1 Title: *Salmonella* Typhimurium induces NAIP/NLRC4- and NLRP3/ASC-independent,
2 caspase-1/4-dependent inflammasome activation in human intestinal epithelial cells
3
4 Running title: Human epithelial inflammasome responses to *Salmonella*
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19 Abstract

20 *Salmonella enterica* serovar Typhimurium is a gram-negative pathogen that causes
21 diseases ranging from gastroenteritis to systemic infection and sepsis. *Salmonella* uses
22 type III secretion systems (T3SSs) to inject effectors into host cells. While these
23 effectors are necessary for bacterial invasion and intracellular survival, intracellular
24 delivery of T3SS products also enables detection of *Salmonella* by cytosolic immune
25 sensors. Upon detecting translocated *Salmonella* ligands, these sensors form
26 multimeric complexes called inflammasomes, which activate caspases that lead to
27 proinflammatory cytokine release and pyroptosis. In particular, the *Salmonella* T3SS
28 needle, inner rod, and flagellin proteins activate the NAIP/NLRC4 inflammasome in
29 murine intestinal epithelial cells (IECs), which leads to restriction of bacterial replication
30 and extrusion of infected IECs into the intestinal lumen, thereby preventing systemic
31 dissemination of *Salmonella*. While these processes are studied quite well in mice, the
32 role of the NAIP/NLRC4 inflammasome in human IECs remains unknown.
33 Unexpectedly, we found the NAIP/NLRC4 inflammasome is dispensable for early
34 inflammasome responses to *Salmonella* in both human intestinal epithelial cell lines and
35 organoids. Additionally, the NLRP3 inflammasome and the adaptor protein ASC are not
36 required for inflammasome activation in Caco-2 cells. Instead, we observed a partial
37 requirement for caspase-1, and a necessity for caspase-4 and GSDMD pore-forming
38 activity in mediating inflammasome responses to *Salmonella* in Caco-2 cells. These
39 findings suggest that unlike murine IECs, human IECs do not rely on NAIP/NLRC4, and
40 also do not use NLRP3/ASC. Instead, they primarily use caspases-1 and -4 to mediate
41 early inflammasome responses to SPI-1-expressing *Salmonella*.

42 Introduction

43 Enteric bacterial pathogens such as *Salmonella* enterica serovar Typhimurium
44 (hereafter referred to as *Salmonella*) are leading causes of global morbidity and
45 mortality from diarrheal diseases (1). Contracted upon ingestion of contaminated food or
46 water, *Salmonella* colonizes the intestinal tract, where it uses evolutionarily conserved
47 molecular syringes called type III secretion systems (T3SS) to inject effectors, or
48 virulence factors, into the host cell cytosol (2). *Salmonella* contains two T3SS: the SPI-1
49 T3SS is expressed early in infection and enables *Salmonella* to invade host cells, while
50 the SPI-2 T3SS is expressed at later timepoints during infection and allows *Salmonella*
51 to replicate within host cells such as intestinal epithelial cells (IECs) (2). IECs thus serve
52 as both the targets of, as well as the first line of physical and innate immune defense
53 against enteric pathogens like *Salmonella*. Most studies of *Salmonella*'s interactions
54 with the innate immune system have been conducted in mice. However, key differences
55 in innate immune genes encoded by mice and humans make it unclear whether mice
56 fully recapitulate how humans respond to *Salmonella*. Here, we interrogated how
57 human IECs sense and respond to *Salmonella* infection.

58 The mammalian immune system can recognize invading intracellular pathogens
59 through cytosolic sensors such as nucleotide-binding domain, leucine-rich repeat (NLR)
60 receptors. Upon detecting a bacterial ligand or activity, these receptors oligomerize to
61 form multimeric signaling complexes called inflammasomes (3). Inflammasomes recruit
62 and activate cysteine proteases, such as caspase-1 and caspase-8 (3, 4). Some
63 inflammasomes require an adaptor protein called apoptosis-associated speck-like
64 protein containing a CARD (ASC) to mediate their interaction with caspases (3). Active

65 caspases can process the proinflammatory cytokines IL-1α, IL-1β, and IL-18 (3), and
66 the pore-forming protein GSDMD (5). This leads to GSDMD-dependent release of the
67 processed cytokines and an inflammatory form of cell death known as pyroptosis (3).
68 Release of these proinflammatory cytokines alerts nearby cells of the infection, while
69 pyroptosis can eliminate the pathogen's replicative niche within the infected host cell.
70 Various cellular insults during infection can trigger activation of different
71 inflammasomes. Inflammasome activation is critical for control of *Salmonella* infection in
72 mice (6). In both murine and human macrophages and murine IECs, *Salmonella*
73 infection activates a family of inflammasome sensors termed NAIPs, which detect the
74 *Salmonella* SPI-1 T3SS needle, inner rod, and flagellin proteins (7–12). Activation of the
75 NAIP/NLRC4 inflammasome specifically in murine IECs restricts bacterial replication,
76 causes extrusion of infected cells from the intestinal epithelial layer, and prevents
77 dissemination of *Salmonella* to systemic sites (13–15). In contrast to mice, humans
78 encode a single NAIP inflammasome sensor, which promiscuously recognizes the
79 T3SS needle, inner rod, and flagellin proteins in human macrophages (10, 16, 17). The
80 role of the NAIP/NLRC4 inflammasome in human IECs during *Salmonella* infection
81 remains unknown.
82 *Salmonella* infection can also induce the NLRP3 inflammasome, which can be
83 activated by a variety of stimuli during infection, including potassium efflux (18). In
84 murine macrophages, the NLRP3 inflammasome is thought to be important for late
85 timepoints during *Salmonella* infection (19). In murine macrophages and murine and
86 human intestinal epithelial cells, the caspase-11 (mice) or caspase-4/5 (humans)
87 inflammasome is activated late in *Salmonella* infection (19–23). Caspase-4/5/11 detect

88 cytosolic LPS and form the noncanonical inflammasome, which can secondarily activate
89 the NLRP3 inflammasome (24, 25). In human macrophages, *Salmonella* infection
90 triggers recruitment of both NLRC4 and NLRP3 to the same macromolecular complex
91 (26). However, whether the NLRP3 inflammasome plays a functional role during
92 *Salmonella* infection of human IECs has not been previously tested.

93 Human IECs infected with *Salmonella* undergo caspase-4 inflammasome
94 activation at late time points following infection (10hpi), when there is a considerable
95 population of replicating cytosolic bacteria (20, 22). However, early inflammasome
96 responses to SPI-1-expressing *Salmonella* have not been previously investigated. In
97 this study, we have found that human IECs undergo inflammasome activation in
98 response to SPI-1-expressing *Salmonella*. However, T3SS ligands and flagellin were
99 not sufficient to activate the inflammasome in human Caco-2 cells or intestinal
100 organoids. Additionally, using a combination of pharmacological inhibitors and
101 CRISPR/Cas9 technology, we found that the NAIP inflammasome, the NLRP3
102 inflammasome, and the adaptor protein ASC are all dispensable for early
103 inflammasome responses to SPI-1-expressing *Salmonella* in Caco-2 cells. Instead, we
104 observed that caspase-1 is partially required, whereas caspase-4 is necessary for
105 inflammasome activation in Caco-2 cells in response to *Salmonella* infection. Our
106 findings delineate the role of several inflammasomes in human IECs *Salmonella*
107 infection. Importantly, these findings indicate how widely inflammasome responses to
108 infection can vary between species as well as cell types.

109 Materials and Methods

110

111 Ethics statement

112 All studies involving human peripheral blood mononuclear cells (PBMCs) and human
113 intestinal organoids were performed in compliance with the requirements of the US
114 Department of Health and Human Services and the principles expressed in the
115 Declaration of Helsinki. Both human PBMCs and organoids are considered to be a
116 secondary use of deidentified human specimens and are exempt via Title 55 Part 46,
117 Subpart A of 46.101 (b) of the Code of Federal Regulations. All experiments performed
118 with murine organoids were done so in compliance with the regulatory standards of, and
119 were approved by the Oregon Health & Science University Institutional Animal Care and
120 Use Committee.

121

122 Bacterial strains and growth conditions

123 *Salmonella enterica* serovar Typhimurium SL1344 WT and Δ*sipB* (27) isogenic
124 strains were routinely grown shaking overnight at 37°C in Luria-Bertani (LB) broth with
125 streptomycin (100 µg/ml). Cells were infected with *Salmonella* grown under SPI-1-
126 inducing conditions (28).

127 *Listeria monocytogenes* WT and isogenic strains on the 10403S background
128 were routinely grown shaking overnight at 30°C in brain heart infusion (BHI) broth (29).
129 *S.* Typhimurium ligands PrgJ or PrgI were translationally fused to the truncated N-
130 terminus of ActA and under the control of the *actA* promoter (29). The *Listeria* strain
131 expressing *S.* Typhimurium SsaI was constructed using a codon-optimized gene

132 fragment (IDT) cloned into the pPL2 vector and introduced into *Listeria* as previously
133 described (29, 30).
134
135 Bacterial infections
136 Where indicated, cells were primed with 100ng/ml or 400ng/ml of Pam3CSK4
137 (Invivogen) for 3 hours prior to infection. To induce SPI-1 expression, overnight cultures
138 of *Salmonella* were diluted into LB broth containing 300 mM NaCl and grown for 3 hours
139 standing at 37°C (28). Overnight cultures of *Listeria* were diluted and grown shaking for
140 3 hours in BHI. All cultures were pelleted at 6,010 × *g* for 3 minutes, washed once with
141 PBS, and resuspended in PBS. Cells were infected with *Salmonella* at a multiplicity of
142 infection (MOI) of 60 or *Listeria* at the indicated MOI for each experiment in the figure
143 legend. Infected cells were centrifuged at 290 × *g* for 10 minutes and incubated at
144 37°C. 1 hour post-infection, cells were treated with 100ng/mL or 50ng/mL of gentamicin
145 to kill any extracellular *Salmonella* or *Listeria* respectively. Infections proceeded at 37°C
146 for the indicated length of time for each experiment. Control cells were mock-infected
147 with PBS for all experiments.
148
149 Cell culture of intestinal epithelial cell lines
150 All cell lines were obtained from American Type Culture Collection (ATCC).
151 Caco-2 cells (HTB-37; ATCC) were maintained in DMEM supplemented with 10%
152 (vol/vol) heat-inactivated FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin. T84
153 cells (CCL-248; ATCC) were maintained in DMEM F-12 supplemented with 5% (vol/vol)
154 heat-inactivated FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin.

155 One day prior to infection or treatment, cells were dissociated with 0.25%
156 Trypsin-EDTA (Gibco) diluted 1:1 with 1X PBS. Cells were incubated with trypsin at
157 37°C for 15 minutes, after which the trypsin was neutralized with serum-containing
158 media. Cells were replated in media without antibiotics in a 24-well plate at a
159 concentration of 3×10^5 cells/well. Where indicated, cells were primed with 100ng/mL or
160 400ng/ml Pam3CSK4 (Invivogen) or 500ng/ml LPS (Sigma-Aldrich) for 3h or 16h prior
161 to anthrax toxin treatment or bacterial infections.
162
163 Culture of intestinal organoids in spheroid culture
164 Spheroids derived from human duodenum or colon were kindly provided by
165 Jared Fisher at Oregon Health & Science University. Human and murine organoids
166 were cultivated in special conditioned medium as described by Myoshi & Stappenbeck
167 (31). Briefly, spheroids were grown in Corning® Matrigel® Basement Membrane Matrix
168 (VWR). Spheroids were dissociated with Trypsin-EDTA (1X PBS, 0.25% trypsin, 0.5
169 mM EDTA) at 37°C for 5 minutes. Subsequently, washing medium (DMEM/F12 with
170 HEPES (Sigma), 10% FBS (heat-inactivated), 1X P/S) was added to stop trypsinization.
171 After washing the cells with washing medium at $200 \times g$ for 5 minutes at room
172 temperature (RT), the supernatant was completely aspirated and the cell pellet
173 resuspended in Matrigel®. 20 µL matrigel-cell drops were placed into 24-well plates and
174 polymerized for 5 minutes at 37°C upside down. Afterwards, cells were cultured in
175 organoid medium consisting of 50% primary culture medium (Advanced DMEM/F12
176 (Gibco), 20% FBS, 1X P/S, 1X L-Glu) and 50% conditioned medium (L-WRN-cell

177 supernatant) supplemented with 10 μM of ROCK inhibitor Y27632 (Sigma) and 10 μM
178 TGF-β inhibitor SB431542 (Millipore) (for murine organoids only).
179
180 ELISAs
181 Supernatants harvest from infected cells were assayed using ELISA kits for
182 human IL-18 (R&D Systems) and IL-8 (R&D Systems).
183
184 Immunoblot analysis
185 Cell lysates were harvested for immunoblot analysis by adding 1X SDS/PAGE
186 sample buffer to cells following infection. Cells were incubated and infected in serum-
187 free media to collect supernatant samples. Supernatant samples were centrifuged at
188 200 × *g* to pellet any cell debris. The supernatant was then treated with trichloroacetic
189 acid (TCA) (25 μL of TCA for 500 μL of supernatant) overnight at 4°C. The next day, the
190 samples were centrifuged at maximum speed (15871 × *g*) for 15 minutes at 4°C.
191 Precipitated supernatant pellets were washed with ice-cold acetone, centrifuged at
192 maximum speed (15871 × *g*) for 10 minutes at 4°C, and resuspended in 1X SDS/PAGE
193 sample buffer. All protein samples (lysates and supernatants) were boiled for 5 minutes.
194 Samples were separated by SDS/PAGE on a 12% (vol/vol) acrylamide gel, and
195 transferred to PVDF Immobilon-P membranes (Millipore). Primary antibodies specific for
196 human IL-18 (MBL International PM014) and β-actin (4967L; Cell Signaling) and HRP-
197 conjugated secondary antibody anti-rabbit IgG (7074S; Cell Signaling) were used. ECL
198 Western Blotting Substrate or SuperSignal West Femto (both from Pierce Thermo
199 Scientific) were used as the HRP substrate for detection.

