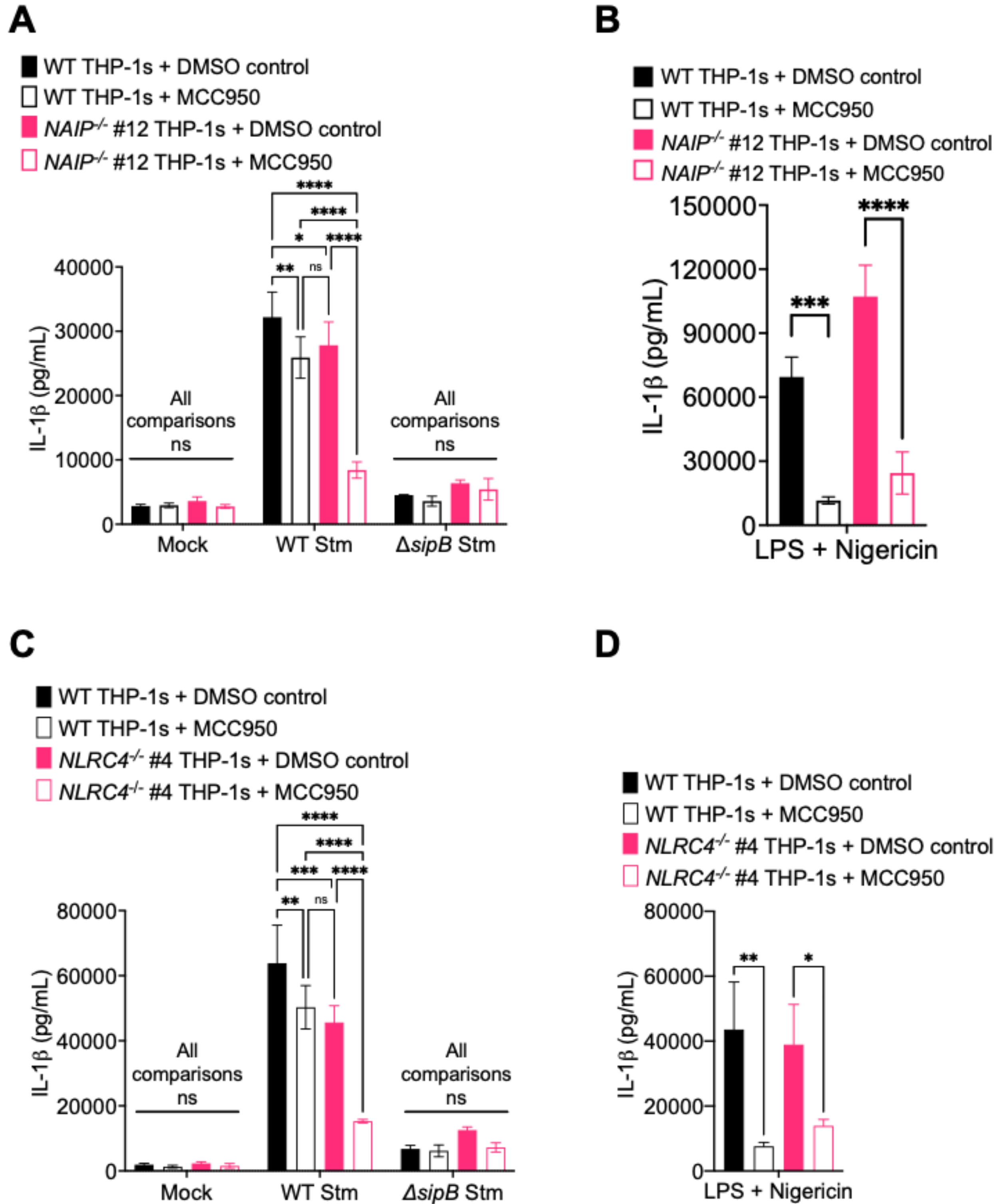

977

978 **Fig 3. *Salmonella* induces NAIP/NLRC4- and NLRP3-dependent inflammasome**

979 **activation in human macrophages.** WT, *NAIP*$^{-/-}$, or *NLRC4*$^{-/-}$ THP-1 monocyte-derived

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980 macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. One hour prior to
981 infection, cells were treated with 1 µM 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 Δ*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 µM nigericin for 6 hours. Release of IL-
985 1β 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.

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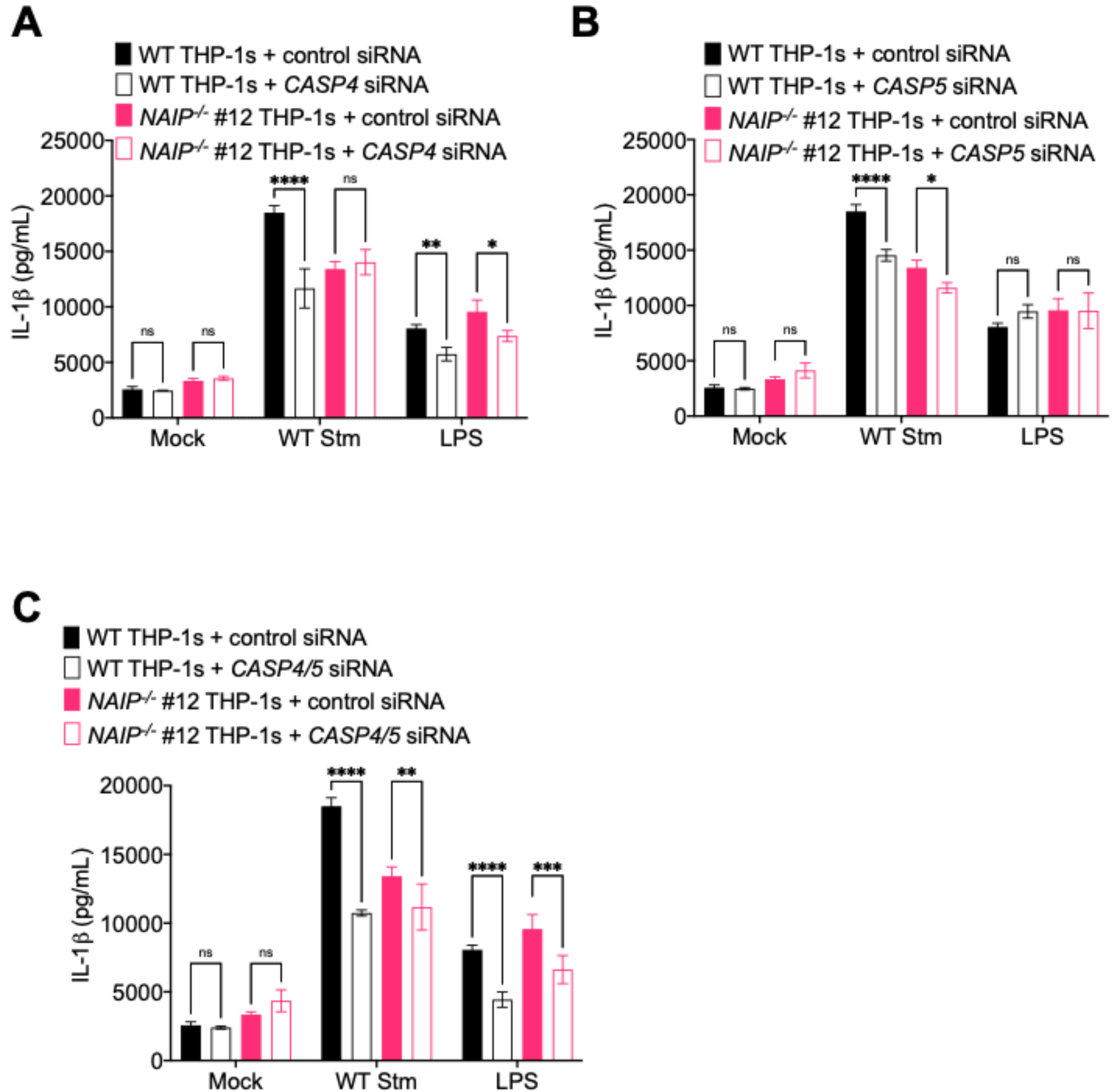

