Title: Inflammasomes primarily restrict cytosolic *Salmonella* replication within human macrophages

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Abstract

Salmonella enterica serovar Typhimurium is a facultative intracellular pathogen that utilizes its type III secretion systems (T3SSs) to inject virulence factors into the host cell and colonize the host. In turn, a subset of cytosolic immune receptors respond to T3SS ligands by forming multimeric signaling complexes called inflammasomes, which activate caspases that induce interleukin-1 (IL-1) family cytokine release and an inflammatory form of cell death called pyroptosis. Human macrophages mount a multifaceted inflammasome response to *Salmonella* infection that ultimately restricts intracellular bacterial replication. However, how inflammasomes restrict *Salmonella* replication remains unknown. We find that caspase-1 is essential for mediating inflammasome responses to *Salmonella* and subsequent restriction of bacterial replication within human macrophages, with caspase-4 contributing as well. We also demonstrate that the downstream pore-forming protein gasdermin D (GSDMD) and ninjurin-1 (NINJ1), a mediator of terminal cell lysis, play a role in controlling *Salmonella* replication in human macrophages. Notably, in the absence of inflammasome responses, we observed hyperreplication of *Salmonella* within the cytosol of infected cells, and we also observed increased bacterial replication within vacuoles, suggesting that inflammasomes control *Salmonella* replication primarily within the cytosol and also within vacuoles. These findings reveal that inflammatory caspases and pyroptotic factors mediate inflammasome responses that restrict the subcellular localization of intracellular *Salmonella* replication within human macrophages.

47 Introduction

48 Intracellular bacterial pathogens create and maintain replicative niches within
49 host cells. To survive inside the host, these pathogens must remodel the host cellular
50 landscape and overcome immune defenses. *Salmonella enterica* serovar Typhimurium
51 (*Salmonella*) is a facultative intracellular pathogen and a major cause of food-borne
52 illness worldwide (Majowicz et al., 2010). Following ingestion, *Salmonella* colonizes the
53 intestinal tract, where it can invade, replicate, and survive in host cells, including
54 macrophages (Crowley et al., 2016; R. L. Santos & Bäumler, 2004). *Salmonella*
55 employs type III secretion systems (T3SSs) that act as molecular syringes to inject
56 virulence factors, or effectors, into the host cell cytosol (Agbor & McCormick, 2011;
57 Crowley et al., 2016). Specifically, *Salmonella* relies on two distinct T3SSs encoded on
58 *Salmonella* Pathogenicity Islands 1 and 2 (SPI-1 and SPI-2) to invade and replicate
59 within host cells, respectively (Mills et al., 1995; Shea et al., 1996; Hensel et al., 1998;
60 Galan & Zhou, 2000; Galán, 1999; Galán & Collmer, 1999; Galan & Curtiss, 1989;
61 Ochman et al., 1996; Cirillo et al., 1998). The SPI-2 T3SS translocates effectors to
62 facilitate biogenesis and maintenance of the *Salmonella*-containing vacuole (SCV),
63 wherein *Salmonella* resides and replicates (Cirillo et al., 1998; Takeuchi, 1967;
64 Takeuchi & Sprinz, 1967; Kihlström & Latkovic, 1978; Garcia-del Portillo et al., 1993;
65 Garcia-del Portillo & Finlay, 1995; Steele-Mortimer et al., 1999; Hansen-Wester et al.,
66 2002; Steele-Mortimer, 2008; Jennings et al., 2017; Brumell, Goosney, et al., 2002;
67 Brumell, Tang, et al., 2002; Beuzón et al., 2000; Hensel et al., 1998). In epithelial cells,
68 *Salmonella* escapes its vacuolar niche and hyperreplicates in the host cell cytosol
69 (Knodler, Crowley, et al., 2014; Knodler et al., 2010; Knodler, Nair, et al., 2014; Malik-

70 Kale et al., 2012). While T3SSs are essential for *Salmonella* to infect host cells, they
71 also inject flagellin (Sun et al., 2007) and structural components of the T3SS into the
72 host cell cytosol, which conversely leads to cytosolic immune detection of *Salmonella*.
73 The mammalian innate immune system detects violations of cytosolic sanctity,
74 such as the presence of intracellular bacterial pathogens, through cytosolic pattern
75 recognition receptors (PRRs) (Janeway, 1989; Medzhitov & Janeway, 2002). A subset
76 of these PRRs includes the nucleotide-binding domain, leucine-rich repeat (NLR) family
77 proteins. NLRs respond to their cognate stimuli by oligomerizing and inducing the
78 assembly of multiprotein complexes termed inflammasomes, which activate
79 inflammatory caspases (Broz & Dixit, 2016; Lamkanfi & Dixit, 2009, 2014; Martinon et
80 al., 2002). Canonical inflammasomes recruit and activate caspase-1, which cleaves and
81 activates pro-inflammatory interleukin-1 (IL-1) family cytokines and the pore-forming
82 protein gasdermin D (GSDMD) (Kuida et al., 1995; Li et al., 1995; Agard et al., 2010;
83 Thornberry et al., 1992; Shi et al., 2015; Kayagaki et al., 2015). Alternatively,
84 noncanonical inflammasomes are formed by caspase-11 in mice and two orthologs in
85 humans, caspase-4 and caspase-5, in response to cytosolic lipopolysaccharide (LPS)
86 (Casson et al., 2015; Hagar et al., 2013; Kayagaki et al., 2011, 2013; Lagrange et al.,
87 2018; Schmid-Burgk et al., 2015; Shi et al., 2014). These caspases directly process and
88 activate GSDMD (Agard et al., 2010; Kayagaki et al., 2015; Shi et al., 2015). Liberated
89 GSDMD N-terminal fragments oligomerize to create pores in the host plasma
90 membrane, through which IL-1 family cytokines and other alarmins are released to
91 promote a lytic form of inflammatory cell death termed pyroptosis (Agard et al., 2010;
92 Ding et al., 2016; Kayagaki et al., 2015; Shi et al., 2015).

93 *Salmonella* activates several inflammasomes in human macrophages. One such
94 inflammasome is the NLR family, apoptosis inhibitory protein (NAIP)/NLR family, CARD
95 domain-containing protein 4 (NLRC4) inflammasome (Bierschenk et al., 2019; Gram et
96 al., 2021; Naseer, Egan, et al., 2022). NAIP detects the cytosolic presence of T3SS
97 structural components and flagellin (Grandjean et al., 2017; Kofoed & Vance, 2011;
98 Kortmann et al., 2015; Miao, Mao, et al., 2010; Molofsky et al., 2006; Rauch et al., 2016;
99 Rayamajhi et al., 2013; Ren et al., 2006; Reyes Ruiz et al., 2017; Sun et al., 2007; Yang
100 et al., 2013; Zhao et al., 2011, 2016). Upon ligand recognition, NAIP recruits NLRC4,
101 which oligomerizes to form the active NAIP/NLRC4 inflammasome (Diebolder et al.,
102 2015; Z. Hu et al., 2015; Zhang et al., 2015). *Salmonella* also activates the NLR pyrin
103 domain-containing protein 3 (NLRP3) inflammasome and the noncanonical
104 inflammasome in human macrophages (Bierschenk et al., 2019; Casson et al., 2015;
105 Gram et al., 2021; Naseer, Egan, et al., 2022). NLRP3 responds to diverse stimuli,
106 including ionic fluxes as a result of host plasma membrane damage (Franchi et al.,
107 2007; Hornung et al., 2008; Mariathasan et al., 2006; Muñoz-Planillo et al., 2013;
108 Perregaux & Gabel, 1994), and can be secondarily activated by the noncanonical
109 inflammasome (Baker et al., 2015; Casson et al., 2015; Kayagaki et al., 2013; Pilla et
110 al., 2014; Rathinam et al., 2012; Rühl & Broz, 2015; Schmid-Burgk et al., 2015; Shi et
111 al., 2014).
112 Inflammasome activation is critical for host defense against *Salmonella*. In mice,
113 the NAIP/NLRC4 inflammasome is required to control *Salmonella* infection (Carvalho et
114 al., 2012; Franchi et al., 2012; Hausmann et al., 2020; Miao et al., 2006; Miao, Mao, et
115 al., 2010; Rauch et al., 2016, 2017; Sellin et al., 2014; Zhao et al., 2016). In murine

116 intestinal epithelial cells (IECs), NAIP/NLRC4 inflammasome activation triggers
117 pyroptosis and expulsion of infected cells, and is both necessary and sufficient in IECs
118 to restrict *Salmonella* replication and prevent bacterial dissemination to distal organ
119 sites (Hausmann et al., 2020; Rauch et al., 2017; Sellin et al., 2014). Unlike murine
120 IECs, human epithelial cells do not rely on NAIP/NLRC4 or caspase-1, but instead rely
121 on caspase-4 to control *Salmonella* replication, in part through pyroptosis and cell
122 extrusion (Holly et al., 2020; Knodler, Crowley, et al., 2014; Knodler et al., 2010;
123 Naseer, Zhang, et al., 2022). Moreover, in murine macrophages, inflammasome
124 activation controls the replication of a mutant strain of *Salmonella* that aberrantly
125 invades the cytosol, but only slightly limits replication of wild-type (WT) *Salmonella*
126 (Thurston et al., 2016). In contrast, we found that human macrophages rely on
127 NAIP/NLRC4- and NLRP3-dependent inflammasome responses to control intracellular
128 WT *Salmonella* replication (Naseer, Egan, et al., 2022). However, how inflammasome
129 signaling restricts *Salmonella* replication in human macrophages remains unknown.
130 In this study, we show that caspase-1 is required for inflammasome responses
131 and the restriction of intracellular *Salmonella* replication early during infection in human
132 macrophages, and that caspase-4 contributes to the restriction of *Salmonella* later
133 during infection. We find that the cell lysis mediators GSDMD and ninjurin-1 (NINJ1)
134 contribute to bacterial restriction. Importantly, we observed that while human
135 macrophages unable to undergo inflammasome responses showed slightly elevated
136 bacterial replication within SCVs, they became permissive to hyperreplication of
137 *Salmonella* within the cytosolic compartment. Thus, inflammasome activation appears
138 to preferentially restrict cytosolic *Salmonella* replication. Our results offer insight into