200

201 Propidium iodide (PI) uptake assay

202 7.5×10^4 Caco-2 cells per well were plated in a black, flat-bottom 96-well plate
203 (Cellstar) in PI uptake media containing 1X HBSS without phenol red, 20 mM HEPES,
204 and 10% (vol/vol) heat-inactivated FBS. Cells were infected at an MOI=60 and control
205 wells were treated with 1% Triton. After infection, cells were centrifuged at 290 × *g* for
206 10 minutes following infection. 5 μM propidium iodide (PI, P3566, Invitrogen) diluted in
207 PI uptake media was added to the cells. The plate was sealed with adhesive optical
208 plate sealing film (Microseal, Bio-Rad) and placed in a Synergy H1 microplate reader
209 (BioTek) pre-heated to 37°C. PI fluorescence was measured every 10 minutes for the
210 indicated number of hours.

211

212 Anthrax toxin-mediated delivery of bacterial ligands into organoids

213 Organoids were seeded in 96-well plates with transparent bottom and opaque
214 walls in 5μl Matrigel domes. After two days in organoid media, organoids were grown for
215 an additional 3 days in differentiation media: DMEM/F12 supplemented with 20%
216 murine R-Spondin1 supernatant, 10% murine Noggin supernatant, 50 ng/mL
217 recombinant murine EGF (Fisher Scientific), 1X P/S, 1X L-Glu, 10 mM HEPES
218 (HiMedia), 1X N2 (Life Technologies), 1X B27 (Life Technologies), and 1 mM N-
219 acetylcysteine (Fisher Scientific). 5 μM DAPT was added for the last 24 hours.
220 Differentiated organoids were then treated with 16μg/ml PA and 8μg/ml LFn-FlaA or 0.1
221 μg/ml LFn-PrgJ in differentiation media containing 10μg/ml propidium iodide for 4 hours.
222 Total lysis wells were treated with 1%Triton.

223

224 Expression of inflammasome genes in human small intestinal organoids

225 To analyze inflammasome expression under different media conditions,
226 spheroids were cultured in differentiation medium: DMEM/F12 supplemented with 20%
227 supernatant from R-Spondin1 expressing L-cells, 10% supernatant from Noggin
228 expressing cells, 50 ng/mL recombinant murine EGF (Fisher Scientific), 1X P/S, 1X L-
229 Glu, 10 mM HEPES (HiMedia), 1X N2 (Life Technologies), 1X B27 (Life Technologies),
230 1 mM N-acetylcysteine (Fisher Scientific), and 5 μM DAPT. After 4 days of incubation at
231 37°C, Matrigel domes were dissolved in PBS-EDTA (5 mM) for 1 hour at 4°C on an
232 orbital shaker. After centrifugation at 300 × *g* for 5 minutes at 4°C, the cell pellet was
233 resuspended in TRIzol™ to analyze mRNA expression.

234

235 Isolation of peripheral blood mononuclear cells (PBMCs)

236 To compare expression levels of inflammasome components in human intestinal
237 epithelial cells with immune cells, PBMC-derived cDNA was kindly provided by William
238 Messer at Oregon Health & Science University. Briefly, PBMCs were isolated using
239 density gradient centrifugation. After overlay of Lymphoprep™ (Alere Technologies AS)
240 with blood mixed 1:2 with 1X PBS (pH 7.4, Gibco), the sample was centrifuged at 800 ×
241 *g* for 20 minutes at room temperature (RT). Residual erythrocytes were lysed with 1X
242 RBC lysis buffer (10X, BioLegend), followed by three washing steps with fresh PBS for
243 10 minutes at 250 × *g*. Subsequently cells were harvested in TRIzol™ Reagent
244 (Thermo Fisher Scientific) for mRNA analysis.

245

246 RNA extraction, cDNA synthesis, and real-time quantitative polymerase chain reaction
247 (RT-qPCR) of organoid and PBMC samples

248 After thawing the TRIzol™ samples, chloroform was added and the tubes
249 centrifuged for 15 minutes at 12,000 × *g* at 4°C. The aqueous phase was transferred to
250 a new tube containing linear polyacrylamide (Gene-Elute™ LPA, Sigma). To allow RNA
251 precipitation, the samples were incubated with isopropanol for 10 minutes and
252 subsequently centrifuged for 10 minutes at 12,000 × *g*. After aspiration of the
253 supernatant, the RNA pellet was washed once with 75% ethanol for 5 minutes at 7,500
254 × *g* at 4°C. The supernatant was aspirated and the dried pellet resuspended in
255 Molecular Biology Grade Water (Corning). After determination of RNA content and
256 quality (260/280 and 260/230 ratios), 1 µg of RNA was reversely transcribed into cDNA.
257 Reaction steps were performed in a Biorad T100 Cycler. First, residual DNA was
258 removed using RQ1 RNAse-free DNase (Promega) in RQ1 DNase 1X Reaction buffer
259 (Promega) for 30 minutes at 37°C. After stopping the reaction with RQ1 DNase Stop
260 Solution (Promega) for 10 minutes at 65°C, Oligo dTs (Sigma) and dNTPs (Sigma) were
261 added for 5 minutes at 65°C. Reverse transcription was performed with SuperScript™
262 IV Reverse Transcriptase (Invitrogen), 5 mM DTT and SuperScript™ IV Reaction Buffer
263 (Invitrogen) for 10 minutes at 50-55°C. Subsequently the enzyme was inactivated at
264 80°C for 10 minutes.

265 To analyze RNA expression, cDNA was mixed with 10 µM forward and reverse
266 primers and PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) according to
267 manufacturer's instructions. The following primers were used:

268 *RPLP0* Forward: 5'- GGCACCATTGAAATCCTGAGTGATGTG -3'

	Reverse:	5'- TTGCGGACACCCTCCAGGAAG -3'
NAIP	Forward:	5'- AAGCATCCGCCCAGCTCTTGA -3'
	Reverse:	5'- TATTGCCCTCCAGATCCACAGACAGTTC -3'
NLRC4	Forward:	5'- CATAGTCAAGTCTCTGTCAAGTGAACCCTGT -3'
	Reverse:	5'- GCTGTTCTAGCACGTTCATCCTGTCG -3'
CASP1	Forward:	5'- GAGGCATTTGCACACCGCCC -3'
	Reverse:	5'- GGATCTCTTCACTTCCTGCCCACA -3'

Amplification was analyzed in real-time with StepOne Software v2.3. In brief, samples were incubated for 10 minutes at 95°C, followed by 40 cycles of heating to 95°C for 15 seconds and cooling to 60°C for 1 minute. To monitor specificity of the run, the melt curves were determined by keeping the samples at 95°C for 15 seconds, cooling to 60°C for 1 min and then increasing the temperature every 15 seconds by 0.3°C up to 95°C. Expression levels relative to the housekeeping gene (*RPLP0*) were calculated using the formula $x = 2^{-\Delta ct}$.

<u>RNA extraction, cDNA synthesis, and RT-qPCR of Caco-2, T84, and THP-1 samples</u>

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). To calculate relative gene expression, mRNA levels of target

genes were normalized to housekeeping gene *HPRT* and the formula $x = 2^{-\Delta CT}$ was used. The following primers from PrimerBank (PrimerBank identification listed within parentheses) were used (32–34):

HPRT (164518913c1)	Forward:	5'- CCTGGCGTCGTGATTAGTGAT -3'
	Reverse:	5' - AGACGTTCAGTCCTGTCCATAA -3'
NAIP (119393877c3)	Forward:	5' - CCCATTAGACGATCACACCAGA -3'
	Reverse:	5' - GGAGTCACTTCCGCAGAGG -3'
NLRC4 (312433959c2)	Forward:	5' - TGCATCATTGAAGGGGAATCTG -3'
	Reverse:	5' - GATTGTGCCAGGTATATCCAGG -3'
NLRP3 (208879435c1)	Forward:	5' - GATCTTCGCTGCGATCAACAG -3'
	Reverse:	5' - CGTGCATTATCTGAACCCCAC -3'
PYCARD (313482805c1)	Forward:	5' - TGGATGCTCTGTACGGGAAG -3'
	Reverse:	5' - CCAGGCTGGTGTGAAACTGAA -3'
CASP1 (380254454c1)	Forward:	5' - TTTCCGCAAGGTTCGATTTTCA -3'
	Reverse:	5' - GGCATCTGCGCTCTACCATC -3'

<u>Inhibitor experiments</u>

Cells were treated 1 hour prior to infection at the indicated concentrations with inhibitors: varying concentrations of MCC950 (Sigma Aldrich PZ0280), 20 μM of pan-caspase inhibitor Z-VAD(OMe)-FMK (SM Biochemicals SMFMK001), 25 μM of caspase-1 inhibitor Ac-YVAD-cmk (Sigma Aldrich SML0429), 30 μM of disulfiram (Sigma).

315 siRNA-mediated knockdown of genes

316 The following Silencer Select siRNA oligos were purchased from Ambion (Life
317 Technologies): *CASP4* (s2412), *CASP5* (s2417), two Silencer Select negative control
318 siRNAs (Silencer Select Negative Control No. 1 siRNA and Silencer Select Negative
319 Control No. 2 siRNA). Three days before infection, 30 nM of siRNA was transfected into
320 Caco-2s using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific)
321 following the manufacturer's protocol. Cells were primed with 400 ng/ml of Pam3CSK4
322 (Invivogen) for 3 hours prior to infection.

323

324 Statistical analysis

325 Prism 9.1.1 (GraphPad Software) was used to graph all data and for all statistical
326 analyses. Statistical significance for experiments was determined using the appropriate
327 test and are indicated in each figure legend. Differences were considered statistically
328 significant if the p value was <0.05.

329 **Results**

330

331 *Salmonella* infection induces inflammasome activation in human intestinal epithelial

332 cells

333 Once inside the host, *Salmonella* upregulates expression of its SPI-1 T3SS,

334 which delivers effectors that enable *Salmonella* to invade intestinal epithelial cells (2).