989

990 **Fig 4. *Salmonella* induces NAIP- and CASP4/5-dependent inflammasome**
991 **activation in human macrophages.** WT or *NAIP*$^{-/-}$ THP-1 monocyte-derived
992 macrophages were treated with siRNA targeting a control scrambled siRNA, siRNA
993 targeting *CASP4* or *CASP5*, or siRNA targeting both *CASP4* and *CASP5* for 48 hours.
994 Cells were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were then infected
995 with PBS (Mock) or WT *S*. Typhimurium for 6 hours. Release of IL-1β into the

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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.

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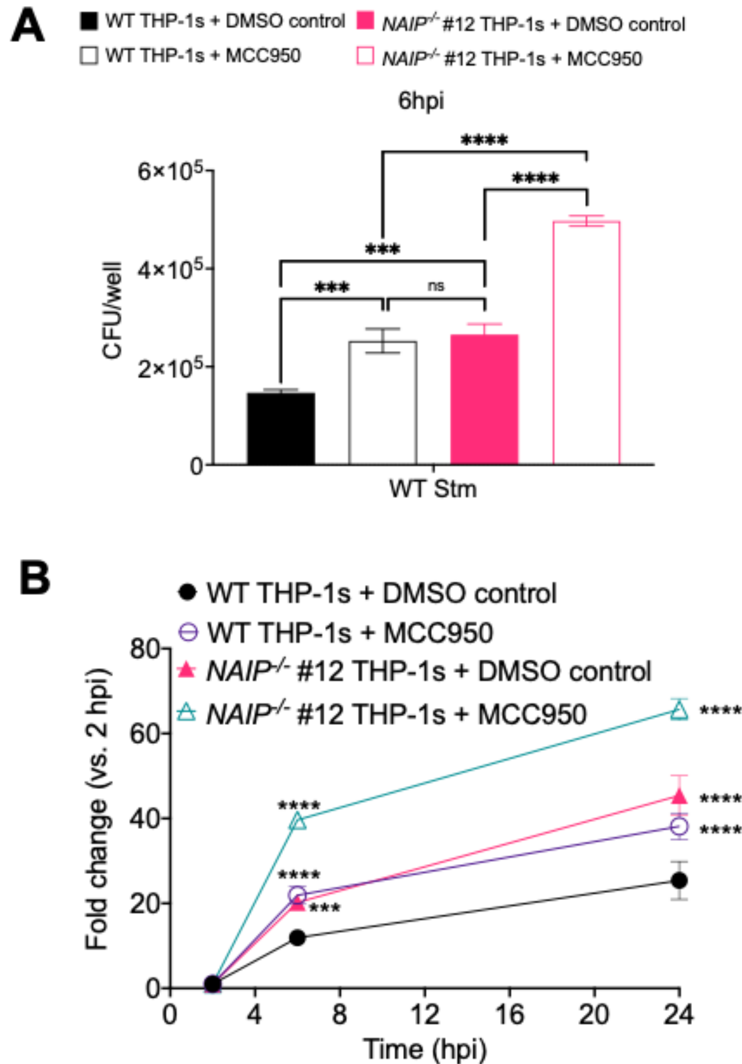

1000

1001 **Fig 5. The NAIP and NLRP3 inflammasomes restrict *Salmonella* replication within**
1002 **human macrophages.** WT or *NAIP*$^{-/-}$ THP-1 monocyte-derived macrophages were
1003 primed with 100 ng/mL Pam3CSK4 for 16 hours. One hour prior to infection, cells were
1004 treated with 1 μM MCC950, a chemical inhibitor of the NLRP3 inflammasome or DMSO
1005 as a control. Cells were then infected with PBS (Mock) or WT *S*. Typhimurium. Cells
1006 were lysed at the indicated time points and bacteria were plated to calculate CFU. (A)
1007 CFU/well of bacteria at 6 hpi (B) Fold change in CFU/well of bacteria at indicated time
1008 point, relative to 2 hpi CFU/well. ns – not significant, ***p < 0.001, ****p < 0.0001 by

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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.

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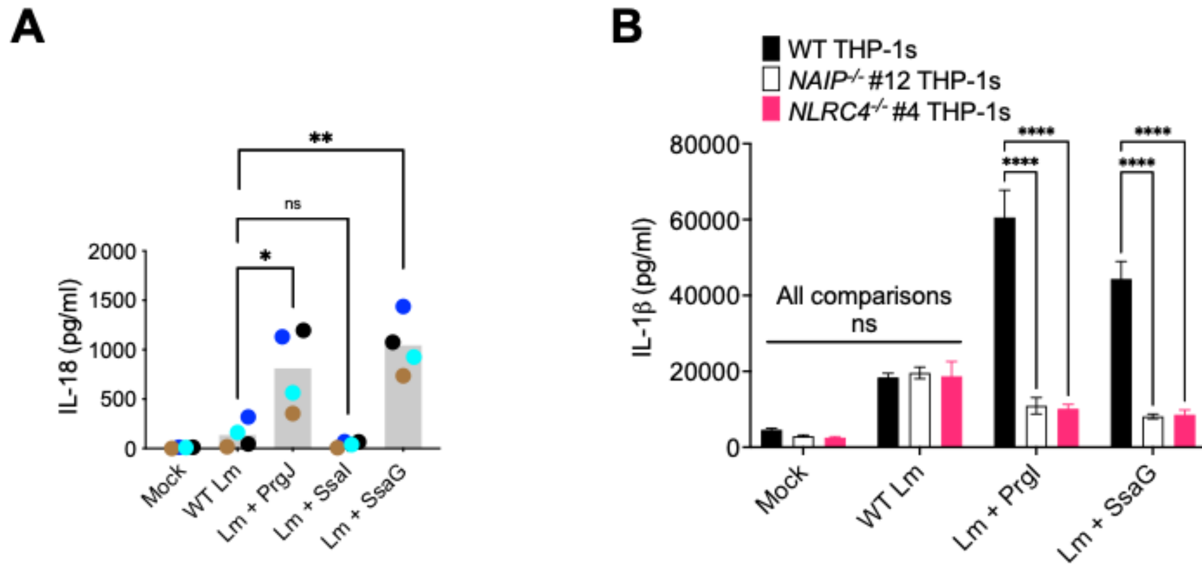

1011

1012 **Fig 6. *Salmonella* SPI-2 needle protein SsaG activates the NAIP/NLRC4**

1013 **inflammasome in human macrophages.** (A) Primary hMDMs from four healthy human