139 how inflammasomes control *Salmonella* in human macrophages and restrict the distinct
140 intracellular spatial niches that *Salmonella* occupy in these cells.
141
142 Results
143 Caspase-1 promotes the control of *Salmonella* replication within human macrophages
144 Human macrophages undergo NAIP/NLRC4- and NLRP3-dependent
145 inflammasome activation during *Salmonella* infection (Bierschenk et al., 2019; Gram et
146 al., 2021; Naseer, Egan, et al., 2022), which restricts intracellular *Salmonella* replication
147 (Naseer, Egan, et al., 2022). Nonetheless, how inflammasome activation controls
148 *Salmonella* replication in human macrophages remains unclear. Inflammasomes recruit
149 and activate caspase-1 in both murine and human cells (Franchi et al., 2006; Man,
150 Hopkins, et al., 2014; Mariathasan et al., 2004; Miao et al., 2006; Ross et al., 2022;
151 Zamboni et al., 2006), and caspase-1 promotes the control of *Salmonella* both *in vivo*
152 and *in vitro* (Broz et al., 2010, 2012; Crowley et al., 2020; Hausmann et al., 2020; Holly
153 et al., 2020; Lara-Tejero et al., 2006; Miao, Leaf, et al., 2010; Rauch et al., 2017;
154 Raupach et al., 2006; Sellin et al., 2014; Thurston et al., 2016). Thus, we sought to test
155 whether caspase-1 restricts *Salmonella* replication in human macrophages.
156 To first interrogate the impact of caspase activity on *Salmonella* replication in
157 human macrophages, we pretreated macrophages derived from the human monocytic
158 cell line, THP-1, with Ac-YVAD-cmk (YVAD), a chemical inhibitor of caspase-1 activity,
159 or Z-VAD-FMK (ZVAD), a pan-caspase inhibitor. Upon infection with wild-type (WT)
160 *Salmonella*, WT THP-1 cells pretreated with either YVAD or ZVAD had significantly
161 reduced levels of IL-1β secretion and cell death compared to infected WT cells

162 pretreated with DMSO, the vehicle control (Figure 1 – figure supplement 1A-B). Since
163 pretreatment with YVAD largely phenocopies ZVAD, these data suggest that caspase-1
164 is the primary caspase that responds to *Salmonella* infection in THP-1 cells. We next
165 assessed intracellular *Salmonella* burdens by determining bacterial colony forming units
166 (CFUs). The increase in bacterial CFUs was significantly higher in WT cells pretreated
167 either YVAD or ZVAD compared to cells pretreated with DMSO (Figure 1 – figure
168 supplement 1C). Microscopic analysis revealed that WT cells treated with either YVAD
169 or ZVAD prior to infection harbored significantly higher intracellular *Salmonella* burdens
170 compared to cells pretreated with DMSO (Figure 1 – figure supplement 1D). Overall,
171 these results suggest that caspase activity, primarily caspase-1 activity, controls
172 *Salmonella* burdens in human macrophages.

173 Next, to genetically test the requirement of caspase-1, we used two independent
174 *CASP1*$^{-/-}$ THP-1 single cell clones generated through CRISPR/Cas9-mediated deletion
175 (Okondo et al., 2017). In agreement with previous reports (Bierschenk et al., 2019;
176 Gram et al., 2021; Naseer, Egan, et al., 2022), WT THP-1 cells infected with WT
177 *Salmonella* exhibited high levels of IL-1β, IL-18, and IL-1α release as well as cell death
178 at 6 hpi (Figure 1A-B; Figure 1 – figure supplement 2A-B). In contrast, *CASP1*$^{-/-}$ THP-1
179 cells released negligible levels of inflammasome-dependent cytokines and did not
180 undergo substantial cell death upon infection (Figure 1A-B; Figure 1 – figure
181 supplement 2A-B). Infected WT and *CASP1*$^{-/-}$ THP-1 cells released similar levels of the
182 inflammasome-independent cytokine TNF-α (Figure 1 – figure supplement 2C). These
183 results indicate that caspase-1 is required for inflammasome responses to *Salmonella*
184 infection in human macrophages.

185 We next tested whether caspase-1 is required to restrict *Salmonella* replication in
186 human macrophages. At 1 hour post-infection (hpi), we did not observe any significant
187 differences in bacterial uptake between WT and *CASP1*$^{-/-}$ THP-1 cells (Figure 1 – figure
188 supplement 2D). However, at 6 hpi, *CASP1*$^{-/-}$ cells harbored significantly higher bacterial
189 burdens and a significant fold-increase in bacterial CFUs compared to WT cells (Figure
190 1C; Figure 1 – figure supplement 2E). To assess intracellular bacterial burdens on a
191 single-cell level, we quantified the amount of WT *Salmonella* per cell by microscopy. WT
192 cells contained relatively low numbers of *Salmonella* on average (~5 bacteria per cell),
193 while *CASP1*$^{-/-}$ cells harbored significantly higher numbers of *Salmonella* on average
194 (~30 bacteria per cell) at 6 hpi (Figure 1D-E). Overall, these data indicate that caspase-
195 1 limits intracellular *Salmonella* replication in human macrophages.
196
197 <u>Caspase-4 contributes to the control of *Salmonella* replication within human</u>
198 <u>macrophages later during infection</u>
199 Caspase-11 is another member of the inflammatory caspase family, and plays a
200 role in host defense against *Salmonella* in mice (Aachoui et al., 2013; Crowley et al.,
201 2020; Knodler, Crowley, et al., 2014; Sellin et al., 2014). Humans harbor two orthologs
202 of murine caspase-11: caspase-4 and caspase-5 (Shi et al., 2014). Caspases-11/4/5
203 recognize cytosolic LPS and form the noncanonical inflammasome to mediate
204 downstream inflammatory responses (Casson et al., 2015; Hagar et al., 2013; Kayagaki
205 et al., 2011, 2013; Lagrange et al., 2018; Schmid-Burgk et al., 2015; Shi et al., 2014).
206 Caspase-4 contributes to inflammasome responses during *Salmonella* infection of THP-
207 1 macrophages and primary human macrophages (Casson et al., 2015; Naseer, Egan,

208 et al., 2022), and caspases-4/5 are required for inflammasome responses to *Salmonella*
209 in THP-1 monocytes (Baker et al., 2015). In human intestinal epithelial cells (IECs),
210 caspase-4 drives inflammasome responses during *Salmonella* infection and limits
211 intracellular bacterial replication (Holly et al., 2020; Knodler, Crowley, et al., 2014;
212 Naseer, Zhang, et al., 2022). However, whether caspase-4 contributes to restriction of
213 intracellular *Salmonella* replication within human macrophages is unclear.

214 To genetically test the contribution of caspase-4 during *Salmonella* infection in
215 THP-1 macrophages, we used CRISPR/Cas9-mediated deletion to disrupt the *CASP4*
216 gene. We selected and sequence-validated two independent *CASP4*$^{-/-}$ THP-1 single cell
217 clones (Figure 2 – figure supplement 1). We observed a slight decrease in secreted IL-
218 1β levels in *CASP4*$^{-/-}$ THP-1 macrophages infected with WT *Salmonella* compared to
219 infected WT THP-1 macrophages at 6 hpi (Figure 2 – figure supplement 2A). We also
220 observed a slight decrease in cell death at 6 hpi in infected *CASP4*$^{-/-}$ clone #2 compared
221 to infected WT cells, whereas cytotoxicity levels were unaffected in infected *CASP4*$^{-/-}$
222 clone #6 (Figure 2 – figure supplement 2B). However, at 24 hpi, we observed a
223 significant decrease in IL-1 release and cell death in both *CASP4*$^{-/-}$ clones infected with
224 WT *Salmonella* compared to infected WT cells (Figure 2A-B; Figure 2 – figure
225 supplement 3A-B). WT and *CASP4*$^{-/-}$ cells also released similar levels of the
226 inflammasome-independent cytokine TNF-α upon infection with WT *Salmonella* at 24
227 hpi (Figure 2 – figure supplement 3C). Overall, these data indicate that caspase-4
228 contributes to inflammasome responses in THP-1 macrophages later during *Salmonella*
229 infection.

230 Since caspase-4 restricts *Salmonella* replication in human epithelial cells (Holly
231 et al., 2020; Knodler, Crowley, et al., 2014; Naseer, Zhang, et al., 2022), we then asked
232 whether caspase-4 contributes to the control of *Salmonella* replication in human
233 macrophages. Upon examination of the fold-change in bacterial CFUs at 6 hpi, we did
234 not observe any significant differences between WT and *CASP4*$^{-/-}$ THP-1 cells (Figure 2
235 – figure supplement 2C). However, at 24 hpi, *CASP4*$^{-/-}$ cells harbored higher bacterial
236 burdens and a significant fold-increase in bacterial CFUs compared to WT cells (Figure
237 2C; Figure 2 – figure supplement 3D). To confirm these findings, we enumerated the
238 amount of WT *Salmonella* per cell by microscopy. At 6 hpi, WT and *CASP4*$^{-/-}$ THP-1
239 cells contained comparable numbers of *Salmonella* per cell (Figure 2 – figure
240 supplement 2D-E). In contrast, at 24 hpi, *CASP4*$^{-/-}$ cells harbored significantly higher
241 burdens of *Salmonella* per cell on average (~20 bacteria per cell) compared to WT cells
242 (~10 bacteria per cell) (Figure 2D-E). Altogether, these results suggest that caspase-4
243 plays a larger role in controlling *Salmonella* burdens later during infection.
244
245 <u>GSDMD promotes the control of *Salmonella* replication within human macrophages</u>
246 Inflammasome activation triggers a lytic form of cell death known as pyroptosis
247 (Lamkanfi & Dixit, 2014). Death of the infected host cell eliminates *Salmonella*'s
248 intracellular replicative niche and thus, may contribute to restricting intracellular bacterial
249 replication. Upon its cleavage by inflammatory caspases, GSDMD forms pores in the
250 host plasma membrane, resulting in pyroptosis (Agard et al., 2010; Ding et al., 2016;
251 Kayagaki et al., 2015; Shi et al., 2015). Whether GSDMD contributes to the control of
252 *Salmonella* replication in human macrophages is unknown.