335 Human IECs infected with *Salmonella* undergo inflammasome activation at late time

336 points following infection (10hpi) (20, 22). However, early inflammasome responses to

337 SPI-1-expressing *Salmonella* have not been previously investigated. To test if human

338 IECs undergo early inflammasome activation in response to *Salmonella* grown under

339 SPI-1-inducing conditions (28), we infected WT Caco-2 cells, a human colorectal cell

340 line, with WT *Salmonella* (WT Stm) or *Salmonella* lacking its SPI-1 T3SS (Δ*sipB* Stm)

341 and assayed for subsequent inflammasome activation by measuring release and

342 cleavage of the inflammasome-dependent cytokine IL-18 at 6hpi, an early time point

343 following *Salmonella* infection (Fig. 1A, B). Cells infected with WT Stm released

344 significantly increased levels of cleaved IL-18 into the supernatant compared to mock-

345 infected cells (Fig. 1A, B). In contrast, cells infected with Δ*sipB* Stm, which is unable to

346 invade cells, failed to release cleaved IL-18 (Fig. 1A, B). We measured cell death as

347 another readout of inflammasome activation by assaying for uptake of the cell-

348 impermeable dye propidium iodide (PI), which enters cells as they form pores in their

349 plasma membrane and undergo cell death. In cells infected with WT Stm, PI uptake

350 began to occur between 4-6hpi, and gradually increased over time, indicating that

351 infected cells begin to undergo cell death as early as 4-6hpi. As expected, cells infected

352 with Δ*sipB* Stm, which cannot enter the host cell, did not uptake any PI. We found that
353 inflammasome activation in response to *Salmonella* also occurs in another human
354 colorectal cell line, T84 cells (Fig. 1D). T84 cells infected with SPI-1-expressing WT Stm
355 also released IL-18 into the supernatant at 6hpi (Fig. 1D). As a control, we found that in
356 both Caco-2 and T84 cells, the inflammasome-independent cytokine, IL-8, is released in
357 response to WT and Δ*sipB* Stm (Fig. S1A, S1B).
358 Inflammasome activation leads to cleavage of the pore-forming protein GSDMD
359 (5, 35). The N-terminal fragment of cleaved GSDMD inserts into the host cell plasma
360 membrane and oligomerizes to create a pore through which cellular components such
361 as cleaved IL-1 and IL-18 can be released (36–39). These pores eventually cause
362 rupture of the cell through osmotic flux, resulting in pyroptosis (3). To determine if pore
363 formation by GSDMD is required for release of IL-18 and PI uptake in human IECs
364 infected with *Salmonella*, we pretreated Caco-2 cells with disulfiram, which prevents
365 cleaved GSDMD from inserting into the plasma membrane and thus abrogates pore
366 formation (40). Treatment with disulfiram led to loss of IL-18 release and PI uptake
367 during *Salmonella* infection (Fig. 1E, 1F). Importantly, release of the inflammasome-
368 independent cytokine IL-8 was not affected by disulfiram treatment (Fig. S1C).
369 Collectively, these results suggest that as early as 4-6hpi, human IECs infected with WT
370 Stm grown under SPI-1-inducing conditions undergo inflammasome activation, and
371 GSDMD-mediated pore formation is required to observe IL-18 release and PI uptake in
372 response to *Salmonella* in human IECs.
373

374 Bacterial T3SS ligands do not activate the inflammasome in human intestinal epithelial
375 cells

376 We next sought to determine the bacterial ligands that trigger inflammasome
377 activation in human IECs. The *Salmonella* SPI-1 T3SS inner rod protein (PrgJ), the SPI-
378 1 T3SS needle protein (PrgI), and flagellin activate the NAIP/NLRC4 inflammasome in
379 both murine and human macrophages, (8, 11). Given that *Salmonella* grown under SPI-
380 1-inducing conditions triggered inflammasome activation in human IECs (Fig. 1), we
381 hypothesized that this inflammasome activation was due to NAIP-mediated recognition
382 of *Salmonella* T3SS ligands. First, we asked whether *Salmonella* T3SS ligands are
383 sufficient to activate the inflammasome in human IECs. We used the Gram-positive
384 bacterium *Listeria monocytogenes* to deliver *Salmonella* T3SS ligands into IECs, We
385 have previously used the *Listeria* system to deliver *Salmonella* T3SS ligands into the
386 cytosol of human macrophages to examine NAIP-dependent inflammasome activation
387 (17). In this system, ligands of interest are translationally fused to the N-terminus of
388 truncated ActA, enabling the ligands to be delivered into the host cell cytosol (17, 29).
389 Human macrophages infected with *Listeria* expressing the *Salmonella* SPI-1 T3SS inner
390 rod PrgJ or needle PrgI undergo robust inflammasome activation (17). Surprisingly,
391 IECs infected with *Listeria* expressing either the SPI-1 inner rod (PrgJ) or needle (PrgI)
392 proteins failed to induce IL-18 release (Fig. 2A, B; Fig. S2A, B). Polarized Caco-2 cells
393 and C2Bbe1 cells, a Caco-2 subtype, infected with *Listeria* expressing the SPI-1 inner
394 rod PrgJ also failed to release IL-18 levels above that observed in cells infected with WT
395 Lm (Fig. S2C, D). Collectively, these data indicate that *Listeria* delivery of bacterial
396 T3SS ligands is not sufficient to induce inflammasome activation in human IEC lines.

397 As we did not observe inflammasome activation by bacterial T3SS ligands
398 delivered using *Listeria*, we tested a second delivery method, the *Bacillus anthracis*
399 toxin system, to deliver these bacterial ligands into the cytosol of IECs (41). The anthrax
400 toxin delivery system contains two subunits: a protective antigen (PA) that creates a
401 pore in the host endosomal membrane and a truncated lethal factor (LFn) that is
402 delivered through the PA pore into the cytosol. T3SS ligands that are translationally
403 fused to the N-terminal domain of the *B. anthracis* LFn are delivered into the host cell
404 cytosol upon treatment with both PA and the LFn fusion (41) (collectively referred to as
405 Tox). We delivered the *Salmonella* SPI-1 T3SS inner rod protein (PrgJTox) into Caco-2
406 cells (Fig. S3A), polarized Caco-2 cells (Fig. S3B), C2Bbe1 cells (Fig. S3C), and T84
407 cells (Fig. S3D) and assayed the release of the inflammasome-dependent cytokine IL-
408 18. In all cell types, we failed to observe IL-18 secretion in response to PrgJTox that
409 was above the PA alone control (Fig. S3). Thus, our data indicate that human IEC lines
410 did not undergo inflammasome activation in response to bacterial T3SS ligands
411 delivered with the anthrax toxin system.
412 To determine if this absence of inflammasome responses to T3SS ligands was
413 limited to immortalized IECs or extended to non-immortalized IECs as well, we delivered
414 flagellin (FlaTox) or the SPI-1 T3SS inner rod PrgJ (PrgJTox) into human intestinal
415 organoids and measured levels of cell death (Fig. 2C). Both human small intestinal
416 organoids and colonoids failed to undergo cell death when treated with FlaTox or
417 PrgJTox (Fig. 2C). In contrast, murine organoids underwent robust cell death in
418 response to FlaTox or PrgJTox, as expected, indicating that these preparations of

419 FlaTox and PrgJTox had the expected biological activity, but were not able to activate
420 the inflammasome in primary or transformed human intestinal cells (Fig. 2C).
421
422 Human intestinal epithelial cells express low levels of *NAIP* and *NLRC4* compared to
423 human myeloid cells
424 Given the role of NAIP/NLRC4 in detecting and responding to *Salmonella* and
425 bacterial T3SS ligands in murine IECs as well as murine and human macrophages (7–
426 12, 14, 16, 17), our findings that human IECs do not respond to T3SS ligands were
427 surprising. Thus, we next asked if expression of *NAIP* and *NLRC4* in human IECs is
428 comparable in Caco-2 cells, T84 cells, and human THP-1 macrophages (Fig. 3A, B).
429 Consistent with their poor responsiveness to cytosolic delivery of NAIP ligands, both
430 Caco-2 and T84 cells had very low expression of *NAIP* and *NLRC4* mRNA compared to
431 human macrophages. Moreover, primary small intestinal organoids also expressed very
432 low levels of *NAIP* and *NLRC4* compared to human PBMCs, indicating that this low
433 expression was a general feature of human IECs (Fig. 3C, D). Collectively, these data
434 suggest that human IECs express very low levels of NAIP and NLRC4, and this may
435 partially explain why human IECs do not mount inflammasome responses to T3SS
436 ligands.
437
438 NAIP is not required for inflammasome activation in response to *Salmonella* infection of
439 human intestinal cells
440 In murine macrophages and IECS as well as human macrophages, NAIP/NLRC4
441 contributes to inflammasome responses during *Salmonella* infection (11, 13, 14, 42).

442 Even low levels of *NAIP* expression are sufficient to induce inflammasome responses in
443 human macrophages (17). To formally test if the inflammasome activation we observe
444 in human IECs infected with *Salmonella* (Fig. 1) requires NAIP, we used the Clustered
445 Regularly Interspersed Palindromic Repeat (CRISPR) system, in conjunction with the
446 RNA-guided exonuclease Cas9, to disrupt the *NAIP* gene in Caco-2 cells (Fig. S4). We
447 sequenced two independent single cell clones each of *NAIP*$^{-/-}$ Caco-2 cells (*NAIP*$^{-/-}$ #7,
448 *NAIP*$^{-/-}$ #8) to confirm appropriate targeting of *NAIP* in each line (Fig. S4). Caco-2 cells
449 are polyploid, and we therefore found multiple mutant alleles for each CRISPR clone we
450 sequenced (Fig. S4). For the two *NAIP*$^{-/-}$ clones, all of the changes resulted in a
451 premature stop codon (Fig. S4).
452 To test whether inflammasome activation during *Salmonella* infection of human
453 IECs requires NAIP, we infected WT or *NAIP*$^{-/-}$ Caco-2 cells with WT Stm or Δ*sipB* Stm
454 and assayed for inflammasome activation by measuring IL-18 release and PI uptake
455 (Fig. 4). As expected, WT Caco-2 cells infected with WT Stm released significantly
456 increased levels of IL-18 and underwent cell death (Fig. 4A, 4B) and this response was
457 dependent on the presence of the SPI-1 T3SS, as cells infected with Δ*sipB* Stm failed to
458 undergo inflammasome activation (Fig. 4A, 4B). In *NAIP*$^{-/-}$ Caco-2 cells infected with WT
459 Stm, we did not observe a decrease in inflammasome activation compared to WT Caco-
460 2 cells (Fig. 4A, 4B). Both WT and *NAIP*$^{-/-}$ Caco-2 cells secreted similar levels of the
461 inflammasome-independent cytokine IL-8 (Fig. 4C). Overall, these data indicate that
462 NAIP is not required for inflammasome responses to SPI-1-expressing *Salmonella* in
463 human IECs.
464

465 NLRP3 and ASC are dispensable for inflammasome activation in response to
466 *Salmonella* in human intestinal epithelial cells

467 Since the NAIP/NLRC4 inflammasome did not have a role in responding to
468 *Salmonella* grown under SPI-1-inducing conditions in human IECs, we sought to
469 determine whether another host cytosolic sensor could be responsible for
470 inflammasome activation. One candidate is the NLRP3 inflammasome, which can be
471 activated by a variety of stimuli during infection, including potassium efflux (18). In
472 murine macrophages, the NLRP3 inflammasome is activated late during *Salmonella*
473 infection (19). *Salmonella* infection also activates the NLRP3 inflammasome in human
474 macrophages (26, 43). To determine if the inflammasome activation we observed in
475 human IECs is dependent on the NLRP3 inflammasome, we infected WT Caco-2 cells
476 that were pre-treated with MCC950, a potent chemical inhibitor of the NLRP3
477 inflammasome (44) and measured IL-18 release (Fig. 5A). As expected, cells treated
478 with the DMSO control underwent robust inflammasome activation in response to WT
479 Stm infection (Fig. 5A). Interestingly, cells treated with varying concentrations of
480 MCC950 also exhibited similar levels of inflammasome activation as DMSO-control
481 treated cells (Fig. 5A), suggesting that the NLRP3 inflammasome is not required for
482 inflammasome responses to *Salmonella* in human IECs. We found that like *NAIP* and
483 *NLRC4*, mRNA expression of *NLRP3* is also very low in Caco-2 cells compared to THP-
484 1 macrophages (Fig. 5B), which may explain why NLRP3 inhibition does not prevent
485 inflammasome activation in human IECs.

486 In addition to the NAIP/NLRC4 and NLRP3 inflammasomes, there are many
487 other inflammasomes that can get activated in response to bacterial infections. The

488 majority of these inflammasomes, including AIM2, IFI16, NLRP3, NLRP6, NLRP7, and
489 pyrin, use an adaptor protein called ASC to recruit and activate downstream caspases
490 (4). To determine if ASC-dependent inflammasomes participate in the response to
491 *Salmonella* in human IECs, we tested if ASC is required for inflammasome activation.
492 Using CRISPR/Cas9, we disrupted *PYCARD*, the gene that encodes for ASC, in Caco-2
493 cells (Fig. S5). We sequence-validated two independent single cell clones of *PYCARD*$^{-/-}$
494 Caco-2 cells (*PYCARD*$^{-/-}$ #4, *PYCARD*$^{-/-}$ #6) (Fig. S5). Most of the mutations resulted in
495 a premature stop codon or the mutated protein bore no resemblance to the WT protein
496 sequence (Fig. S5). mRNA expression of *PYCARD* was also abrogated in the KO
497 clones relative to WT Caco-2 cells (Fig. S6A). We did not detect any ASC protein
498 expression even in WT Caco-2 cells (Fig. S6B).
499 We next infected WT or *PYCARD*$^{-/-}$ Caco-2 cells with WT Stm or Δ*sipB* Stm and
500 assayed for inflammasome activation (Fig. 5C, D). As expected, WT Caco-2 cells
501 infected with WT Stm released significantly increased levels of IL-18 and underwent cell
502 death (Fig. 5C, D). This was dependent on the presence of the SPI-1 T3SS, as cells
503 infected with Δ*sipB* Stm failed to undergo inflammasome activation (Fig. 5C, D). In
504 *PYCARD*$^{-/-}$ Caco-2 cells infected with WT Stm, we did not observe a decrease in
505 inflammasome activation compared to WT Caco-2 cells, indicating that ASC is
506 dispensable for IL-18 release and cell death in Caco-2 cells in response to Stm. Both
507 WT and *PYCARD*$^{-/-}$ Caco-2 cells exhibited similar levels of IL-8 release, an
508 inflammasome-independent cytokine (Fig. 5E). In addition, expression of *PYCARD*
509 mRNA in Caco-2 cells was very low compared to THP-1 macrophages (Fig. 5F).