1014 donors were infected with PBS (Mock), WT *Listeria (*WT Lm*)*, *Listeria* expressing PrgJ

1015 (Lm + PrgJ), SsaI (Lm + SsaI), or SsaG (Lm + SsaG) for 16 hours at MOI=5. Release of

1016 IL-18 into the supernatant was measured by ELISA. Each dot represents the mean of

1017 individual donors derived from triplicate wells. The grey bar represents the mean of all

1018 donors. (B) WT or *NAIP*$^{-/-}$, *NLRC4*$^{-/-}$ THP-1 monocyte-derived macrophages were

1019 primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were treated with PBS (Mock),

1020 WT *Listeria (*WT Lm*)*, *Listeria* expressing PrgI (Lm + PrgI), or SsaG (Lm + SsaG) for 6

1021 hours at MOI=20. Release of IL-1β into the supernatant was measured by ELISA. ns –

1022 not significant, * $p < 0.05$, ** $p < 0.01$, ****$p < 0.0001$ paired t-test (A) or by Dunnett's

1023 multiple comparisons test (B). Data shown are representative of at least three

1024 independent experiments.

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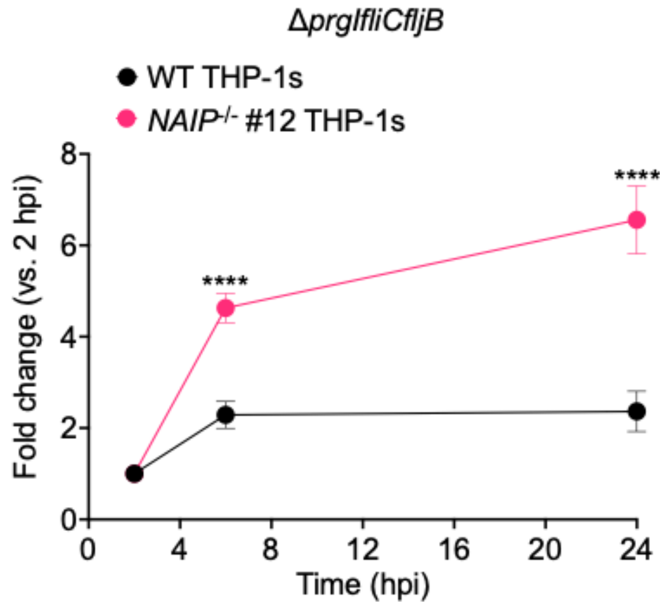

1025

1026 **Fig 7. NAIP/NLRC4 inflammasome recognition of the SPI-2 T3SS restricts**

1027 ***Salmonella*** **replication in human macrophages.** WT or *NAIP*$^{-/-}$ THP-1 monocyte-

1028 derived macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were

1029 then infected with PBS (Mock) or Δ*prgIfliCfljB S*. Typhimurium. Cells were lysed at the

1030 indicated time points and bacteria were plated to calculate CFU. Fold change in

1031 CFU/well of bacteria at indicated time point, relative to 2 hpi CFU/well. ns – not

1032 significant, ****p* < 0.001, *****p* < 0.0001 by Tukey's multiple comparisons test.

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1033 Supporting Information

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

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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.

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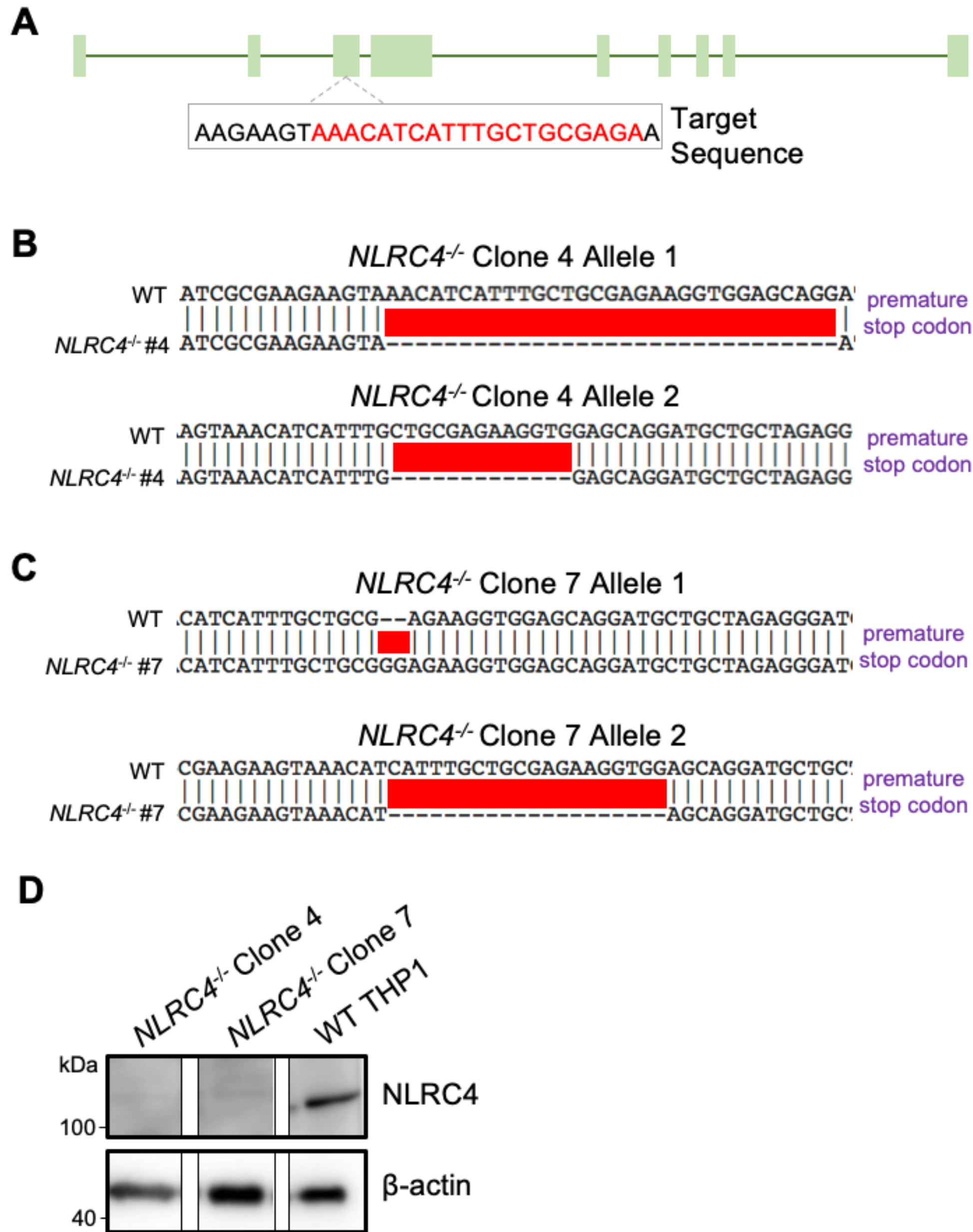

1044

1045 **S2 Fig. Validation of *NLRC4* mutant THP-1 single cell clones generated with**
1046 **CRISPR/Cas9-mediated genome editing.** (A) Schematic representation of the *NLRC4*
1047 gene with exons (filled boxes) and introns (lines). gRNA target sequence is highlighted
1048 in red. (B-C) Sequence alignments of WT THP-1 and *NLRC4*$^{-/-}$ clones are shown for
1049 both alleles per clone. Red boxes highlight the mutated region. Purple text represents

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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.