253 First, we asked whether GSDMD pore formation plays a role in controlling
254 *Salmonella* replication in human macrophages. To do this, we pretreated THP-1
255 macrophages and primary human monocyte-derived macrophages (hMDMs) with the
256 chemical inhibitor disulfiram. Disulfiram prevents cleaved GSDMD from inserting into
257 the host plasma membrane, thereby limiting GSDMD-mediated pore formation (J. J. Hu
258 et al., 2020). Disulfiram treatment led to the loss of IL-1β release and cytotoxicity in
259 macrophages infected with WT *Salmonella*, compared to treatment with the vehicle
260 control, DMSO (Figure 3 – figure supplement 1A-B, D-E). Next, we examined the effect
261 of GSDMD-mediated pore formation on intracellular *Salmonella* burdens. The fold-
262 increase in bacterial CFUs at 6 hpi was significantly higher in cells treated with
263 disulfiram compared to cells treated with DMSO (Figure 3 – figure supplement 1C, F).
264 Collectively, these results suggest that GSDMD-mediated pore formation promotes the
265 restriction of intracellular *Salmonella* replication.
266 Consistent with our findings with disulfiram, we observed a significant decrease
267 in IL-1 cytokine release in *GSDMD*$^{-/-}$ THP-1 macrophages (Okondo et al., 2017;
268 Taabazuing et al., 2017) infected with WT *Salmonella* compared to infected WT THP-1
269 macrophages (Figure 3A; Figure 3 – figure supplement 2A-B). These results suggest
270 that human macrophages undergo GSDMD-dependent IL-1 release during infection.
271 Interestingly, loss of GSDMD did not completely abrogate the release of IL-1,
272 suggesting that there is also GSDMD-independent IL-1 release (Figure 3A; Figure 3 –
273 figure supplement 2A-B). Infected WT and *GSDMD*$^{-/-}$ THP-1 cells secreted similar levels
274 of the inflammasome-independent cytokine TNF-α (Figure 3 – figure supplement 2C).
275 Importantly, infected *GSDMD*$^{-/-}$ cells failed to undergo substantial cell death, in contrast

276 to infected WT cells, suggesting that GSDMD facilitates cell death during *Salmonella*
277 infection (Figure 3B). Overall, these results indicate that GSDMD plays an important
278 role in mediating inflammasome responses during *Salmonella* infection of human
279 macrophages.

280 We next examined whether GSDMD controls intracellular *Salmonella* replication
281 in human macrophages. While we did not observe any differences in bacterial uptake
282 between WT and *GSDMD*$^{-/-}$ cells at 1 hpi (Figure 3 – figure supplement 2D), we did
283 observe significantly higher bacterial CFUs in *GSDMD*$^{-/-}$ cells compared to WT cells at 6
284 hpi (Figure 3 – figure supplement 2E). Moreover, the increase in bacterial CFUs at 6 hpi
285 was significantly higher in *GSDMD*$^{-/-}$ cells than in WT cells (Figure 3C). Next, we used
286 microscopy to further interrogate intracellular *Salmonella* burdens. As expected, WT
287 cells contained low numbers of *Salmonella* per cell (~5 bacteria per cell) (Figure 3D-E).
288 However, *GSDMD*$^{-/-}$ cells harbored significantly higher numbers of *Salmonella* per cell
289 on average (~20 bacteria per cell) at 6 hpi (Figure 3D-E), suggesting that GSDMD
290 restricts intracellular *Salmonella* burdens. Altogether, these data indicate that GSDMD
291 promotes the control of *Salmonella* replication in human macrophages.
292

293 <u>NINJ1 contributes to the control of *Salmonella* replication within human macrophages</u>

294 Terminal cell lysis is regulated downstream of GSDMD cleavage and pore
295 formation (Bjanes et al., 2021; Kayagaki et al., 2021). While GSDMD pores release a
296 subset of molecules, including IL-1 family cytokines, the extensive release of cellular
297 contents is mediated by plasma membrane rupture (Ding et al., 2016; Ruan et al.,
298 2018). Recently, NINJ1 was identified as an executioner of terminal cell lysis (Bjanes et

299 al., 2021; Borges et al., 2022; Degen et al., 2023; Kayagaki et al., 2021, 2023). NINJ1 is
300 a transmembrane protein that oligomerizes in the host plasma membrane to induce lytic
301 rupture of the cell after the initiation of pyroptosis and other forms of regulated cell death
302 (Degen et al., 2023; Kayagaki et al., 2021). Cells can be protected from plasma
303 membrane rupture through treatment with the amino acid glycine (Fink & Cookson,
304 2006; Frank et al., 2000; Heilig et al., 2018; Verhoef et al., 2005), which interferes with
305 the clustering of NINJ1 (Borges et al., 2022). Thus, upon incubation with glycine, cells
306 can still form GSDMD pores but cannot undergo terminal cell lysis (Fink & Cookson,
307 2006; Heilig et al., 2018; Tsuchiya et al., 2021; Verhoef et al., 2005). Whether NINJ1-
308 dependent cell lysis might contribute to control of *Salmonella* replication in human
309 macrophages remains unknown.

310 First, to determine whether glycine exerts a cytoprotective effect on THP-1
311 macrophages, we pretreated WT and *NAIP*$^{-/-}$ THP-1 cells with glycine prior to infection
312 with WT *Salmonella* and assayed for downstream inflammasome responses. Notably,
313 WT and *NAIP*$^{-/-}$ cells pretreated with glycine exhibited significantly decreased cell death
314 and a small defect in IL-1β release following infection compared to infected cells treated
315 with the vehicle control (Figure 4 – figure supplement 1A-B). Release of the
316 inflammasome-independent cytokine TNF-α was unaffected by glycine treatment
317 (Figure 4 – figure supplement 1C). Altogether, these results indicate that glycine
318 prevents cell lysis and limits IL-1 release in THP-1 macrophages upon *Salmonella*
319 infection.

320 Given glycine's cytoprotective effect on infected THP-1 macrophages, we next
321 sought to determine whether glycine impacts intracellular *Salmonella* burdens in human

322 macrophages. THP-1 macrophages pretreated with glycine retained significantly higher
323 intracellular bacterial burdens at 6 hpi compared to cells given the vehicle control
324 (Figure 4 – figure supplement 1D). We did not detect any significant differences in
325 bacterial uptake (Figure 4 – figure supplement 1E). Moreover, WT and *NAIP*$^{-/-}$ THP-1
326 cells pretreated with glycine exhibited a greater increase in *Salmonella* CFUs compared
327 to cells treated with the vehicle control (Figure 4 – figure supplement 1F), suggesting
328 that glycine treatment impedes intracellular control of *Salmonella*. Therefore,
329 cytoprotection by glycine appears to interfere with the restriction of *Salmonella* burdens
330 in human macrophages.

331 Next, to genetically test the role of NINJ1, we transfected WT THP-1
332 macrophages with small interfering RNA (siRNA) targeting *NINJ1* or control scrambled
333 siRNA. WT cells treated with control siRNA exhibited robust IL-1β release and
334 cytotoxicity upon infection with WT *Salmonella* (Figure 4A-B). However, knockdown of
335 *NINJ1* resulted in a significant decrease, yet not complete abrogation, of cytotoxicity in
336 infected cells (Figure 4B). Knockdown of *NINJ1* also led to defect in IL-1β secretion
337 (Figure 4A). In these experiments, we observed efficient siRNA-mediated knockdown of
338 *NINJ1* ranging from 77% to 85%. Collectively, these data suggest a critical role for
339 NINJ1 in contributing to IL-1 release and cell death during *Salmonella* infection in
340 human macrophages.

341 We then enumerated bacterial CFUs to assess whether NINJ1 impacts
342 intracellular *Salmonella* replication. At 1 hpi, we did not observe any differences in
343 bacterial uptake between WT THP-1 cells treated with control siRNA or *NINJ1* siRNA
344 (Figure 4C). However, at 6 hpi, we found that cells treated with *NINJ1* siRNA contained

345 higher intracellular bacterial burdens than cells treated with control siRNA (Figure 4D).
346 Furthermore, there was a greater increase in bacterial CFUs at 6 hpi in *NINJ1* siRNA-
347 treated cells compared to control siRNA-treated cells (Figure 4E). Together, these
348 findings suggest that NINJ1 contributes to intracellular bacterial control.
349
350 Inflammasome activation primarily controls cytosolic *Salmonella* replication in human
351 macrophages
352 In epithelial cells, WT *Salmonella* can replicate in both vacuolar and cytosolic
353 compartments, specifically hyperreplicating in the cytosol (Knodler, Crowley, et al.,
354 2014; Knodler et al., 2010; Knodler, Nair, et al., 2014; Malik-Kale et al., 2012). However,
355 in murine macrophages, WT *Salmonella* appears to replicate exclusively in vacuoles
356 (Beuzón et al., 2002; Thurston et al., 2016). *Salmonella* lacking the SPI-2 effector SifA
357 (Δ*sifA*), which is required for SCV membrane stability, frequently enter the cytosol and
358 cannot replicate efficiently in murine macrophages (Beuzón et al., 2000, 2002; Thurston
359 et al., 2016). Whether *Salmonella* replicates within vacuoles or the cytosol of human
360 macrophages remains largely unknown. One study suggested that a small but
361 significant proportion of *Salmonella* are exposed to the cytosol in THP-1 macrophages
362 (Fisch et al., 2020). Our recently published data and current findings indicate that when
363 human macrophages fail to undergo robust inflammasome responses, *Salmonella*
364 hyperreplicates to large numbers within these cells (Naseer, Egan, et al., 2022). The
365 hyperreplication that we observed in human macrophages was reminiscent of previous
366 reports describing *Salmonella* hyperreplication within the cytosol of IECs (Knodler,
367 Crowley, et al., 2014; Knodler et al., 2010; Knodler, Nair, et al., 2014; Malik-Kale et al.,

368 2012). Thus, we hypothesized that inflammasome activation limits *Salmonella*
369 replication in both SCVs and the host cell cytosol, and that in the absence of
370 inflammasome responses, *Salmonella* hyperreplicates to large numbers within the host
371 cell cytosol and also exhibits increased replication within SCVs.

372 First, to test whether inflammasome activation restricts the ability of *Salmonella*
373 to replicate in vacuolar and cytosolic compartments of human macrophages, we used a
374 chloroquine (CHQ) resistance assay. As a weak base, CHQ accumulates in endosomal
375 compartments, including the SCV, without entering the cytosol of host cells (Klein,
376 Powers, et al., 2017; Knodler, Nair, et al., 2014; Steinberg, 1994). Thus, vacuolar
377 bacteria are CHQ-sensitive, while cytosolic bacteria are CHQ-resistant (Knodler, Nair, et
378 al., 2014). We infected THP-1 macrophages with WT *Salmonella* and treated the
379 infected cells with CHQ. Then, we determined the bacterial CFUs at 6 hpi, quantifying
380 the numbers of vacuolar bacteria (CHQ-sensitive) and cytosolic bacteria (CHQ-
381 resistant). We found that WT THP-1 cells contained mostly vacuolar *Salmonella* while
382 also retaining some cytosolic *Salmonella* (Figure 5A), suggesting that a subset of
383 *Salmonella* dwells in the cytosol of THP-1 macrophages, in agreement with a previous
384 study (Fisch et al., 2020). In *NAIP*$^{-/-}$ and *CASP1*$^{-/-}$ THP-1 cells, we observed slightly
385 higher burdens of vacuolar *Salmonella* compared to WT cells (Figure 5A). Strikingly, we
386 also observed significantly larger numbers of cytosolic *Salmonella* in *NAIP*$^{-/-}$ and
387 *CASP1*$^{-/-}$ cells compared to WT cells (Figure 5A). These data indicate that
388 inflammasome signaling restricts *Salmonella* primarily within the host cell cytosol but
389 also within SCVs in human macrophages.