510 Collectively, these data indicate that NLRP3 and ASC-dependent inflammasomes are
511 not required for inflammasome responses to *Salmonella* infection in human IECs.
512
513 Caspase-1 is partially required for inflammasome activation in response to *Salmonella*
514 in human intestinal epithelial cells
515 Inflammasomes recruit various caspases which can then cleave and process IL-1
516 and IL-18 cytokines and mediate pyroptosis (45). For example, the murine NAIP/NLRC4
517 inflammasome can recruit both caspase-1 and caspase-8 in response to *Salmonella*
518 infection of murine IECs (14). In human macrophages, NAIP/NLRC4, NLRP3, caspase-
519 1, and caspase-8 are recruited to the same macromolecular complex during *Salmonella*
520 infection (26). Notably, although expression of *CASP1* mRNA in Caco-2 cells is lower
521 than that in THP-1 macrophages (Fig. 6A), *CASP1* expression in Caco-2 cells is still
522 higher than expression of other inflammasome genes we have assessed so far
523 (compare axes in Fig. 6A to Fig. 3A, 3B, 5B, 5F). Similarly, expression of *CASP1* in
524 small intestinal organoids, although lower than that observed in human PBMCs, is
525 higher than expression of other inflammasome-related genes (compare axes in Fig. 6B
526 to Fig. 3C, 3D). This suggests that caspase-1 may be an important contributor to
527 inflammasome responses in IECs.
528 To test the contribution of caspases to inflammasome responses during
529 *Salmonella* infection in human IECs, we pretreated Caco-2 cells with pharmacological
530 inhibitors targeting caspases of interest (ZVAD: pan-caspase inhibitor or YVAD: inhibitor
531 for caspase-1). We then infected cells with WT Stm or Δ*sipB* Stm and assayed for
532 inflammasome activation by measuring IL-18 release (Fig. 6C). DMSO-treated cells

533 infected with WT Stm released IL-18, whereas cells treated with the inhibitors had a
534 significant defect in IL-18 release. Treatment with ZVAD, the pan-caspase inhibitor,
535 resulted in a lower level of IL-18 release compared to treatment with the caspase-1
536 inhibitor YVAD, suggesting that in addition to caspase-1, other caspases are also
537 important during *Salmonella* infection of human IECs. As expected, cells infected with
538 Δ*sipB* Stm demonstrated no inflammasome activation regardless of inhibitor treatment.
539 These results suggest that caspase-1 contributes to inflammasome responses to
540 *Salmonella* in human IECs.

541 Since pharmacological inhibitors preferentially targeting individual caspases can
542 have cross-reactivity with other caspases, we used CRISPR/Cas9 to disrupt *CASP1* in
543 Caco-2 cells (Fig. S7, S8). We sequenced and validated two independent single cell
544 clones of *CASP1*$^{-/-}$ Caco-2 cells (*CASP1*$^{-/-}$ #7, *CASP1*$^{-/-}$ #12) (Fig. S7, S8). All mutations
545 resulted in a premature stop codon (Fig. S7, S8). We further confirmed decreased
546 mRNA expression of *CASP1* in *CASP1*$^{-/-}$ #7 Caco-2 cells (Fig. S7B). We were unable to
547 detect protein expression in both WT Caco-2 cells and *CASP1*$^{-/-}$ #7 Caco- 2 cells by
548 western blot (Fig. S7C), indicating that caspase-1 is poorly expressed in Caco-2 cells.

549 To test whether inflammasome activation during *Salmonella* infection requires
550 caspase-1 in human IECs, we infected WT or *CASP1*$^{-/-}$ Caco-2 cells with WT or Δ*sipB*
551 Stm and assayed for subsequent inflammasome activation (Fig. 6D, E). As expected,
552 WT Caco-2 cells infected with WT Stm released significant levels of IL-18 and
553 underwent cell death (Fig. 6D, E). This response was dependent on the presence of the
554 SPI-1 T3SS, as cells infected with Δ*sipB* Stm failed to undergo inflammasome activation
555 (Fig. 6D). Consistent with YVAD inhibitor treatment, *CASP1*$^{-/-}$ Caco-2 cells infected with

556 WT Stm showed a statistically significant decrease in, but not complete abrogation of,
557 IL-18 release at 6hpi (Fig. 6D). There was also a slight delay in uptake of PI in *CASP1*$^{-/-}$
558 #7 Caco-2 cells relative to WT Caco-2 cells (Fig. 6E). In contrast, both WT and KO
559 Caco-2 cells exhibited similar levels of IL-8 release, an inflammasome-independent
560 cytokine (Fig. 6F). Overall, these data suggest that caspase-1 is partially required for
561 the inflammasome response to *Salmonella* infection in human IECs.
562
563 <u>Caspase-4 is required for inflammasome responses to *Salmonella* in human intestinal</u>
564 <u>epithelial cells</u>
565 In mice, in addition to caspase-1, a second caspase, caspase-11, responds to
566 *Salmonella* infection (19, 21). Caspase-11 and its human orthologs caspases-4/5 detect
567 cytosolic LPS and form the noncanonical inflammasome (24, 25). Caspases-4/5 are
568 important for late inflammasome responses to *Salmonella* infection of human intestinal
569 epithelial cells, specifically when *Salmonella* escapes to the cytoplasm from its
570 *Salmonella*-containing vacuole (SCV) (20). To test the contribution of the caspase-4/5
571 inflammasome earlier during infection with *Salmonella* grown under SPI-1-inducing
572 conditions, we transfected Caco-2 cells with siRNAs targeting *CASP4, CASP5* or both.
573 We then infected the cells with WT or Δ*sipB* Stm and assayed for inflammasome
574 activation by measuring IL-18 release (Fig. 7A). As expected, cells treated with a control
575 scrambled siRNA exhibited IL-18 secretion upon infection with WT Stm, but failed to
576 undergo IL-18 secretion when infected with Δ*sipB* Stm (Fig. 7A). However, knockdown
577 of *CASP4*, either alone, or in conjunction with *CASP5*, resulted in nearly complete
578 abrogation of IL-18 secretion in cells infected with WT Stm compared to control siRNA-

579 treated cells, suggesting that caspase-4 is required for inflammasome activation (Fig.
580 7A). Knockdown of *CASP5* alone resulted in a partial and significant decrease in IL-18
581 secretion, indicating that while caspase-5 may be playing a role, it is not absolutely
582 required (Fig. 7A). We observed moderately high (67 – 75%) siRNA-mediated
583 knockdown efficiencies (Fig. S9A). Release of the inflammasome-independent cytokine
584 IL-8 was comparable across conditions (Fig. S9B).

585 To definitively test the requirement of caspase-4 in early inflammasome
586 responses to SPI-1-expressing *Salmonella*, we disrupted *CASP4* in Caco-2 cells using
587 CRISPR/Cas9 (Fig. S10, S11). We sequenced and validated two independent single
588 cell clones of *CASP4*$^{-/-}$ Caco-2 cells (*CASP4*$^{-/-}$ #3, *CASP4*$^{-/-}$ #4) (Fig. S10). All mutations
589 resulted in a premature stop codon (Fig. S10). Both clones exhibited decreased mRNA
590 expression of *CASP4* and no protein expression relative to WT Caco- 2 cells (Fig. S11).

591 We then infected WT or *CASP4*$^{-/-}$ Caco-2 cells with WT or Δ*sipB* Stm and
592 assayed for subsequent IL-18 secretion and cell death as readouts for inflammasome
593 activation (Fig. 7B – E). As expected, WT Caco-2 cells infected with WT Stm released
594 significant levels of cleaved IL-18 and underwent cell death (Fig. 7B – E), whereas cells
595 infected with Δ*sipB* Stm failed to release IL-18 (Fig. 7B). Consistent with our findings
596 with siRNA knockdown of *CASP4*, *CASP4*$^{-/-}$ Caco-2 cells infected with WT Stm failed to
597 release IL-18 and exhibited a delay in PI uptake at 4-6 hpi (Fig. 7B – E). Interestingly,
598 *CASP4*$^{-/-}$ Caco-2 cells began to undergo cell death later in infection, suggesting that
599 while caspase-4 is required for inducing cell death early in infection, it may not be
600 absolutely required at later timepoints of infection. Like WT cells, *CASP4*$^{-/-}$ cells still
601 released substantial levels of the inflammasome-independent cytokine IL-8 following

602 infection (Fig. S9C). Collectively, these data suggest that caspase-4 is required for early

603 inflammasome responses to SPI-1-expressing *Salmonella* infection in human IECs.

604 Discussion

605 In this study, we have demonstrated that Caco-2 cells undergo inflammasome
606 activation in response to early infection with SPI-1-expressing *Salmonella* that requires
607 the presence of the SPI-1 T3SS, and that GSDMD-mediated pore formation is required
608 for release of IL-18 and cell death (Fig. 1, S1). Unexpectedly however, individual
609 bacterial T3SS ligands or flagellin were not sufficient to induce inflammasome activation
610 in Caco-2 cells or intestinal organoids (Fig. 2, S2, S3). Additionally, we found that
611 neither the NAIP nor NLRP3 canonical inflammasomes (Fig. 4, S4, 5), are required for
612 inflammasome responses during *Salmonella* infection, which may be due to low mRNA
613 expression of *NAIP*, *NLRC4*, and *NLRP3* in human IECs and human intestinal
614 organoids (Fig. 3, 5). Moreover, we found that ASC, a shared adaptor protein that
615 several inflammasomes use to recruit caspases, is also not required for inflammasome
616 activation (Fig. 5, S5, S6), suggesting that inflammasomes that require ASC for their
617 function are likely not playing a role in human IECs during *Salmonella* infection.
618 Interestingly, we found that caspase-1 is partially required for inflammasome activation
619 in human IECs (Fig. 6, S7, S8), leaving the unanswered question of what upstream
620 sensor is activating caspase-1. Finally, we found that caspase-4 is necessary for early
621 inflammasome responses to SPI-1-expressing *Salmonella* (Fig. 7, S9 – S11).

622 NAIP/NLRC4 inflammasome activation in IECs is critical for control of *Salmonella*
623 infection in mice (13–15). It was therefore surprising that NAIP was dispensable in
624 human IECs during *Salmonella* infection (Fig. 3, S3 – S6). Perhaps a lack of robust
625 NAIP/NLRC4 inflammasome responses in human IECs partially underlies why some
626 intestinal bacterial pathogens with T3SSs, such as *Shigella flexneri,* enterohemorrhagic

627 *Escherichia coli,* and enteropathogenic *E. coli,* can cause disease in humans but not
628 mice. Indeed, mice are normally resistant to *S. flexneri,* but mice lacking the
629 NAIP/NLRC4 inflammasome can be robustly colonized by *S. flexneri* and develop a
630 disease resembling human shigellosis (46).

631 The lack of NAIP activation may be due to low expression of *NAIP* and *NLRC4*
632 mRNA in human IEC lines as well as human small intestinal organoids (Fig. 3). Single
633 cell transcriptome analysis of cells intestinal cells from human donors demonstrate that
634 only a small subset of intestinal cells express *NAIP* at detectable levels, while *NLRC4*
635 expression is below the limit of detection (47). However, it is possible that a population
636 of IECs exist that express *NAIP* and *NLRC4* at low levels that may be below the limit of
637 detection of single-cell transcriptome analysis, but may be sufficient to activate the
638 inflammasome in response to infection under physiological conditions. Indeed, single
639 cell transcriptome analysis of murine small intestines reveal that expression of *NLRC4*
640 is also very low in murine IECs (48), but murine IECs undergo robust NAIP/NLRC4
641 inflammasome activation despite this relatively low expression (13–15). Despite our
642 findings that NAIP is dispensable in Caco-2 cells to respond to *Salmonella* infection and
643 human intestinal organoids do not mount inflammasome responses to T3SS ligands, it
644 is possible that under physiological conditions, this inflammasome still plays an
645 important role during bacterial infection or disease pathogenesis. Indeed, human
646 patients with activating NLRC4 mutations exhibit gastrointestinal symptoms (49–52).

647 A recent study comparing inflammasome responses of human and murine IECs
648 during *Salmonella* infection reported that while caspase-1 was required for
649 inflammasome activation in murine IECs, it was dispensable in late inflammasome

650 responses to *Salmonella* in human IECs (22). We found caspase-1 to be partially
651 required for inflammasome activation in IECs infected with *Salmonella* (Fig. 6, S7, S8).
652 Differences in our experimental conditions likely explain this difference in findings. Our
653 *Salmonella* was grown under SPI-1-inducing conditions, and we assayed for
654 inflammasome activation at a slightly earlier timepoint. Perhaps caspase-1 plays a role
655 in early inflammasome responses to SPI-1-expressing *Salmonella* in human IECs, and
656 it is less important at later timepoints when *Salmonella* has shifted to expressing its SPI-
657 2 T3SS.