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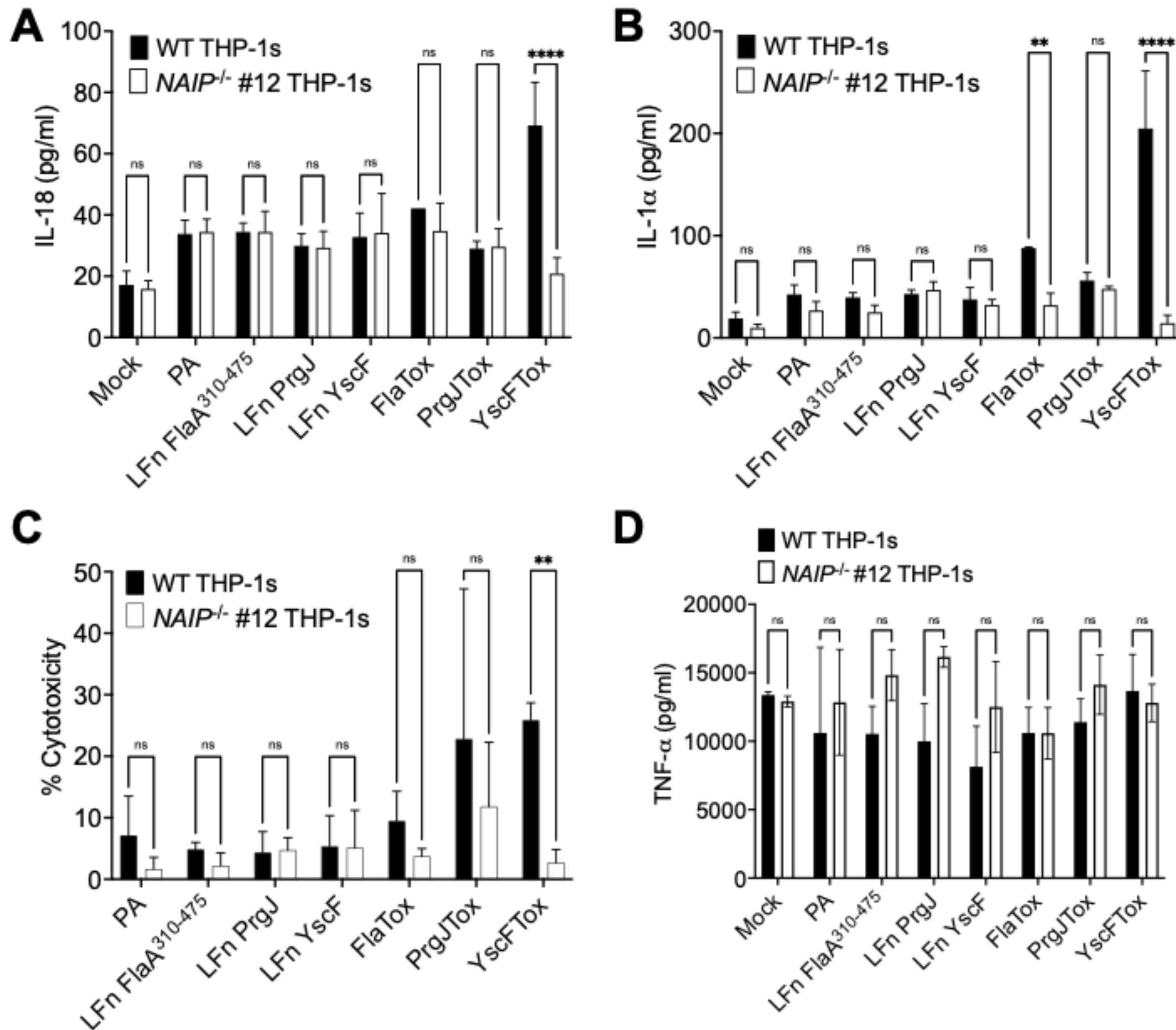

1053

1054 **S3 Fig. (related to Figure 1) NAIP is necessary for inflammasome responses to**

1055 **T3SS ligands in human macrophages.** WT or *NAIP*$^{-/-}$ THP-1 monocyte-derived

1056 macrophages were primed with 100 ng/ml Pam3CSK4 for 16 hours. Cells were then

1057 treated with PBS (Mock), PA alone, LFn FlaA$^{310–475}$ alone, LFn PrgJ alone, LF YscF

1058 alone, PA+LFn FlaA$^{310–475}$ (FlaTox), PA+LFn PrgJ (PrgJTox), or PA+LFn YscF

1059 (YscFTox) for 6 hours. (A, B, D) Release of cytokines IL-18, IL-1α, and TNF-α into the

1060 supernatant were measured by ELISA. (C) Cell death (percentage cytotoxicity) was

1061 measured by lactate dehydrogenase release assay and normalized to Mock-treated

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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.

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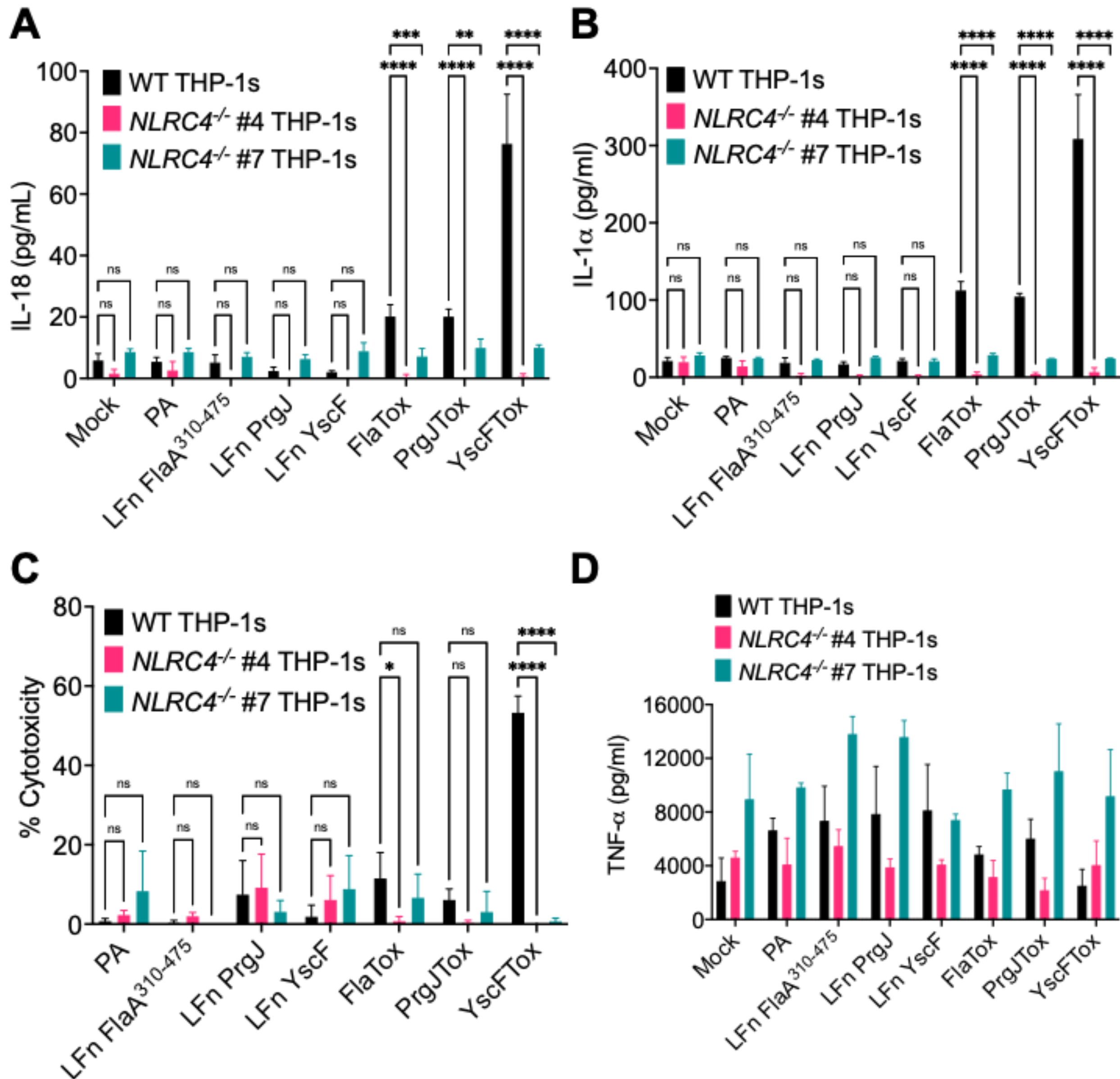