390 The SPI-1 T3SS can damage the SCV (Roy et al., 2004), and the SPI-1 T3SS
391 and its effectors contribute to *Salmonella* replication in the cytosol of epithelial cells
392 (Chong et al., 2019; Klein, Grenz, et al., 2017; Knodler, Nair, et al., 2014). So, we next
393 assessed whether the SPI-1 T3SS was required for the cytosolic exposure of
394 *Salmonella* in human macrophages. We infected WT and *CASP1*$^{-/-}$ THP-1 macrophages
395 with *Salmonella* lacking the SPI-1 T3SS translocon protein SipB (Δ*sipB*), thus
396 preventing SPI-1 T3SS effector translocation into host cells. While we observed a
397 significant increase in the amount of vacuolar and cytosolic Δ*sipB Salmonella* in
398 *CASP1*$^{-/-}$ cells compared to WT cells at 6 hpi, the numbers of cytosolic Δ*sipB* were
399 significantly lower than vacuolar Δ*sipB* in both WT and *CASP1*$^{-/-}$ cells (Figure 5B). We
400 also observed lower cytosolic Δ*sipB Salmonella* burdens compared to WT *Salmonella* in
401 both WT and *CASP1*$^{-/-}$ THP-1 cells (Figure 5A-B). Therefore, these results suggest that
402 there is a partial dependence on the SPI-1 T3SS for *Salmonella*'s cytosolic access in
403 human macrophages.
404 Since CFU assays are population-based, we next used single-cell methods to
405 interrogate the subcellular localization of WT *Salmonella* in human macrophages. We
406 relied on a strain of WT *Salmonella* that constitutively expresses mCherry and maintains
407 a reporter plasmid, pNF101, that expresses *gfp-ova* under the control of a promoter
408 responsive to the host cytosolic metabolite glucose-6-phosphate (Lau et al., 2019). We
409 scored the number of GFP-positive/mCherry-positive bacteria (cytosolic) and GFP-
410 negative/mCherry-positive bacteria (vacuolar) in WT, *NAIP*$^{-/-}$ and *CASP1*$^{-/-}$ THP-1
411 macrophages at 8 hpi by microscopy. We observed low numbers of both vacuolar and
412 cytosolic populations of *Salmonella* in WT THP-1 cells (Figure 5C-D). In contrast, *NAIP*$^{-}$

413 $^{/-}$ and *CASP1*$^{-/-}$ THP-1 cells maintained significantly larger numbers of vacuolar
414 *Salmonella*, with a substantial increase in the numbers of cytosolic *Salmonella* (Figure
415 5C-D). Taken together, these results suggest that inflammasome responses primarily
416 restrict *Salmonella* replication within the host cell cytosol and also control bacterial
417 replication within SCVs in human macrophages.

418 We next asked whether inflammasome responses also restrict the replication of
419 *Salmonella* within the cytosol and SCV in primary human macrophages. We pretreated
420 hMDMs with either ZVAD, to inhibit inflammasome responses mediated by caspase
421 activity, or the vehicle control DMSO, and then infected the cells with WT *Salmonella*
422 constitutively expressing mCherry and harboring pNF101. We observed that hMDMs
423 treated with ZVAD contained significantly more cytosolic *Salmonella* than hMDMs
424 treated with DMSO (Figure 5 – figure supplement 1A-B). We observed no significant
425 differences between the vacuolar burdens of *Salmonella* in hMDMs pretreated with
426 ZVAD or DMSO (Figure 5 – figure supplement 1A-B). Thus, inflammasome responses
427 appear to primarily control *Salmonella* replication within the cytosol of primary human
428 macrophages.

429

430 <u>Inflammasome activation modulates the cytosolic exposure of *Salmonella* in human</u>
431 <u>macrophages</u>

432 Finally, to characterize the effect of inflammasomes on the subcellular niches of
433 *Salmonella* in human macrophages at higher resolution, we used transmission electron
434 microscopy (TEM). In WT THP-1 cells, TEM analysis revealed that the majority of
435 bacteria resided in a membrane-bound compartment (vacuolar, white arrows) (Figure

436 6A; Figure 6 – figure supplement 1B). In *CASP1*$^{-/-}$ THP-1 cells, we observed a more
437 mixed population of vacuolar bacteria and bacteria that were exposed to the host cell
438 cytosol to varying extents (Figure 6B; Figure 6 – figure supplement 1B). We observed
439 *Salmonella* free-living in the cytosol without a vacuolar membrane (fully cytosolic, black
440 asterisk) as well as *Salmonella* exposed to the cytosol within discontinuous vacuolar
441 membranes (partially cytosolic, cyan arrows) (Figure 6B; Figure 6 – figure supplement
442 1B). We observed a greater proportion of cytosol-exposed *Salmonella* in *CASP1*$^{-/-}$ cells
443 compared to WT cells (Figure 6 – figure supplement 1A). Strikingly, these TEM results
444 revealed distinct intracellular populations of *Salmonella* in human macrophages,
445 providing further evidence that inflammasome signaling controls the replicative niches
446 that *Salmonella* occupies in human macrophages.
447 We next sought to further characterize the cytosolic exposure of *Salmonella* in
448 *CASP1*$^{-/-}$ THP-1 cells using electron tomography (ET), a technique that can reveal three-
449 dimensional (3D) detail about SCV membranes. Indeed, we confirmed the presence of
450 *Salmonella* in SCVs with varying degrees of vacuolar membrane discontinuities,
451 yielding various extents of cytosolic exposure (Figure 6C; Figure 6 – figure supplement
452 1C; Video 1). Altogether, our TEM and ET data reveal the full spectrum of cytosolic
453 exposure for *Salmonella* in *CASP1*$^{-/-}$ cells as a result of vacuolar membrane
454 discontinuities. Overall, our findings indicate that inflammasome responses play a role
455 in modulating the subcellular populations of *Salmonella*, thereby controlling the number
456 of *Salmonella* able to replicate within the cytosol and SCV in human macrophages.
457
458 <u>Discussion</u>

459 Our data reveal that inflammatory caspases and downstream cell lysis mediators
460 are required to restrict *Salmonella* replication in human macrophages. Furthermore, our
461 findings indicate that inflammasomes restrict *Salmonella* hyperreplication within the
462 cytosol of human macrophages. Caspase-1 is required for inflammasome responses
463 and control of intracellular *Salmonella* replication early during infection. In contrast,
464 caspase-4 contributed minimally early during infection and instead played a larger role
465 in inflammasome responses and restriction of *Salmonella* replication at later timepoints.
466 We also found that GSDMD and NINJ1 were required for cell death, IL-1 cytokine
467 release, and control of *Salmonella* replication. Finally, in the absence of these
468 inflammasome components and effectors in human macrophages, we observed a
469 hyperreplicating population of *Salmonella* within the cytosol, as well as increased
470 bacterial loads within the SCV, suggesting that inflammasome activation prevents
471 hyperreplication of *Salmonella* within the cytosol and increased replication within SCVs.
472 There are multiple downstream consequences of inflammasome activation,
473 including pyroptosis, cytokine secretion, phagolysosomal fusion, the formation of pore-
474 induced intracellular traps (PITs), and the generation of reactive oxygen species (ROS),
475 each of which could be responsible for the control of intracellular *Salmonella* replication
476 in human macrophages. Previous studies have suggested that inflammatory caspases
477 limit the intracellular replication of WT *Salmonella* in human epithelial cells and murine
478 macrophages through host cell death (Aachoui et al., 2013; Holly et al., 2020; Knodler,
479 Crowley, et al., 2014). Interestingly, in murine macrophages, the restriction of a mutant
480 strain of *Salmonella* that frequently enters the cytosol is dependent on caspase-1/11 but
481 independent of IL-1 signaling and host cell death (Thurston et al., 2016). Moreover,

482 caspase-1/11-dependent production of mitochondrial ROS and hydrogen peroxide
483 contributes to the control of WT *Salmonella* replication in SCVs in murine macrophages
484 (Man, Ekpenyong, et al., 2014). Future studies investigating the downstream
485 consequences of inflammasome activation will help elucidate the mechanisms by which
486 inflammatory caspases and cell lysis mediators control intracellular *Salmonella*
487 replication in human macrophages.

488 Whereas caspase-1 is the primary caspase that mediates inflammasome
489 responses and control of *Salmonella* burdens in human macrophages, our data indicate
490 that caspase-4 also plays a role at a later stage of infection. Whether caspase-4 is
491 activated by vacuolar *Salmonella* or hyperreplicating cytosolic *Salmonella* in human
492 macrophages remains an open question. Other host immune factors may also
493 contribute to caspase-4-dependent inflammasome responses to *Salmonella*. Guanylate
494 binding proteins (GBPs) can modulate inflammasome responses to intracellular LPS
495 and impact intracellular bacterial replication (Degrandi et al., 2007; Fisch et al., 2020;
496 Kim et al., 2011; Kutsch et al., 2020; Meunier et al., 2014; Pilla et al., 2014; J. C. Santos
497 et al., 2020; Tietzel et al., 2009; Wandel et al., 2020).