658 The identity of the host sensor that is acting upstream of caspase-1 in human
659 IECs remains unknown. Humans have 22 NLRs that could serve as potential cytosolic
660 sensors of bacterial structures and activity (53). Most of these NLRs do not contain a
661 CARD domain, and they would therefore require an adaptor protein such as ASC to
662 recruit caspases. Given our finding that ASC is not required for inflammasome
663 activation in human IECs (Fig. 5, S5, S6), it is likely that the putative host sensor
664 upstream of caspase-1 contains its own CARD domain. Alternatively, it may interact
665 with a different CARD domain-containing adaptor protein than ASC. Future studies will
666 focus on identifying host factors that different caspases interact with during *Salmonella*
667 infection of human IECs.

668 In agreement with previous studies (20, 22), we found caspase-4 to be required
669 for inflammasome activation in response to *Salmonella* in human IECs (Fig 7, S9 –
670 S11). Importantly, caspase-4 activation restricts bacterial replication, and specifically
671 restricts replication of a subpopulation of cytosolic *Salmonella* (20, 22). It is unclear
672 whether *Salmonella*'s access into the cytosol is mediated by the bacteria or host, and

673 future studies will explore the mechanisms by which cytosolic populations of *Salmonella*
674 arise and how they influence inflammasome activation in human IECs.
675 In mice, inflammasome activation mediates control of *Salmonella* by restricting
676 bacterial replication, extruding infected cells, and preventing systemic dissemination of
677 *Salmonella* (13, 15, 20). Human IECs have been shown to undergo extrusion, but
678 whether this occurs in a caspase-4-dependent manner remains unknown (54). While we
679 know that human IECs and enteroids restrict bacterial replication in a caspase-4-
680 dependent manner (20, 22), it remains unknown if inflammasome activation results in
681 other mechanisms of control of infection. In mice, IL-18 release during *Salmonella*
682 infection recruits natural killer (NK) cells that are critical for early mucosal inflammatory
683 responses (55). We observe robust release of the inflammasome-dependent cytokine
684 IL-18, but the downstream role of this cytokine in the human intestine during *Salmonella*
685 infection has not been explored. Additionally, while our study focused exclusively on
686 *Salmonella* infection, it is worth exploring if enteric pathogens with similar lifestyles to
687 *Salmonella*, such as *Shigella*, or ones with different lifestyles, such as the extracellular
688 pathogen *Yersinia*, elicit similar responses. Future studies that interrogate downstream
689 consequences of inflammasome activation in human IECs in response to various
690 enteric pathogens could shed light on human mucosal inflammatory responses to
691 bacterial pathogens.
692 Overall, our data indicate that *Salmonella* infection of human IECs triggers
693 inflammasome pathways that are distinct from that in mice. Pathways that are activated
694 in mice and important for control of infection, such as the NAIP and NLRP3
695 inflammasomes, were unexpectedly not required for inflammasome responses in

696 human IECs under the conditions we investigated. Instead, inflammasome responses in
697 human IECs required caspases-1 and -4. Our findings provide a foundation for future
698 studies aimed at uncovering the relative contribution of different caspases, and the
699 downstream responses that they mediate in human IECs.

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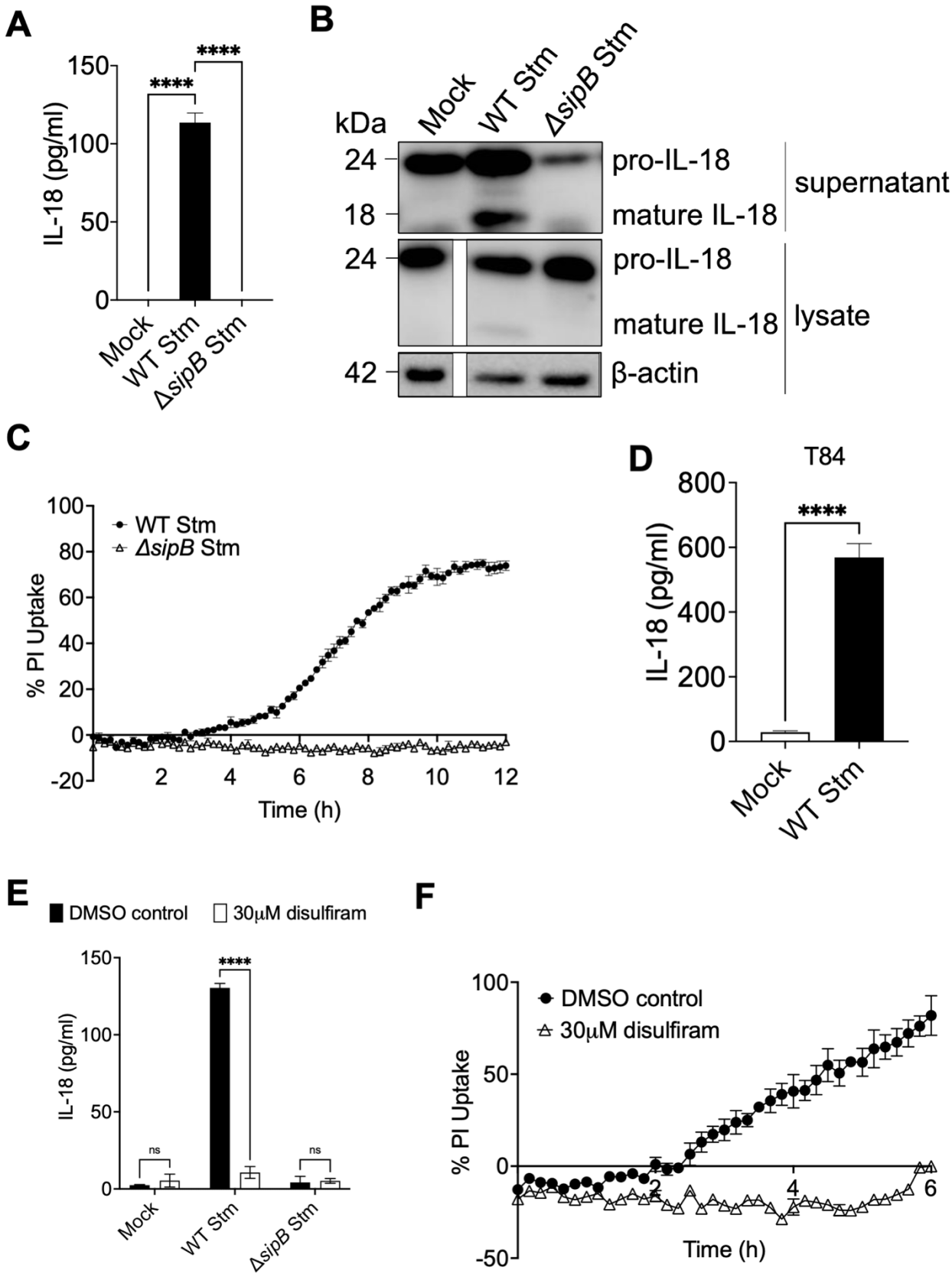
889 Figures


A
IL-18 (pg/ml)
150
100
50
0
****
****
Mock
WT Stm
ΔsipB Stm
B
kDa
Mock
WT Stm
ΔsipB Stm
24
18
24
42
pro-IL-18
mature IL-18
pro-IL-18
mature IL-18
β-actin
supernatant
lysate
C
% PI Uptake
WT Stm
ΔsipB Stm
Time (h)
D
T84
IL-18 (pg/ml)
800
600
400
200
0
****
Mock
WT Stm
E
DMSO control
30μM disulfiram
IL-18 (pg/ml)
150
100
50
0
****
ns
ns
Mock
WT Stm
ΔsipB Stm
F
% PI Uptake
DMSO control
30μM disulfiram
Time (h)

890

891 **Figure 1: *Salmonella* infection induces inflammasome activation in human**
892 **intestinal epithelial cells.** Caco-2 cells (A – C, E, F) or T84 cells (D) were infected with
893 PBS (Mock), WT *S*. Typhimurium, or Δ*sipB S*. Typhimurium. (A, D) Release of IL-18
894 into the supernatant was measured by ELISA at 6hpi. (B) Lysates and supernatants
895 collected 6hpi were immunoblotted for IL-18 and β-actin. (C) Cell death was measured
896 as percentage uptake of propidium iodide, normalized to cells treated with 1% Triton.
897 (E, F) Caco-2 cells were treated with 30 μM disulfiram or DMSO as a vehicle control 1
898 hour prior to infection. Cells were then infected with PBS (Mock), WT *S.* Typhimurium,
899 or Δ*sipB S*. Typhimurium. (E) Release of IL-18 into the supernatant was measured by
900 ELISA at 6hpi. (F) Cell death as percentage uptake of propidium iodide, normalized to
901 cells treated with 1% Triton. ns – not significant, **** $p < 0.0001$ by Dunnett's multiple
902 comparisons test (A), or by unpaired t-test (C, D, F) or by Šídák's multiple comparisons
903 test (E). Error bars represent the standard deviation or the standard error of the mean
904 (SEM) (C, F) of triplicate wells from one experiment. Data shown are representative of
905 at least three independent experiments.