1064

1065 **S4 Fig. (related to Figure 1) NLRC4 is necessary for inflammasome responses to**

1066 **T3SS ligands in human macrophages.** WT or two independent clones of *NLRC4*$^{-/-}$

1067 THP-1 monocyte-derived macrophages were primed with 100 ng/ml Pam3CSK4 for 16

1068 hours. Cells were then treated with PBS (Mock), PA alone, LFn FlaA$^{310–475}$ alone, LFn

1069 PrgJ alone, LFn YscF alone, PA+LFn FlaA$^{310–475}$ (FlaTox), PA+LFn PrgJ (PrgJTox), or

1070 PA+LFn YscF (YscFTox) for 6 hours. (A, B, D) Release of cytokines IL-18, IL-1α, and

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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.

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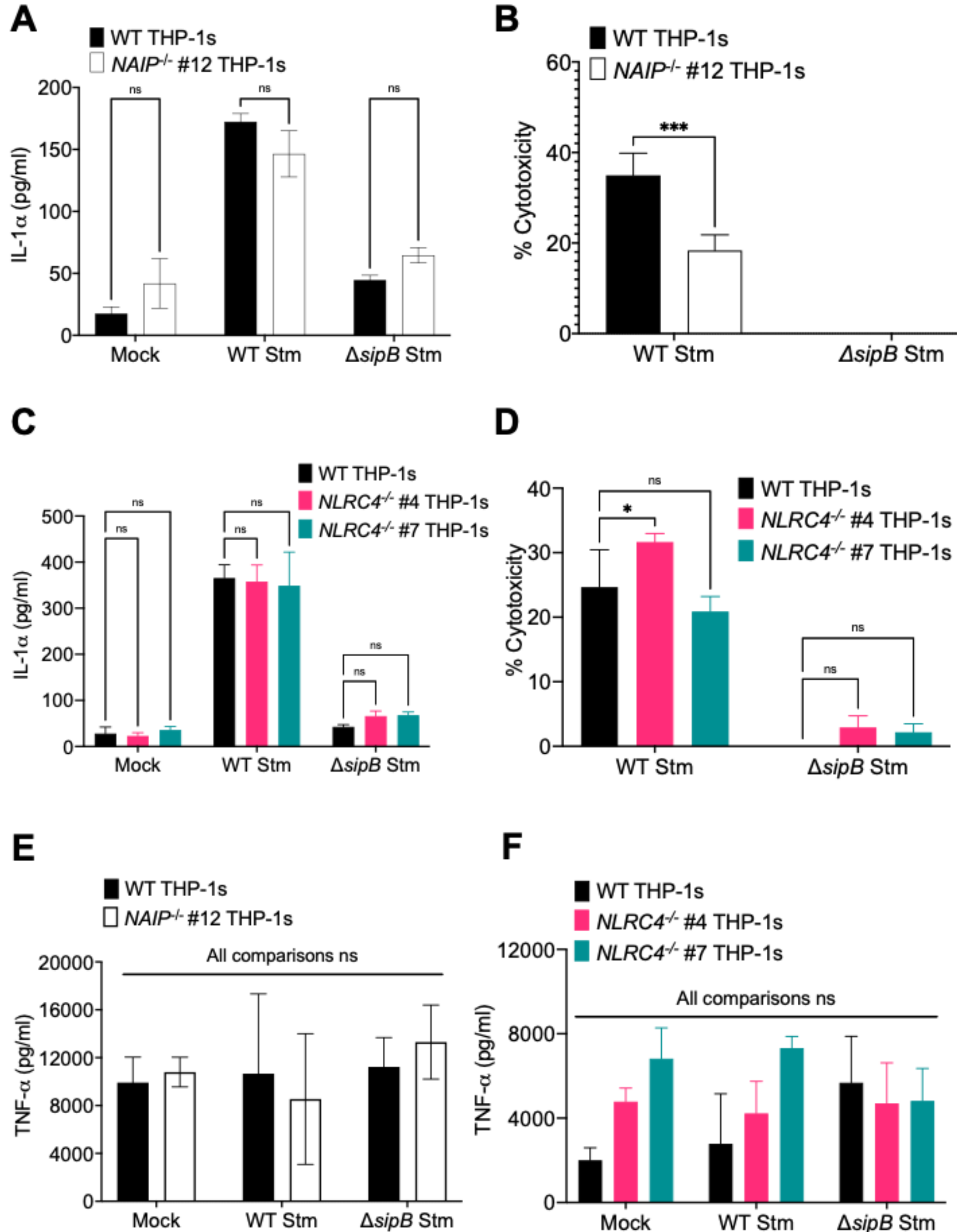

1076

1077 **S5 Fig. (related to Figure 2) NAIP and NLRC4 are partially required for**

1078 **inflammasome activation during *Salmonella* infection in human macrophages.**

1079 WT, *NAIP$^{-/-}$*, or two independent clones of *NLRC4$^{-/-}$* THP-1 monocyte-derived

1080 macrophages were primed with 100 ng/uL Pam3CSK4 for 16 hours. Cells were then

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1081 infected with PBS (Mock), WT *S*. Typhimurium, or Δ*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 uM
1083 nigericin for 6 hours. (A, C, E, F) Release of cytokines IL-1α and TNF-α into the
1084 supernatant were measured by ELISA. (B, D) Cell death (percentage cytotoxity) 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.