498 The impact of GSDMD on the restriction of intracellular *Salmonella* replication
499 may be due to downstream responses, such as IL-1 release and pyroptosis. Moreover,
500 pyroptosis can induce PIT formation, which traps and damages intracellular bacteria,
501 rendering them more susceptible to immune defenses, such as neutrophil-mediated
502 killing (Jorgensen et al., 2016). It is possible that pyroptosis leads to the development of
503 PITs in human macrophages during *Salmonella* infection, thereby limiting *Salmonella*'s
504 intracellular replication. In addition, the cleaved N-terminal fragment of GSDMD can

505 directly kill bacteria (Ding et al., 2016; Liu et al., 2016; Wang et al., 2019), so perhaps
506 GSDMD directly targets *Salmonella* to mediate restriction. It remains unknown whether
507 other cell lysis factors, like NINJ1, together with GSDMD, directly bind the SCV,
508 affecting vacuolar integrity and thus influencing intracellular *Salmonella* replication.
509 Our data indicate that inflammasome responses primarily control *Salmonella*
510 replication within the cytosol of human macrophages and also within SCVs. How
511 inflammasome activation inhibits *Salmonella* replication in the cytosol of human
512 macrophages remains unknown. *Salmonella* hyperreplicates in the cytosol of human
513 epithelial cells, which fail to undergo caspase-1-dependent inflammasome responses
514 (Holly et al., 2020; Knodler, Crowley, et al., 2014; Knodler et al., 2010; Knodler, Nair, et
515 al., 2014; Naseer, Zhang, et al., 2022). Our study suggests that *CASP1*$^{-/-}$ THP-1
516 macrophages behave similarly to human IECs, as they support *Salmonella*
517 hyperreplication in the cytosol in the absence of caspase-1. Inflammasome activation
518 could curtail cytosolic replication of *Salmonella* through host cell death, direct GSDMD
519 targeting of cytosolic *Salmonella,* and/or another effector mechanism. Alternatively,
520 inflammasome activation could regulate the cytosolic access of *Salmonella* through host
521 factors that directly damage the SCV, such as pore-forming proteins like GSDMD.
522 The bacterial factors that facilitate *Salmonella*'s cytosolic lifestyle in human
523 macrophages remain largely unknown. Our findings indicate that the SPI-1 T3SS is
524 partially required for *Salmonella* to access the cytosol in THP-1 macrophages. In
525 epithelial cells, the SPI-1 T3SS SopB and SipA enable *Salmonella* to efficiently colonize
526 and replicate in the cytosol (Chong et al., 2019; Klein, Grenz, et al., 2017). Future

527 studies are needed to elucidate whether these same effectors play a role in supporting
528 *Salmonella*'s cytosolic replication in human macrophages.
529 Of note, our data indicate that there is considerable single-cell heterogeneity in
530 terms of total intracellular bacterial burdens as well as proportions of vacuolar and
531 cytosolic *Salmonella* in human macrophages using single-cell microscopic analysis.
532 Several factors could account for this phenotypic heterogeneity, including expression
533 levels of inflammasome components, the amount of translocated *Salmonella* ligands, or
534 the extent of inflammasome activation, all of which might differ at a single cell level and
535 are masked in bulk-population assays. Notably, heterogeneity in *Salmonella* gene
536 expression, intracellular *Salmonella* populations, and intracellular *Salmonella*
537 proliferation and viability has been previously observed in human epithelial cells and
538 murine macrophages (Helaine et al., 2010, 2014; Knodler, Nair, et al., 2014; Malik-Kale
539 et al., 2012; Powers et al., 2021).
540 Collectively, our results reveal that inflammasome responses restrict intracellular
541 *Salmonella* replication, particularly within the cytosol of human macrophages. These
542 findings are in contrast to mouse macrophages, where inflammasomes are activated
543 but only marginally restrict the intracellular replication of WT *Salmonella*. Our findings
544 provide insight into how human macrophages leverage inflammasomes to restrict
545 *Salmonella* intracellular replication. Moreover, our work offers a basis for future studies
546 to investigate how inflammasome activation modulates the subcellular localization of
547 bacterial replicative niches within host cells.
548
549 <u>Materials and Methods</u>

550 Ethics statement

551 All experiments on primary human monocyte-derived macrophages (hMDMs) were
552 performed in compliance with the requirements of the US Department of Health and
553 Human Services and the principles expressed in the Declaration of Helsinki. hMDMs
554 were derived from samples obtained from the University of Pennsylvania Human
555 Immunology Core, and they are considered to be a secondary use of deidentified
556 human specimens and are exempt via Title 55 Part 46, Subpart A of 46.101 (b) of the
557 Code of Federal Regulations.

558

559 Cell culture of THP-1 cells

560 THP-1 cells (TIB-202; American Type Culture Collection) were maintained in RPMI
561 supplemented with 10% (vol/vol) heat-inactivated FBS, 0.05 nM β-mercaptoethanol, 100
562 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified incubator. Two
563 days prior to experimentation, the cells were replated in media without antibiotics in a
564 48-well plate at a concentration of 2×10^5 cells/well or in a 24-well plate at a
565 concentration of 3.5×10^5 cells per well and incubated with phorbol 12-myristate 13-
566 acetate (PMA) for 24 hours to allow differentiation into macrophages. Then,
567 macrophages were primed with 100 ng/mL Pam3CSK4 (Invivogen) for 16 to 20 hours
568 prior to bacterial infections. For fluorescence microscopy experiments, cells were plated
569 on glass coverslips in a 24-well plate.

570

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

572 Purified human monocytes from deidentified healthy human donors were obtained from
573 the University of Pennsylvania Human Immunology Core. The monocytes were cultured
574 in RPMI supplemented with 10% (vol/vol) heat-inactivated FBS, 2 mM L-glutamine, 100
575 IU/mL penicillin, 100 µg/ml streptomycin, and 50 ng/ml recombinant human M-CSF
576 (Gemini Bio-Products). Cells were cultured for 4 days in 10 mL of media in at a
577 concentration of 4-5 × 10^5 cells/mL in 10 cm-dishes. Then, 10 mL of fresh growth media
578 was added for an additional 2 days to promote complete differentiation into
579 macrophages. One day prior to infection, the cells were rinsed with cold PBS, gently
580 detached with trypsin-EDTA (0.05%) and replated in media without antibiotics and with
581 25 ng/mL human M-CSF in a 48-well plate at a concentration of 1 × 10^5 cells per well or
582 in a 24-well plate at a concentration of 2 × 10^5 cells per well. For fluorescence
583 microscopy, cells were replated on glass coverslips in a 24-well plate. For experiments
584 involving LPS, cells were primed with 500 ng/mL LPS (Sigma-Aldrich) for 3 hours prior
585 to bacterial infections.
586
587 Inhibitor experiments
588 Human macrophages were treated 1 hour prior to infection at the indicated
589 concentrations with the following inhibitors: 20 µM of the pan-caspase inhibitor Z-
590 VAD(OMe)-FMK (SM Biochemicals; SMFMK001), 25 µM of the caspase-1 inhibitor Ac-
591 YVAD-cmk (Sigma-Aldrich; SML0429), and 40 µM of the GSDMD inhibitor disulfiram
592 (Sigma). DMSO treatment was used as a vehicle control with these inhibitors. To
593 prevent cell lysis, cells were treated with 20 mM glycine (Fisher Scientific) for 30
594 minutes prior to infection. Distilled water was used as a vehicle control.

595

596 Bacterial strains and growth conditions

597 *Salmonella enterica* serovar Typhimurium SL1344 WT was routinely grown shaking
598 overnight at 37°C in Luria-Bertani (LB) broth with streptomycin (100 µg/mL). For
599 infection of cultured cells, the overnight culture was diluted in LB with streptomycin
600 (100 µg/mL) containing 300 mM NaCl and grown standing for 3 hours at 37°C to induce
601 SPI-1 expression (Lee & Falkow, 1990).

602

603 SL1344 WT *glmS::Ptrc-mCherryST::FRT* pNF101 (Lau et al., 2019) was kindly provided
604 by Dr. Leigh Knodler. This strain constitutively expresses *mCherry* that is
605 chromosomally encoded. It also harbors the *PuhpT-gfpova* plasmid, pNF101, where
606 expression of GFP is under the control of the glucose-6-phosphate responsive *uhpT*
607 promoter derived from *Shigella flexneri*. SL1344 WT *glmS::Ptrc-mCherryST::FRT*
608 pNF101 was grown shaking overnight at 37°C in Luria-Bertani (LB) broth with
609 streptomycin (100 µg/mL) and ampicillin (100 µg/mL). For SPI-1 induction prior to
610 infection, the overnight culture was diluted in LB with streptomycin (100 µg/mL) and
611 ampicillin (100 µg/mL) that also contained 300 mM NaCl and then grown standing for 3
612 hours at 37°C (Lee & Falkow, 1990).

613

614 Bacterial infections

615 Overnight cultures of *Salmonella* were diluted into LB broth with streptomycin
616 (100 µg/mL) containing 300 mM NaCl and grown for 3 hours standing at 37°C to induce
617 SPI-1 gene expression (Lee & Falkow, 1990). Bacterial cultures were then pelleted at

618 6,010 × g for 3 minutes, washed once with PBS, and resuspended in PBS. Human
619 macrophages were infected with *Salmonella* at a multiplicity of infection (MOI) of 20.
620 Infected cells were centrifuged at 290 × g for 10 minutes and incubated at 37°C. At 30
621 minutes post-infection, the cells were treated with 100 ng/mL of gentamicin to kill any
622 extracellular *Salmonella*. Then, the infection proceeded at 37°C for 6 to 8 hours, as
623 indicated. For all experiments, control cells were mock-infected with PBS.
624
625 Bacterial intracellular burden assay
626 Cells were infected with WT *Salmonella* as described above at an MOI of 20. Then, 30
627 minutes post-infection, cells were treated with 100 µg/ml of gentamicin to kill any
628 extracellular bacteria. 1 hour post-infection, the media was replaced with fresh media
629 containing 10 µg/ml of gentamicin. At the indicated time points, the infected cells were
630 lysed with PBS containing 0.5% Triton to collect all intracellular *Salmonella*. Harvested
631 bacteria were serially diluted in PBS and plated on LB agar plates containing
632 streptomycin (100 µg/ml) to enumerate colony forming units (CFUs). Plates were
633 incubated overnight at 37°C and CFUs were subsequently counted.
634
635 Chloroquine (CHQ) Resistance Assay
636 THP-1 macrophages were infected in 48-well plates as described above. For each
637 timepoint, triplicate wells were incubated in the presence of CHQ (500 µM) and
638 gentamicin (100 ng/mL) for 1 hour to quantify the CHQ-resistant bacteria (Bârzu et al.,
639 1997; Fernandez et al., 2001; Klein, Powers, et al., 2017; Knodler, Nair, et al., 2014;
640 Zychlinsky et al., 1994). Another triplicate wells were incubated with gentamicin (100