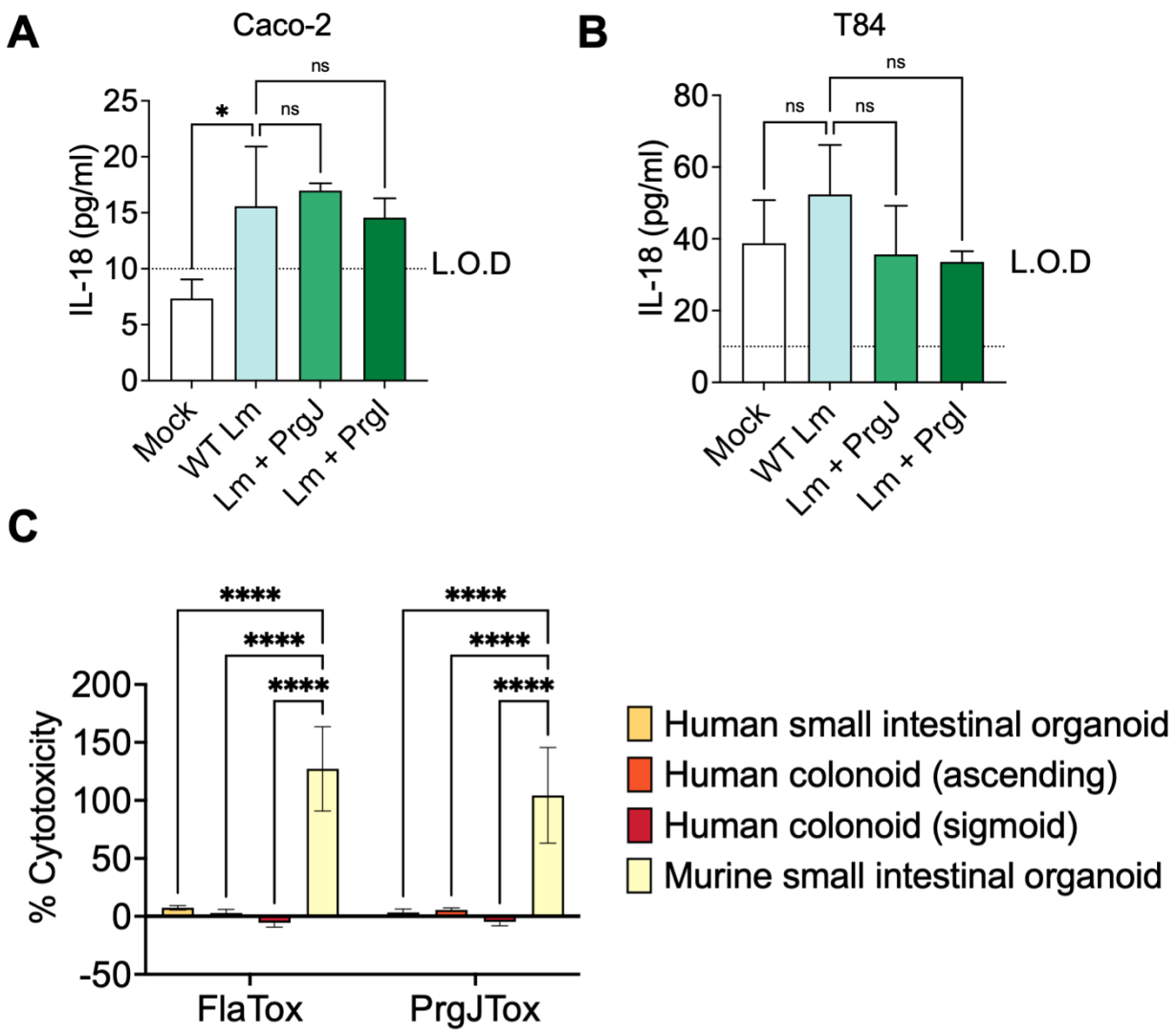
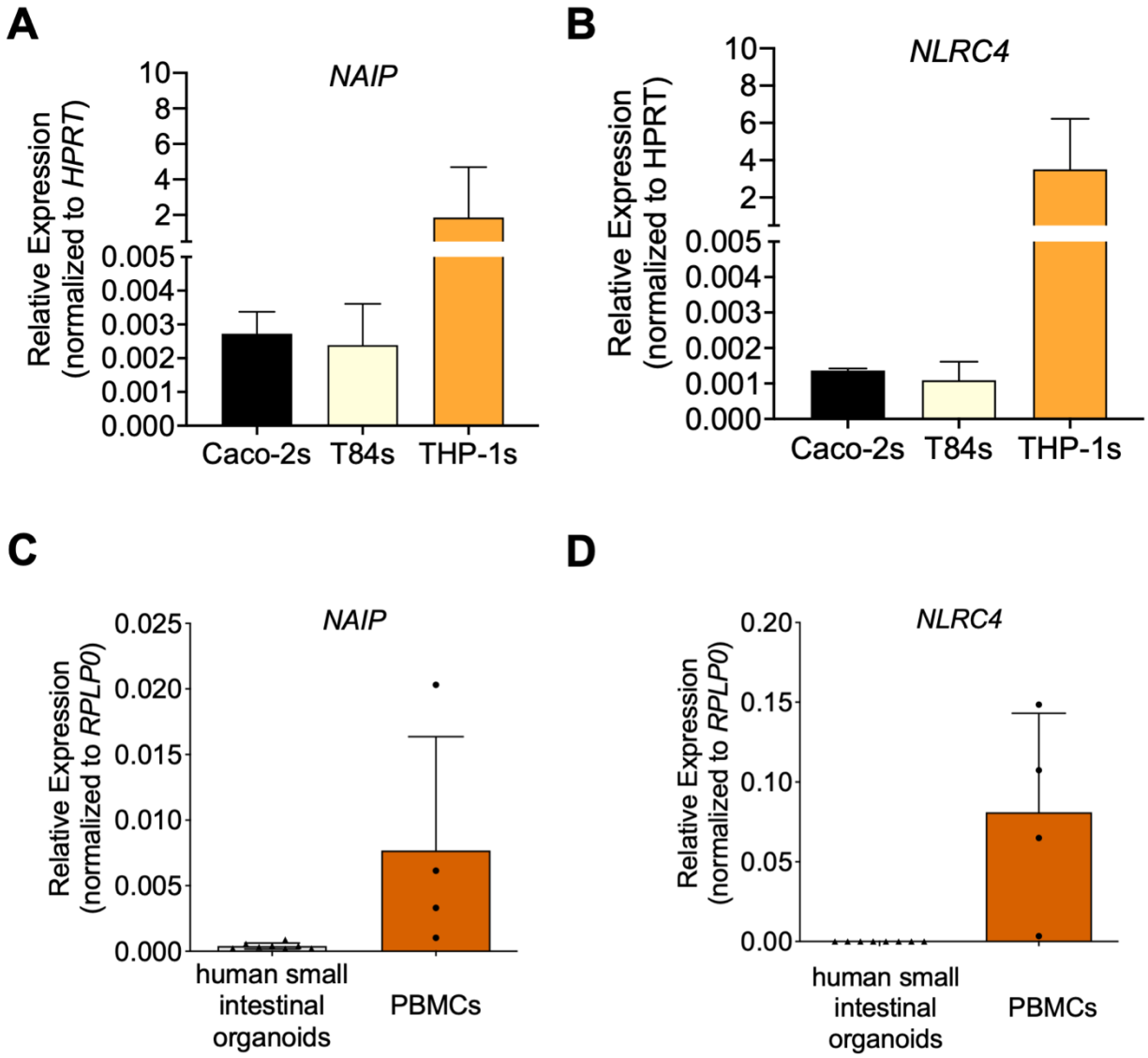


Figure 2: Bacterial T3SS ligands do not activate the inflammasome in human intestinal epithelial cells. (A, B) Caco-2 (A) or T84 cells (B) were primed for 3h with 100 ng/ml of Pam3CSK4 and infected with PBS (Mock), WT *L. monocytogenes* (WT Lm), or *L. monocytogenes* expressing *S.* Typhimurium SPI-1 inner rod (Lm + PrgJ), or SPI-1 needle (Lm + PrgI), at an MOI of 100. Release of IL-18 into the supernatant was measured by ELISA at 16hpi. L.O.D indicates the limit of detection of the assay. (C) Differentiated intestinal organoids were treated with FlaTox (PA + LFn-Fla) or Inner Rod Tox (PA + LFn-Rod) in media containing propidium iodide for 4h. Cell death was

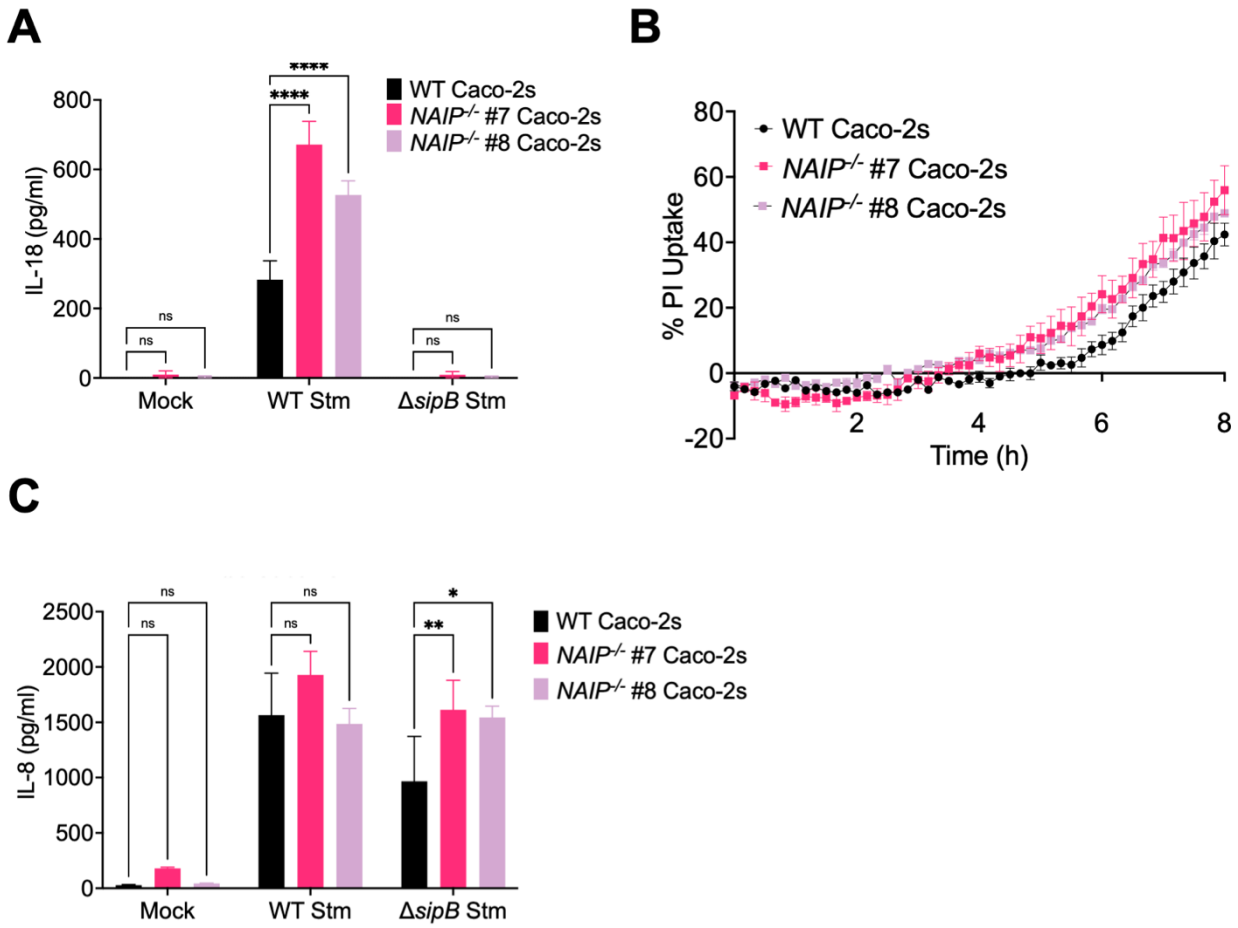
915 measured as percentage uptake of propidium iodide, normalized to organoids treated
916 with 1% Triton. ns – not significant, * $p < 0.05$, **** $p < 0.0001$ by Dunnett's multiple
917 comparisons test. Error bars represent the standard deviation of triplicate wells from
918 one experiment. Data shown are representative of at least three independent
919 experiments.



920

921 **Figure 3: Human intestinal epithelial cells express low levels of NAIP/NLRC4**
922 **compared to human myeloid cells.** Relative mRNA expression of *NAIP* and *NLRC4*
923 compared to the housekeeping control *HPRT* or *RPLP0* as measured by qRT-PCR in

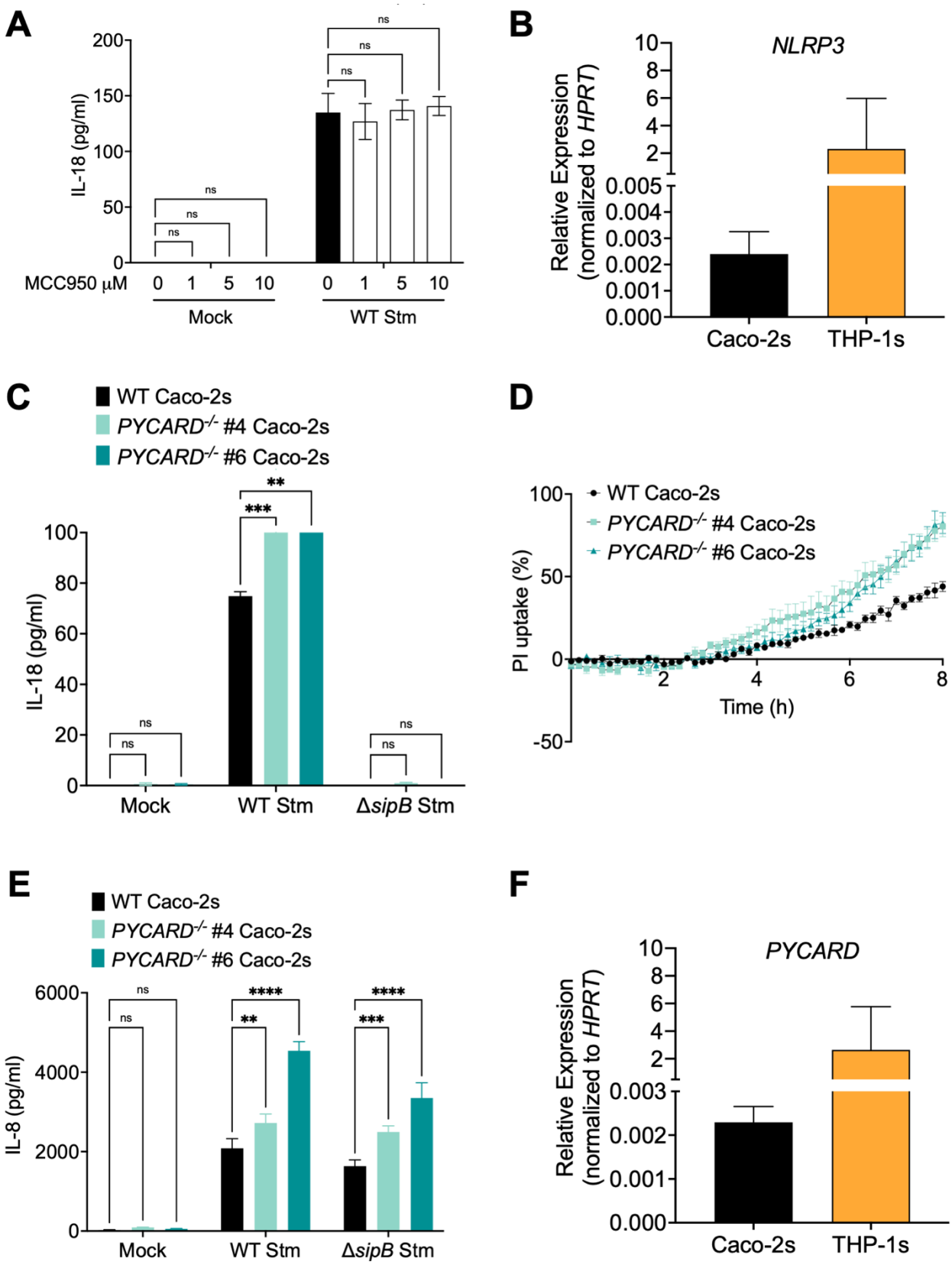
924 (A, B) Caco-2 cells, T84 cells, and THP-1 macrophages, and in (C, D) human peripheral
925 blood mononuclear cells (PBMCs) and human small intestinal organoids. Error bars
926 represent the standard deviation of multiple wells. Data shown are representative of at
927 least three independent experiments.