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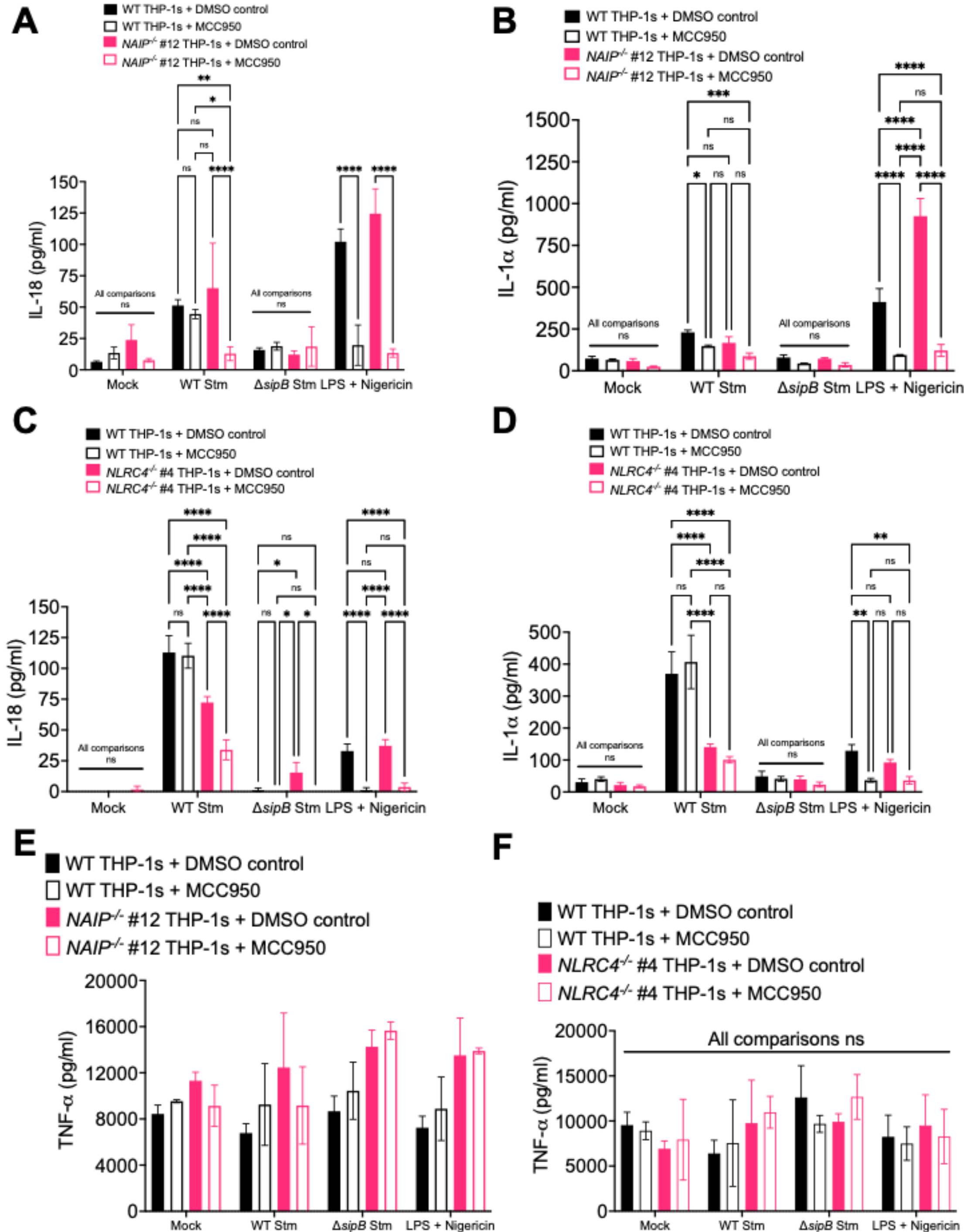

1089

1090 **S6 Fig. (related to Figure 3) *Salmonella* induces NAIP/NLRC4- and NLRP3-**

1091 **dependent inflammasome activation in human macrophages.** WT, *NAIP*$^{-/-}$, or

1092 *NLRC4*$^{-/-}$ THP-1 monocyte-derived macrophages were primed with 100 ng/uL

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1093 Pam3CSK4 for 16 hours. One hour prior to infection, cells were treated with 1 μM
1094 MCC950, a chemical inhibitor of the NLRP3 inflammasome. Cells were then infected
1095 with PBS (Mock), WT *S*. Typhimurium, or Δ*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 uM
1097 nigericin for 6 hours. (A-F) Release of cytokines IL-18, IL-1α, 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.

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A Average knockdown efficiency

THP-1s	*CASP4*	*CASP5*
WT	82.5%	89.5%
NAIP$^{-/-}$ #12	79.5%	79.5%

B Average knockdown efficiency

THP-1s	*CASP4*	*CASP5*
WT	81%	74.5%
NAIP$^{-/-}$ #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.

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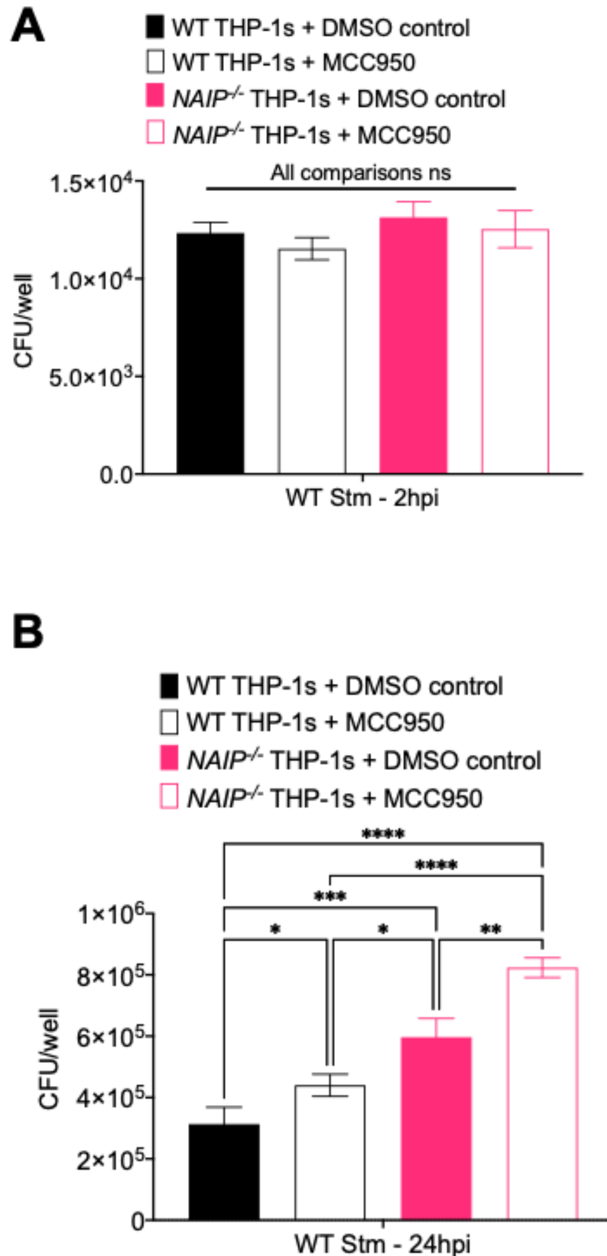

1107

1108 **S8 Fig. (related to Figure 5) NAIP and NLRP3 restrict replication of *Salmonella* in**

1109 **human macrophages.** WT or *NAIP*$^{-/-}$ THP-1 monocyte-derived macrophages were

1110 primed with 100 ng/ml Pam3CSK4 for 16 hours. One hour prior to infection, cells were

1111 treated with 1 μM MCC950 or DMSO as a control. Cells were then infected with WT *S.*

1112 Typhimurium. Cells were lysed at the indicated time points and bacterial were plated to

1113 calculate CFU. (A) CFU/well of bacteria at 2 hpi (B) CFU/well of bacteria at 24 hpi. *p <

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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.