641 ng/mL) only to quantify the total intracellular bacteria. At the indicated time points, the
642 infected cells were lysed with PBS containing 0.5% Triton to collect intracellular
643 *Salmonella*. Harvested bacteria were serially diluted in PBS and plated on LB agar
644 plates containing streptomycin (100 µg/ml) to enumerate colony forming units (CFUs).
645 Plates were incubated overnight at 37°C and CFUs were subsequently counted.
646
647 ELISAs
648 Harvested supernatants from infected human macrophages were assayed for cytokine
649 levels using ELISA kits for human IL-1α (R&D Systems), IL-18 (R&D Systems), IL-1β
650 (BD Biosciences), and TNF-α (R&D Systems).
651
652 LDH cytotoxicity assays
653 Harvested supernatants from infected human macrophages were assayed for
654 cytotoxicity by quantifying the loss of cellular membrane integrity via lactate
655 dehydrogenase (LDH) activity. LDH release was measured using an LDH Cytotoxicity
656 Detection Kit (Clontech) according to the manufacturer's instructions and normalized to
657 mock-infected cells.
658
659 siRNA-mediated knockdown of genes
660 All Silencer Select siRNA oligos were purchased from Ambion (Life Technologies).
661 Individual siRNA targeting *NINJ1* (ID# s9556) was used. The two Silencer Select
662 negative control siRNAs (Silencer Select Negative Control No. 1 siRNA and Silencer
663 Select Negative Control No. 2 siRNA) were used as a control. Three days prior to the

664 infection, 30 nM of siRNA was transfected into the human macrophages using
665 Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) following the
666 manufacturer's protocol. Then, 24 hours after transfection, the media was replaced with
667 fresh media containing antibiotics. Finally, 16 hours before infection, the media was
668 replaced with fresh antibiotic-free media containing 100 ng/ml Pam3CSK4.
669
670 Quantitative RT-PCR Analysis
671 RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) following the manufacturer's
672 protocol. Human macrophages were lysed in 350 µL RLT buffer with β-mercaptoethanol
673 and centrifuged through a QIAshredder spin column (Qiagen). cDNA was synthesized
674 from isolated RNA using SuperScript II Reverse Transcriptase (Invitrogen) following the
675 manufacturer's protocol. Quantitative PCR was conducted with the CFX96 real-time
676 system from Bio-Rad using the SsoFast EvaGreen Supermix with Low ROX (Bio-Rad).
677 To calculate knockdown efficiency, mRNA levels of siRNA-treated cells were
678 normalized to the housekeeping gene *HPRT* and control siRNA-treated cells using the
679 $2^{-\Delta\Delta CT}$ (cycle threshold) method (Livak & Schmittgen, 2001). The following primers from
680 PrimerBank were used. The PrimerBank identifications are *NINJ1* (148922910c1),
681 *CASP4* (73622124c2) and *HPRT* (164518913c1); all primers listed as 5′–3′:
682 *NINJ1* forward: TCAAGTACGACCTTAACAACCCG
683 *NINJ1* reverse: TGAAGATGTTGACTACCACGATG
684 *CASP4* forward: TCTGCGGAACTGTGCATGATG
685 *CASP4* reverse: TGTGTGATGAAGATAGAGCCCAT
686 *HPRT* forward: CCTGGCGTCGTGATTAGTGAT

687 *HPRT* reverse: AGACGTTCAGTCCTGTCCATAA

688

689 Immunoblot analysis

690 Cell lysates were harvested for immunoblot analysis by adding 1X SDS/PAGE sample
691 buffer to cells. All protein samples (lysates) were boiled for 5 minutes. Samples were
692 separated by SDS/PAGE on a 12% (vol/vol) acrylamide gel and transferred to PVDF
693 Immobilon-P membranes (Millipore). Primary antibodies specific for caspase-4 (4450S;
694 Cell Signaling) and β-actin (4967L; Cell Signaling) and HRP-conjugated secondary
695 antibodies anti-mouse IgG (F00011; Cell Signaling) and anti-rabbit IgG (7074S; Cell
696 Signaling) were used. ECL Western Blotting Substrate (Pierce Thermo Scientific) was
697 used as the HRP substrate for detection.

698

699 Fluorescent microscopy of intracellular *Salmonella*

700 Primary hMDMs or THP-1 cells were plated on glass coverslips in a 24-well plate as
701 described above. Cells were either infected with WT *Salmonella* constitutively
702 expressing GFP [SI1344 harboring pFPV25.1 (Valdivia & Falkow, 1996)], or WT
703 *Salmonella* constitutively expressing mCherry with a cytosolic GFP reporter [SL1344
704 *glmS::Ptrc-mCherryST::FRT* pNF101 (Lau et al., 2019)] at an MOI of 20 as described
705 above. At the indicated timepoints following infection, cells were washed 2 times with
706 PBS and then fixed with 4% paraformaldehyde for 10 minutes. Following fixation, cells
707 were mounted on glass slides with DAPI mounting medium (Sigma Fluoroshield).
708 Coverslips were imaged on an inverted fluorescence microscope (IX81; Olympus), and
709 the images were collected using a high-resolution charge-coupled-device camera

710 (FAST1394; QImaging) at a magnification of 100×. All images were analyzed and
711 presented using SlideBook (version 5.0) software (Intelligent Imaging Innovations, Inc.)
712 and ImageJ software. For experiments with WT *Salmonella* constitutively expressing
713 GFP, the proportion of infected cells containing GFP-expressing *Salmonella* (green)
714 were scored by counting 50 infected cells per coverslip. 150 total infected cells were
715 scored for each condition. For experiments with WT *Salmonella* constitutively
716 expressing mCherry with a cytosolic GFP reporter, the proportion of infected cells
717 containing GFP-positive *Salmonella* (cytosolic) and GFP-negative, mCherry-positive
718 *Salmonella* (vacuolar) were scored by counting 50 infected cells per coverslip. 150 total
719 infected cells were scored for each condition.
720
721 Transmission electron microscopy
722 Two days prior to experimentation, 2×10^6 cells THP-1 cells were replated in 10-cm
723 dishes in media without antibiotics and incubated with phorbol 12-myristate 13-acetate
724 (PMA) for 24 hours to allow differentiation into macrophages. Then, macrophages were
725 primed with 100 ng/mL Pam3CSK4 (Invivogen) for 16 hours prior to bacterial infection.
726 Cells were infected with WT *Salmonella* at an MOI of 20 as described above. At 8 hpi,
727 the media was aspirated, and the cells were fixed with 2.5% glutaraldehyde, 2.0%
728 paraformaldehyde in 0.1M sodium cacodylate buffer, pH 7.4. Then, at the Electron
729 Microscopy Resource Laboratory in the Perelman School of Medicine, after subsequent
730 buffer washes, the samples were post-fixed in 2.0% osmium tetroxide with 1.5%
731 K3Fe(CN)6 for 1 hour at room temperature, and rinsed in dH2O. After dehydration
732 through a graded ethanol series, the tissue was infiltrated and embedded in EMbed-812

733 (Electron Microscopy Sciences, Fort Washington, PA). Thin sections were stained with
734 uranyl acetate and SATO lead and examined with a JEOL 1010 electron microscope
735 fitted with a Hamamatsu digital camera and AMT Advantage NanoSprint500 software.
736
737 Electron tomography
738 Electron tomography was performed at room temperature on a ThermoFisher Krios G3i
739 TEM equipped with a 300 keV field emission gun. Imaging was performed using the
740 SerialEM software (Mastronarde, 2005) on a K3 direct electron detector (Xuong et al.,
741 2007) (Gatan Inc., Pleasanton, CA, USA) operated in the electron-counted mode. We
742 additionally used the Gatan Imaging Filter (Gatan Inc., Pleasanton, CA, USA) with a slit
743 width of 20 eV to increase contrast by removing inelastically scattered electrons
744 (Krivanek et al., 1995). After initially assessing the infected macrophages at lower
745 magnifications, tilt series were collected at a magnification of 26,000X (with a
746 corresponding pixel size of 3.38 Å) and a defocus of −6 µm. Precooking the target areas
747 with a total dosage of 1000-1500 e/Å^2 was required to minimize sample shrinking and
748 drifting during tilt series collection. A bi-directional tilt scheme was employed with 2°
749 increments and 120° span (−60° to +60°). The cumulative dose of each tilt-series was in
750 the order of ~500 e−/Å2. Once acquired, tilt series were aligned (using the patch
751 tracking function) and reconstructed into tomograms, both using the IMOD software
752 package (Kremer et al., 1996). After careful assessment of the 3-dimensional (3-D)
753 tomograms, optimal 2-D slices were chosen for figure presentations. Color overlays
754 provide additional assistance with interpretation.
755

756 Statistical analysis

757 Prism 9.5.0 (GraphPad Software) was utilized for the graphing of data and all statistical
758 analyses. Statistical significance for experiments with THP-1 macrophages and hMDMs
759 was determined using the appropriate test and are indicated in each figure legend.
760 Differences were considered statistically significant if the p value was <0.05.

761

762

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767 and *GSDMD*$^{-/-}$ THP-1 cells. We thank the Human Immunology Core of the Penn Center
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775

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1373
1374

1375 Figure Legends

1376 **Figure 1. Caspase-1 promotes the control of *Salmonella* replication within human**
1377 **macrophages**. WT and two independent clones of *CASP1*$^{-/-}$ THP-1 monocyte-derived
1378 macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were then
1379 infected with PBS (Mock), WT *S*. Typhimurium (A, B, C), or WT *S.* Typhimurium
1380 constitutively expressing GFP (D,E) at an MOI = 20. (A) Release of IL-1β into the
1381 supernatant was measured by ELISA at 6 hpi. (B) Cell death (percentage cytotoxicity)
1382 was measured by lactate dehydrogenase release assay and normalized to mock-
1383 infected cells at 6 hpi. (C) Cells were lysed at 1 hpi and 6 hpi, and bacteria were
1384 subsequently plated to calculate CFU. Fold-change in CFU/well was calculated. (D, E)
1385 Cells were fixed at 6 hpi and stained for DAPI to label DNA (blue). (D) The number of
1386 bacteria per cell at 6 hpi was scored by fluorescence microscopy. Each small dot
1387 represents one infected cell. 150 infected cells were scored for each genotype. (E)
1388 Representative images are shown. Scale bar represents 10 μm. Bars represent the
1389 mean for each genotype, and error bars represent the standard deviation of triplicate
1390 wells from one experiment (A, B, C, D). ***$p < 0.001$, ****$p < 0.0001$ by Dunnett's