928

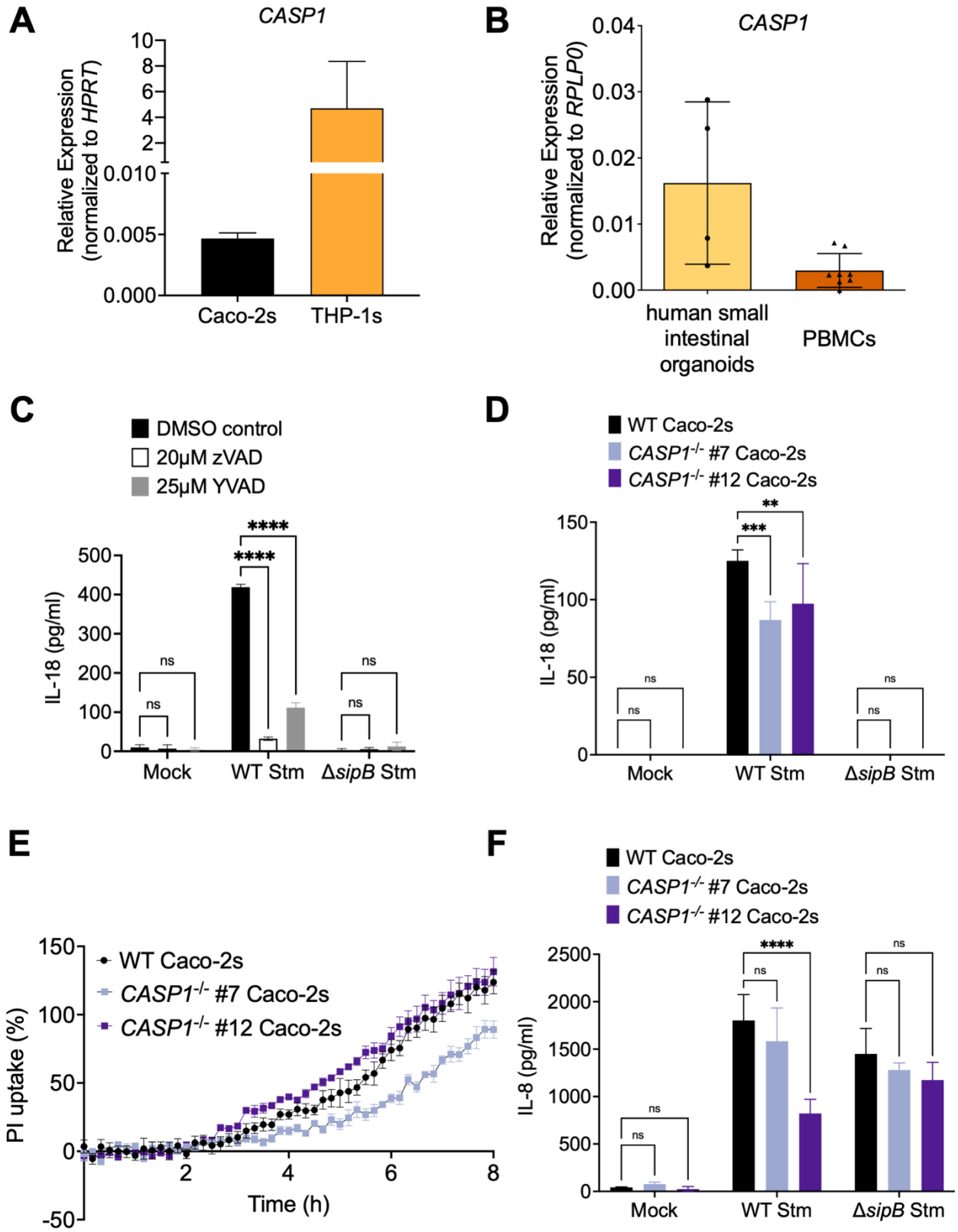
929 **Figure 4: NAIP is not required for inflammasome responses to *Salmonella* in**
930 **human intestinal epithelial cells.** WT or two independent single cell clones of *NAIP*$^{-/-}$
931 Caco-2 cells were infected with PBS (Mock), WT *S*. Typhimurium, or Δ*sipB S*.
932 Typhimurium for 6hrs. (A, C) Release of IL-18 or IL-8 into the supernatant was
933 measured by ELISA. (B) Cell death was measured as percentage uptake of propidium
934 iodide, normalized to cells treated with 1% Triton. (A, C) ns – not significant, * $p < 0.05$,

935 ** $p < 0.01$, *****$p < 0.0001$ by Dunnett's multiple comparisons test. Error bars represent
936 the standard deviation or the standard error of the mean (SEM) (B) of triplicate wells
937 from one experiment. Data shown are representative of at least three independent
938 experiments.


A
IL-18 (pg/ml)
200
150
100
50
0
ns
ns
ns
ns
ns
ns
MCC950 μM
0 1 5 10
0 1 5 10
Mock
WT Stm
B
NLRP3
Relative Expression
(normalized to HPRT)
10
8
6
4
2
0.005
0.004
0.003
0.002
0.001
0.000
Caco-2s
THP-1s
C
WT Caco-2s
PYCARD-/- #4 Caco-2s
PYCARD-/- #6 Caco-2s
IL-18 (pg/ml)
100
80
60
40
20
0
**
***
ns
ns
ns
ns
Mock
WT Stm
ΔsipB Stm
D
WT Caco-2s
PYCARD-/- #4 Caco-2s
PYCARD-/- #6 Caco-2s
PI uptake (%)
100
50
0
-50
2
4
6
8
Time (h)
E
WT Caco-2s
PYCARD-/- #4 Caco-2s
PYCARD-/- #6 Caco-2s
IL-8 (pg/ml)
6000
4000
2000
0
ns
ns
****
**
****
***
Mock
WT Stm
ΔsipB Stm
F
PYCARD
Relative Expression
(normalized to HPRT)
10
8
6
4
2
0.003
0.002
0.001
0.000
Caco-2s
THP-1s

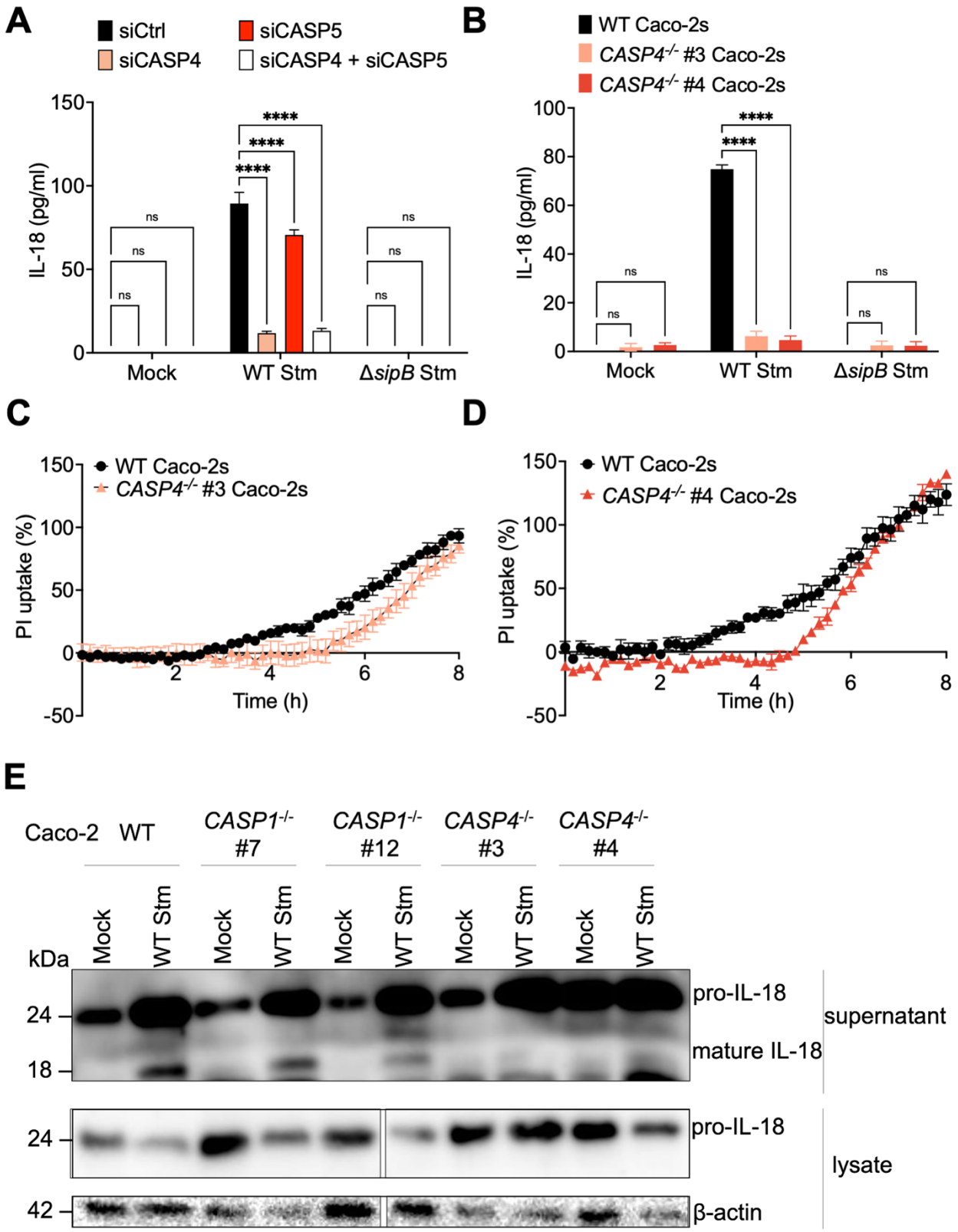
939

940 **Figure 5: NLRP3 and ASC are dispensable for inflammasome responses to**
941 ***Salmonella* in human intestinal epithelial cells.** (A) WT Caco-2 cells were primed for
942 3hrs with 400 ng/ml of Pam3CSK4. One hour prior to infection, cells were treated with
943 the indicated concentrations of MCC950 or DMSO as a vehicle control. Cells were then
944 infected with PBS (Mock) or WT *S*. Typhimurium for 6hrs. Release of IL-18 into the
945 supernatant was measured by ELISA. (B, F) Relative mRNA expression of *NLRP3* and
946 *PYCARD* compared to the housekeeping control *HPRT* as measured by qRT-PCR in
947 Caco-2 cells and THP-1 macrophages. (C, D, E) WT or two independent single cell
948 clones of *PYCARD*$^{-/-}$ Caco-2 cells were infected with PBS (Mock), WT *S*. Typhimurium,
949 or Δ*sipB S*. Typhimurium. (C, D) Release of IL-18 or IL-8 into the supernatant were
950 measured by ELISA at 6hpi. (E) Cell death was measured as percentage uptake of
951 propidium iodide, normalized to cells treated with 1% Triton. (A, C, E) ns – not
952 significant, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by Dunnett's multiple comparisons
953 test. Error bars represent the standard deviation or the standard error of the mean
954 (SEM) (D) of triplicate wells from one experiment. Data shown are representative of at
955 least three independent experiments.