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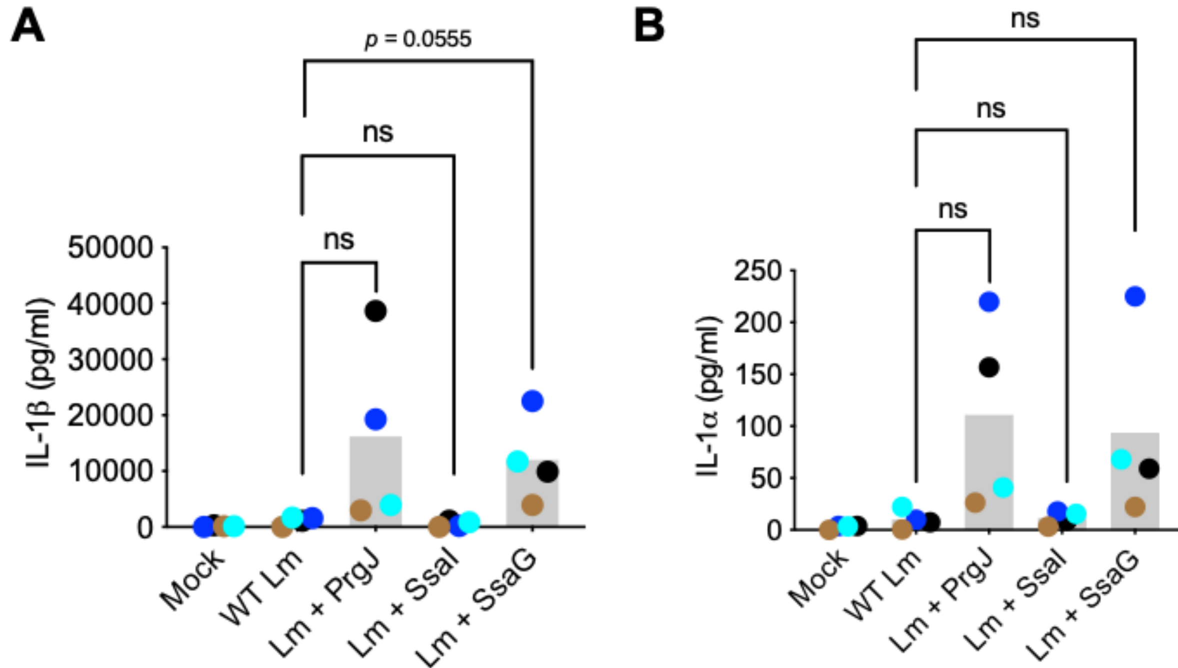

1116

1117 **S9 Fig. (related to Figure 6A) *Salmonella* SPI-2 needle protein SsaG activates the**
1118 **inflammasome in human macrophages.** Primary hMDMs from four healthy human
1119 donors was infected with PBS (Mock), WT *Listeria* (WT Lm), *Listeria* expressing PrgJ
1120 (Lm + PrgJ), SsaI (Lm + SsaI), or SsaG (Lm + SsaG) for 16 hours at MOI=5. Each dot
1121 represents the triplicate mean of one donor. The grey bar represents the mean of all
1122 donors. Release of cytokines IL-1β and IL-1α, was measured by ELISA. *p* values based
1123 on paired t-tests.

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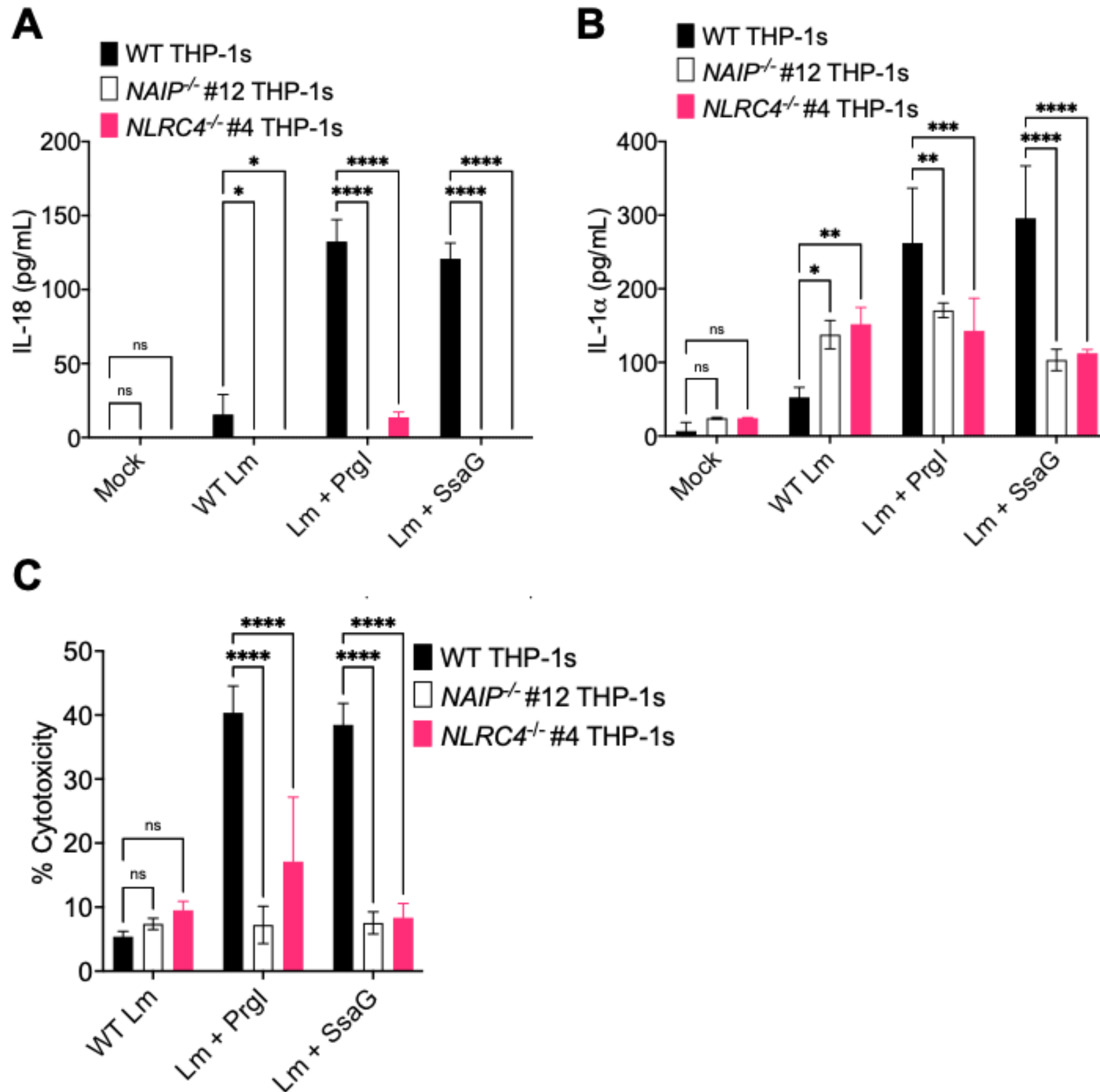