1391 multiple comparisons test (A, B, C, D). Data shown are representative of at least three
1392 independent experiments.
1393
1394 **Figure 2. Caspase-4 contributes to the control of *Salmonella* replication within**
1395 **human macrophages later during infection.** WT and two independent clones of
1396 *CASP4*$^{-/-}$ THP-1 monocyte-derived macrophages were primed with 100 ng/mL
1397 Pam3CSK4 for 16 hours. Cells were then infected with PBS (Mock), WT *S.*
1398 Typhimurium (A, B, C), or WT *S.* Typhimurium constitutively expressing GFP (D, E) at
1399 an MOI = 20. (A) Release of IL-1β into the supernatant was measured by ELISA at 24
1400 hpi. (B) Cell death (percentage cytotoxicity) was measured by lactate dehydrogenase
1401 release assay and normalized to mock-infected cells at 24 hpi. (C) Cells were lysed at 1
1402 hpi and 24 hpi, and bacteria were subsequently plated to calculate CFU. Fold-change in
1403 CFU/well was calculated. (D, E) Cells were fixed at 24 hpi and stained for DAPI to label
1404 DNA (blue). (D) The number of bacteria per cell at 24 hpi was scored by fluorescence
1405 microscopy. Each small dot represents one infected cell. 150 infected cells were scored
1406 for each genotype. (E) Representative images are shown. Scale bar represents 10 μm.
1407 Bars represent the mean for each genotype, and error bars represent the standard
1408 deviation of triplicate wells from one experiment (A, B, C, D). ns – not significant, *$p <$
1409 0.05 by Dunnett's multiple comparisons test (A, B, C, D). Data shown are representative
1410 of at least three independent experiments.
1411
1412 **Figure 3. GSDMD promotes the control of *Salmonella* replication within human**
1413 **macrophages.** WT and *GSDMD*$^{-/-}$ THP-1 monocyte-derived macrophages were primed

1414 with 100 ng/mL Pam3CSK4 for 16 hours. Cells were then infected with PBS (Mock), WT
1415 *S*. Typhimurium (A, B, C), or WT *S.* Typhimurium constitutively expressing GFP (D,E) at
1416 an MOI = 20. (A) Release of IL-1β into the supernatant was measured by ELISA at 6
1417 hpi. (B) Cell death (percentage cytotoxicity) was measured by lactate dehydrogenase
1418 release assay and normalized to mock-infected cells at 6 hpi. (C) Cells were lysed at 1
1419 hpi and 6 hpi, and bacteria were subsequently plated to calculate CFU. Fold-change in
1420 CFU/well was calculated. (D, E) Cells were fixed at 6 hpi and stained for DAPI to label
1421 DNA (blue). (D) The number of bacteria per cell at 6 hpi was scored by fluorescence
1422 microscopy. Each small dot represents one infected cell. 150 infected cells were scored
1423 for each genotype. (E) Representative images are shown. Scale bar represents 10 μm.
1424 Bars represent the mean for each genotype, and error bars represent the standard
1425 deviation of triplicate wells from one experiment (A, B, C, D). **$p < 0.01$, ***$p < 0.001$,
1426 ****$p < 0.0001$ by Šídák's multiple comparisons test (A) or by unpaired t-test (B, C, D).
1427 Data shown are representative of at least three independent experiments.
1428
1429 **Figure 4. NINJ1 contributes to the control of *Salmonella* replication within human**
1430 **macrophages.** WT THP-1 monocyte-derived macrophages were treated with siRNA
1431 targeting a control scrambled siRNA or siRNA targeting *NINJ1* for 72 hours prior to
1432 infection. Cells were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were then
1433 infected with PBS (Mock) or WT *S*. Typhimurium at an MOI = 20. (A) Release of IL-1β
1434 into the supernatant was measured by ELISA at 6 hpi. (B) Cell death (percentage
1435 cytotoxicity) was measured by lactate dehydrogenase release assay and normalized to
1436 mock-infected cells at 6 hpi. (C, D, E) Cells were lysed at 1 hpi and 6 hpi, and bacteria

1437 were subsequently plated to calculate CFU. (C) CFU/well at 1 hpi. (D) CFU/well at 6 hpi.
1438 (E) Fold-change in CFU/well was calculated. Bars represent the mean for each
1439 condition. Error bars represent the standard deviation of triplicate wells from one
1440 experiment. ns – not significant, *p< 0.05, **p< 0.01 by unpaired t-test. Data shown are
1441 representative of at least three independent experiments.
1442
1443 **Figure 5. Inflammasome activation primarily controls cytosolic *Salmonella***
1444 **replication in human macrophages.** WT, *NAIP*$^{-/-}$ (A), and *CASP1*$^{-/-}$ (A, B) THP-1
1445 monocyte-derived macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours.
1446 Cells were then infected with PBS (Mock) or WT *S*. Typhimurium (A) or Δ*sipB S*.
1447 Typhimurium (B) at an MOI = 20. At 5 hpi, cells were left untreated or treated with 500
1448 μM CHQ for 1 hour. Then, at 6 hpi, cells were lysed, and bacteria were subsequently
1449 plated to calculate CFU. CFU/well of vacuolar (CHQ-sensitive) and cytosolic (CHQ-
1450 resistant) bacteria were calculated from the total bacteria. (C,D) WT, *NAIP*$^{-/-}$, and
1451 *CASP1*$^{-/-}$ THP-1 monocyte-derived macrophages were primed with 100 ng/mL
1452 Pam3CSK4 for 16 hours. Cells were then infected with PBS (Mock) or WT *S.*
1453 Typhimurium constitutively expressing mCherry and harboring the GFP cytosolic
1454 reporter plasmid, pNF101, at an MOI = 20. Cells were fixed at 8 hpi and stained for
1455 DAPI to label DNA (blue). (C) The number of GFP-positive, mCherry-positive bacteria
1456 (cytosolic) per cell and the number of GFP-negative, mCherry-positive bacteria
1457 (vacuolar) per cell were scored by fluorescence microscopy. Each small dot represents
1458 one infected cell. 150 total infected cells were scored for each genotype. (D)
1459 Representative images from 8 hpi are shown. Scale bar represents 10 μm. White

1460 arrows indicate cytosolic bacteria (GFP-positive, mCherry-positive). Bars represent the
1461 mean for each genotype (A, B). Error bars represent the standard deviation of triplicate
1462 wells from one experiment (A, B). ns – not significant, *p< 0.05, ****p < 0.0001 by
1463 Dunnett's multiple comparisons test (A) or by Tukey's multiple comparisons test (B).
1464 Data shown are representative of at least three independent experiments (A, B, C).
1465
1466 **Figure 6. Inflammasome activation modulates the cytosolic exposure of**
1467 ***Salmonella* in human macrophages.** (A, B) WT and *CASP1*$^{-/-}$ THP-1 monocyte-
1468 derived macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were
1469 then infected with WT *S*. Typhimurium at an MOI = 20. At 8 hpi, cells were fixed and
1470 collected to be processed for transmission electron microscopy. Representative
1471 transmission electron micrographs are shown. *Salmonella* (green) in an SCV (maroon)
1472 with cytosolic or vacuolar clearance (yellow). Scale bar represents 1 µm [A(i), B(i)] or
1473 400 nm [A(ii), B(ii)]. (A) WT THP-1s, (ii) is an inset from (i). White arrows indicate
1474 vacuolar bacteria. (B) *CASP1*$^{-/-}$ THP-1s, (ii) is an inset from (i). White arrows indicate
1475 vacuolar bacteria, cyan arrows indicate partially cytosolic bacteria, and black asterisk
1476 indicates a fully cytosolic bacterium. (C) *CASP1*$^{-/-}$ THP-1 monocyte-derived
1477 macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were then
1478 infected with WT *S*. Typhimurium at an MOI = 20. At 8 hpi, cells were fixed and
1479 collected to be processed for transmission electron microscopy. Representative
1480 tomogram slices are shown, depicting *Salmonella* (green) in an SCV with a
1481 discontinuous vacuolar membrane (maroon).
1482

1483 **Video 1. Characterization of the cytosolic exposure of *Salmonella* in *CASP1*$^{-/-}$**

1484 **human macrophages.** *CASP1*$^{-/-}$ THP-1 monocyte-derived macrophages were primed

1485 with 100 ng/mL Pam3CSK4 for 16 hours. Cells were then infected with WT *S*.

1486 Typhimurium at an MOI = 20. At 8 hpi, cells were fixed and collected to be processed

1487 for transmission electron microscopy. Representative tomogram shown, depicting

1488 *Salmonella* in an SCV with a discontinuous vacuolar membrane.