A
CASP1
Relative Expression (normalized to HPRT)
10
8
6
4
2
0.010
0.005
0.000
Caco-2s
THP-1s
B
CASP1
Relative Expression (normalized to RPLP0)
0.04
0.03
0.02
0.01
0.00
human small intestinal organoids
PBMCs
C
DMSO control
20μM zVAD
25μM YVAD
IL-18 (pg/ml)
500
400
300
200
100
0
****
****
ns
ns
ns
ns
Mock
WT Stm
ΔsipB Stm
D
WT Caco-2s
CASP1-/- #7 Caco-2s
CASP1-/- #12 Caco-2s
IL-18 (pg/ml)
150
100
50
0
**
***
ns
ns
ns
ns
Mock
WT Stm
ΔsipB Stm
E
WT Caco-2s
CASP1-/- #7 Caco-2s
CASP1-/- #12 Caco-2s
PI uptake (%)
150
100
50
0
-50
2
4
6
8
Time (h)
F
WT Caco-2s
CASP1-/- #7 Caco-2s
CASP1-/- #12 Caco-2s
IL-8 (pg/ml)
2500
2000
1500
1000
500
0
****
ns
ns
ns
ns
ns
Mock
WT Stm
ΔsipB Stm

956

957 **Figure 6: Caspase-1 is partially required for inflammasome responses to**
958 ***Salmonella* in human intestinal epithelial cells.** (A, B) Relative mRNA expression of
959 *CASP1* compared to the housekeeping control *HPRT* as measured by qRT-PCR in
960 Caco-2 cells, THP-1 macrophages, human peripheral blood mononuclear cells
961 (PBMCs), and human small intestinal organoids. (C) WT Caco-2 cells were primed with
962 400 ng/ml of Pam3CSK4 for 3h. One hour prior to infection, cells were treated with 20
963 μM of pan-caspase inhibitor Z-VAD(OMe)-FMK, 25 μM of caspase-1 inhibitor Ac-YVAD-
964 cmk, or DMSO as a vehicle control. Cells were then infected with PBS (Mock), WT *S*.
965 Typhimurium, or Δ*sipB S*. Typhimurium for 6hrs. Release of IL-18 into the supernatant
966 was measured by ELISA. (D – F) WT or two independent single cell clones of *CASP1*$^{-/-}$
967 Caco-2 cells were infected with PBS (Mock), WT *S*. Typhimurium, or Δ*sipB S*.
968 Typhimurium. (D, F) Release of IL-18 or IL-8 into the supernatant were measured by
969 ELISA at 6hpi. (E) Cell death was measured as percentage uptake of propidium iodide,
970 normalized to cells treated with 1% Triton. (C, D, F) ns – not significant, ** $p < 0.01$, ****
971 $p < 0.0001$ by Dunnett's multiple comparisons test. Error bars represent the standard
972 deviation or the standard error of the mean (SEM) (E) of triplicate wells from one
973 experiment. Data shown are representative of at least three independent experiments.


A
siCtrl
siCASP5
siCASP4
siCASP4 + siCASP5
IL-18 (pg/ml)
150
100
50
0
****
****
****
ns
ns
ns
ns
ns
ns
Mock
WT Stm
ΔsipB Stm
B
WT Caco-2s
CASP4-/- #3 Caco-2s
CASP4-/- #4 Caco-2s
IL-18 (pg/ml)
100
80
60
40
20
0
****
****
ns
ns
ns
ns
Mock
WT Stm
ΔsipB Stm
C
WT Caco-2s
CASP4-/- #3 Caco-2s
PI uptake (%)
150
100
50
0
-50
2
4
6
8
Time (h)
D
WT Caco-2s
CASP4-/- #4 Caco-2s
PI uptake (%)
150
100
50
0
-50
2
4
6
8
Time (h)
E
Caco-2 WT
CASP1-/- #7
CASP1-/- #12
CASP4-/- #3
CASP4-/- #4
Mock
WT Stm
Mock
WT Stm
Mock
WT Stm
Mock
WT Stm
Mock
WT Stm
kDa
24
18
pro-IL-18
mature IL-18
supernatant
24
pro-IL-18
lysate
42
β-actin

974

975 **Figure 7: Caspase-4 is required for inflammasome responses to *Salmonella* in**
976 **human intestinal epithelial cells.** (A) WT Caco-2 cells were treated with siRNA
977 targeting *CASP4, CASP5,* or a control scrambled siRNA for 72h. Cells were primed with
978 400 ng/ml of Pam3CSK4 for 3hrs. Cells were then infected with PBS (Mock), WT *S*.
979 Typhimurium*,* or Δ*sipB S*. Typhimurium for 6hrs. Release of IL-18 was measured by
980 ELISA (B – E) WT or two independent clones of *CASP4*$^{-/-}$ or *CASP1*$^{-/-}$ (E only) Caco-2
981 cells were infected with PBS (Mock), WT *S*. Typhimurium, or Δ*sipB S*. Typhimurium for
982 6hrs. (B) Release of IL-18 into the supernatant was measured by ELISA at 6hpi. (C, D)
983 Cell death was measured as percentage uptake of propidium iodide, normalized to cells
984 treated with 1% Triton. (E) Lysates and supernatants collected 6hpi were
985 immunoblotted for IL-18 and β-actin. (A, B) ns – not significant, **** $p < 0.0001$ by
986 Tukey's (A) or Dunnett's (B) multiple comparisons test. Error bars represent the
987 standard deviation or the standard error of the mean (SEM) (C, D) of triplicate wells
988 from one experiment. Data shown are representative of at least three independent
989 experiments.

990 Figure Legends

991 **Figure 1: *Salmonella* infection induces inflammasome activation in human**
992 **intestinal epithelial cells.** Caco-2 cells (A – C, E, F) or T84 cells (D) were infected with
993 PBS (Mock), WT *S*. Typhimurium, or Δ*sipB S*. Typhimurium. (A, D) Release of IL-18
994 into the supernatant was measured by ELISA at 6hpi. (B) Lysates and supernatants
995 collected 6hpi were immunoblotted for IL-18 and β-actin. (C) Cell death was measured
996 as percentage uptake of propidium iodide, normalized to cells treated with 1% Triton.
997 (E, F) Caco-2 cells were treated with 30 μM disulfiram or DMSO as a vehicle control 1
998 hour prior to infection. Cells were then infected with PBS (Mock), WT *S.* Typhimurium,
999 or Δ*sipB S*. Typhimurium. (E) Release of IL-18 into the supernatant was measured by
1000 ELISA at 6hpi. (F) Cell death as percentage uptake of propidium iodide, normalized to
1001 cells treated with 1% Triton. ns – not significant, **** $p < 0.0001$ by Dunnett's multiple
1002 comparisons test (A), or by unpaired t-test (C, D, F) or by Šídák's multiple comparisons
1003 test (E). Error bars represent the standard deviation or the standard error of the mean
1004 (SEM) (C, F) of triplicate wells from one experiment. Data shown are representative of
1005 at least three independent experiments.

1006

1007 **Figure 2: Bacterial T3SS ligands do not activate the inflammasome in human**
1008 **intestinal epithelial cells.** (A, B) Caco-2 (A) or T84 cells (B) were primed for 3h with
1009 100 ng/ml of Pam3CSK4 and infected with PBS (Mock), WT *L. monocytogenes* (WT
1010 Lm), or *L. monocytogenes* expressing *S.* Typhimurium SPI-1 inner rod (Lm + PrgJ), or
1011 SPI-1 needle (Lm + PrgI), at an MOI of 100. Release of IL-18 into the supernatant was
1012 measured by ELISA at 16hpi. L.O.D indicates the limit of detection of the assay. (C)

1013 Differentiated intestinal organoids were treated with FlaTox (PA + LFn-Fla) or Inner Rod
1014 Tox (PA + LFn-Rod) in media containing propidium iodide for 4h. Cell death was
1015 measured as percentage uptake of propidium iodide, normalized to organoids treated
1016 with 1% Triton. ns – not significant, * $p < 0.05$, **** $p < 0.0001$ by Dunnett's multiple
1017 comparisons test. Error bars represent the standard deviation of triplicate wells from
1018 one experiment. Data shown are representative of at least three independent
1019 experiments.
1020
1021 **Figure 3: Human intestinal epithelial cells express low levels of NAIP/NLRC4**
1022 **compared to human myeloid cells.** Relative mRNA expression of *NAIP* and *NLRC4*
1023 compared to the housekeeping control *HPRT* or *RPLP0* as measured by qRT-PCR in
1024 (A, B) Caco-2 cells, T84 cells, and THP-1 macrophages, and in (C, D) human peripheral
1025 blood mononuclear cells (PBMCs) and human small intestinal organoids. Error bars
1026 represent the standard deviation of multiple wells. Data shown are representative of at
1027 least three independent experiments.
1028
1029 **Figure 4: NAIP is not required for inflammasome responses to *Salmonella* in**
1030 **human intestinal epithelial cells.** WT or two independent single cell clones of *NAIP*$^{-/-}$
1031 Caco-2 cells were infected with PBS (Mock), WT *S*. Typhimurium, or Δ*sipB S*.
1032 Typhimurium for 6hrs. (A, C) Release of IL-18 or IL-8 into the supernatant was
1033 measured by ELISA. (B) Cell death was measured as percentage uptake of propidium
1034 iodide, normalized to cells treated with 1% Triton. (A, C) ns – not significant, * $p < 0.05$,
1035 ** $p < 0.01$, *****$p < 0.0001$ by Dunnett's multiple comparisons test. Error bars represent

1036 the standard deviation or the standard error of the mean (SEM) (B) of triplicate wells
1037 from one experiment. Data shown are representative of at least three independent
1038 experiments.
1039
1040 **Figure 5: NLRP3 and ASC are dispensable for inflammasome responses to**
1041 ***Salmonella* in human intestinal epithelial cells.** (A) WT Caco-2 cells were primed for
1042 3hrs with 400 ng/ml of Pam3CSK4. One hour prior to infection, cells were treated with
1043 the indicated concentrations of MCC950 or DMSO as a vehicle control. Cells were then
1044 infected with PBS (Mock) or WT *S*. Typhimurium for 6hrs. Release of IL-18 into the
1045 supernatant was measured by ELISA. (B, F) Relative mRNA expression of *NLRP3* and
1046 *PYCARD* compared to the housekeeping control *HPRT* as measured by qRT-PCR in
1047 Caco-2 cells and THP-1 macrophages. (C, D, E) WT or two independent single cell
1048 clones of *PYCARD*$^{-/-}$ Caco-2 cells were infected with PBS (Mock), WT *S*. Typhimurium,
1049 or Δ*sipB S*. Typhimurium. (C, D) Release of IL-18 or IL-8 into the supernatant were
1050 measured by ELISA at 6hpi. (E) Cell death was measured as percentage uptake of
1051 propidium iodide, normalized to cells treated with 1% Triton. (A, C, E) ns – not
1052 significant, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by Dunnett's multiple comparisons
1053 test. Error bars represent the standard deviation or the standard error of the mean
1054 (SEM) (D) of triplicate wells from one experiment. Data shown are representative of at
1055 least three independent experiments.
1056
1057 **Figure 6: Caspase-1 is partially required for inflammasome responses to**
1058 ***Salmonella* in human intestinal epithelial cells.** (A, B) Relative mRNA expression of

1059 *CASP1* compared to the housekeeping control *HPRT* as measured by qRT-PCR in
1060 Caco-2 cells, THP-1 macrophages, human peripheral blood mononuclear cells
1061 (PBMCs), and human small intestinal organoids. (C) WT Caco-2 cells were primed with
1062 400 ng/ml of Pam3CSK4 for 3h. One hour prior to infection, cells were treated with 20
1063 μM of pan-caspase inhibitor Z-VAD(OMe)-FMK, 25 μM of caspase-1 inhibitor Ac-YVAD-
1064 cmk, or DMSO as a vehicle control. Cells were then infected with PBS (Mock), WT *S*.
1065 Typhimurium, or Δ*sipB S*. Typhimurium for 6hrs. Release of IL-18 into the supernatant
1066 was measured by ELISA. (D – F) WT or two independent single cell clones of *CASP1*$^{-/-}$
1067 Caco-2 cells were infected with PBS (Mock), WT *S*. Typhimurium, or Δ*sipB S*.
1068 Typhimurium. (D, F) Release of IL-18 or IL-8 into the supernatant were measured by
1069 ELISA at 6hpi. (E) Cell death was measured as percentage uptake of propidium iodide,
1070 normalized to cells treated with 1% Triton. (C, D, F) ns – not significant, ** $p < 0.01$, ****
1071 $p < 0.0001$ by Dunnett's multiple comparisons test. Error bars represent the standard
1072 deviation or the standard error of the mean (SEM) (E) of triplicate wells from one
1073 experiment. Data shown are representative of at least three independent experiments.
1074
1075 **Figure 7: Caspase-4 is required for inflammasome responses to *Salmonella* in**
1076 **human intestinal epithelial cells.** (A) WT Caco-2 cells were treated with siRNA
1077 targeting *CASP4, CASP5,* or a control scrambled siRNA for 72h. Cells were primed with
1078 400 ng/ml of Pam3CSK4 for 3hrs. Cells were then infected with PBS (Mock), WT *S*.
1079 Typhimurium*,* or Δ*sipB S*. Typhimurium for 6hrs. Release of IL-18 was measured by
1080 ELISA (B – E) WT or two independent clones of *CASP4*$^{-/-}$ or *CASP1*$^{-/-}$ (E only) Caco-2
1081 cells were infected with PBS (Mock), WT *S*. Typhimurium, or Δ*sipB S*. Typhimurium for

1082 6hrs. (B) Release of IL-18 into the supernatant was measured by ELISA at 6hpi. (C, D)
1083 Cell death was measured as percentage uptake of propidium iodide, normalized to cells
1084 treated with 1% Triton. (E) Lysates and supernatants collected 6hpi were
1085 immunoblotted for IL-18 and β-actin. (A, B) ns – not significant, **** $p < 0.0001$ by
1086 Tukey's (A) or Dunnett's (B) multiple comparisons test. Error bars represent the
1087 standard deviation or the standard error of the mean (SEM) (C, D) of triplicate wells
1088 from one experiment. Data shown are representative of at least three independent
1089 experiments.