1124

1125 **S10 Fig. (related to Figure 6B) NAIP/NLRC4 are necessary for inflammasome**

1126 **responses to the *Salmonella* SPI-2 needle protein SsaG in human macrophages.**

1127 WT, *NAIP*$^{-/-}$, or *NLRC4*$^{-/-}$ THP-1 monocyte-derived macrophages were primed with 100

1128 ng/ml Pam3CSK4 for 16 hours. Cells were then treated with PBS (Mock), WT *Listeria*

1129 (WT Lm), *Listeria* expressing PrgI (Lm + PrgJ) or SsaG (Lm + SsaG) for 6 hours at

1130 MOI=20. (A, B) Release of cytokines IL-18, and IL-1α was measured by ELISA. (C) Cell

1131 death was measured by lactate dehydrogenase (LDH) release. ns – not significant, $*p <$

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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.

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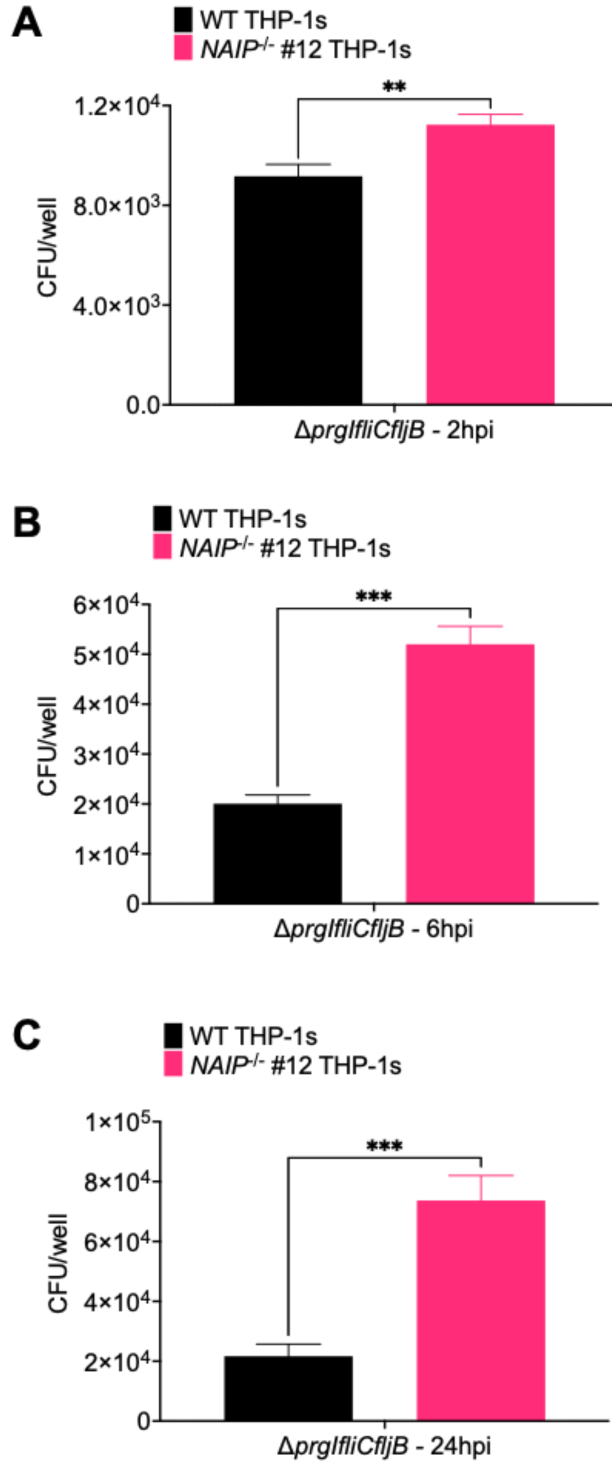

1134

1135 **S11 Fig. (related to Figure 6C) NAIP/NLRC4 inflammasome recognition of the SPI-**

1136 **2 T3SS restricts *Salmonella* replication in human macrophages.** WT or *NAIP*$^{-/-}$

1137 THP-1 monocyte-derived macrophages were primed with 100 ng/ml Pam3CSK4 for 16

bioRxiv preprint doi: https://doi.org/10.1101/2021.06.17.448811; this version posted June 17, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

1138 hours. Cells were then infected with a SPI-1 T3SS/flagellin-deficient strain of *S.*
1139 Typhimurium, Δ*prgIfliCfljB*. (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.

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A

```
PrgJ      MSIATIVPENAVIGQAVNIRSMETDIVSLDDRLLQAFSGSAIATAVDKQTITNRIEDPNL  60
SsaG      -----------------------MDIAQLVD-MLSHMAH-QA-----GQAINDKM-NGND  29
PrgI      --------------MATPWSGYLDDVSAKFDTGVDNLQT-QV-----TEALD-KL-A-AK  37
                                  *:    *  :. :           :::  ::

PrgJ      VTDPKELAISQEMISDYNLYVSMVSTLTRKGVGAVETLLRS--   101
SsaG      LLNPESMIKAQFALQQYSTFINYESSLIKMIKDMLSGIIAKI-   71
PrgI      PSDPALLAAYQSKLSEYNLYRNAQSNTVKVFKDIDAAIIQNFR   80
            :*  :   *  :.:*. : .  *.  :   .    :: .
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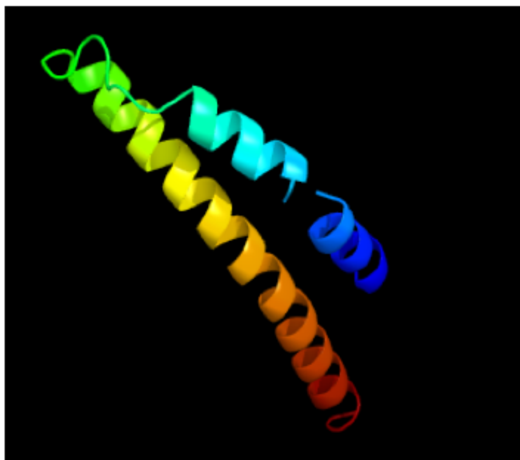
B



1142

1143 **S12 Fig. Sequence alignment and three-dimensional structural prediction of**

1144 **SsaG.** (A) The primary sequences of PrgJ, PrgI, and SsaG were aligned using Multiple

1145 Sequence Alignment by Clustal Omega. * indicates single, *fully conserved* residue, **:**

1146 indicates conservation between groups of *strongly* similar properties, and **.** indicates

1147 conservation between groups of *weakly* similar properties. Small, hydrophobic residues

1148 are indicated in red (AVFPMILW). Acidic residues are indicated in blue (DE). Basic

1149 residues are indicated in magenta (RK). The remaining residues are indicated in green

1150 (STYHCNGQ). (B) The three-dimensional structure of SsaG was predicted with high

1151 confidence and high coverage using the PHYRE2 server. The structure is colored from

1152 N to C terminus using the colors of the rainbow (red, orange, yellow, green, and blue).