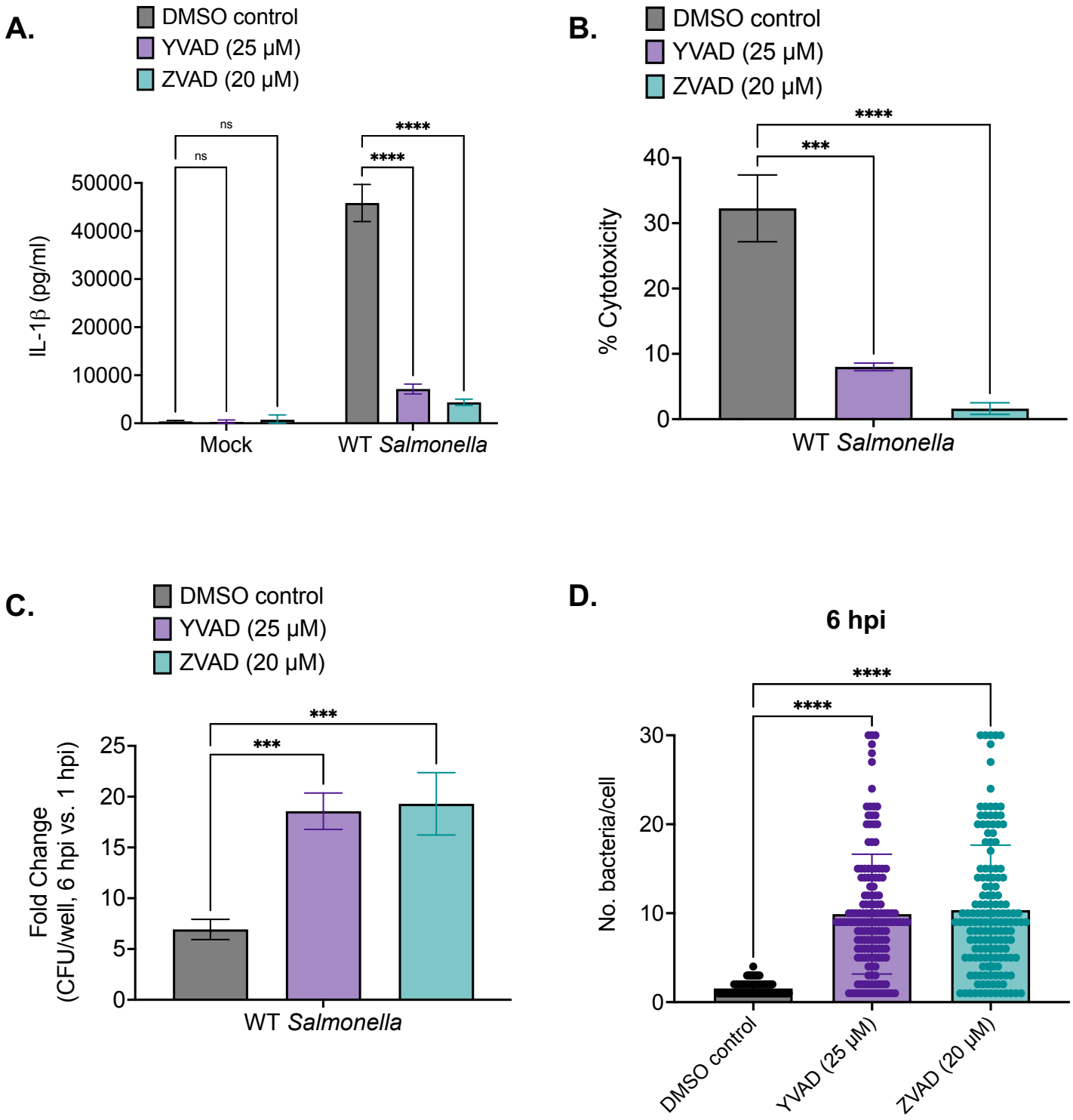


Fig. 1—Fig. Sup. 1

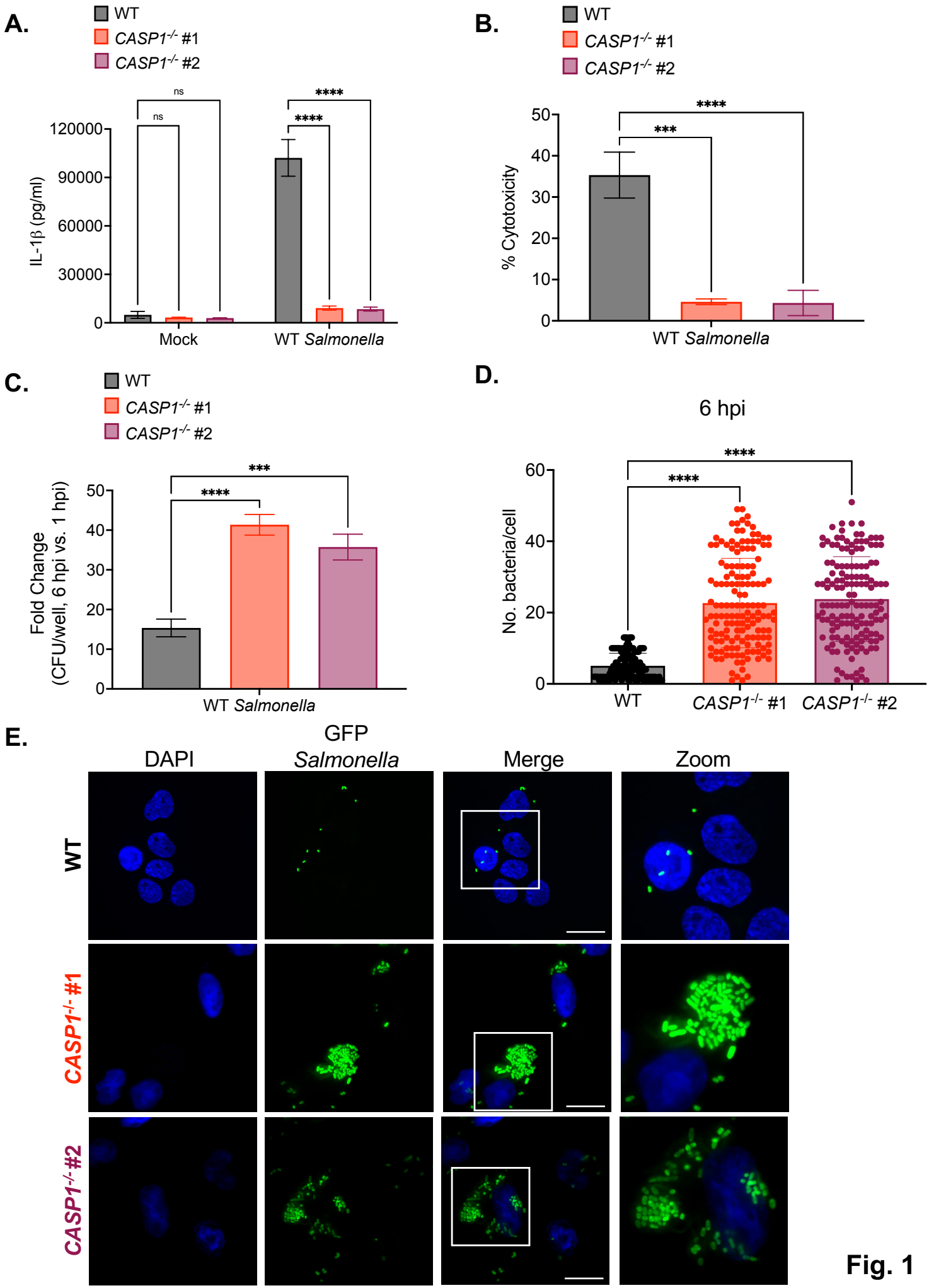
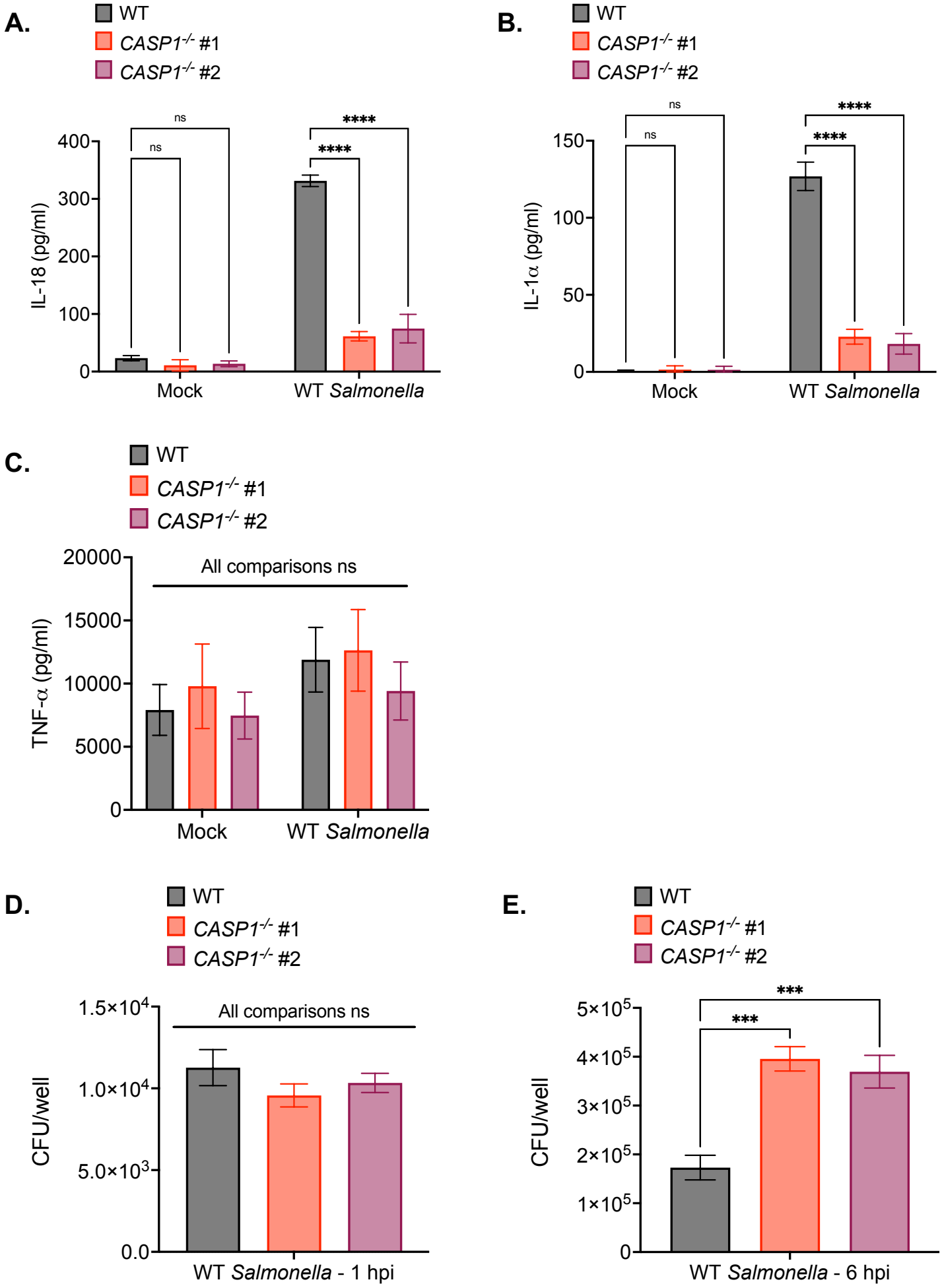

A.
WT
CASP1-/- #1
CASP1-/- #2
IL-1β (pg/ml)
120000
90000
60000
30000
0
ns
ns
****
****
Mock
WT Salmonella
B.
WT
CASP1-/- #1
CASP1-/- #2
% Cytotoxicity
50
40
30
20
10
0
****
***
WT Salmonella
C.
WT
CASP1-/- #1
CASP1-/- #2
Fold Change
(CFU/well, 6 hpi vs. 1 hpi)
50
40
30
20
10
0
***
****
WT Salmonella
D.
6 hpi
No. bacteria/cell
60
40
20
0
****
****
WT
CASP1-/- #1
CASP1-/- #2
E.
DAPI
GFP
Salmonella
Merge
Zoom
WT
CASP1-/- #1
CASP1-/- #2

Fig. 1



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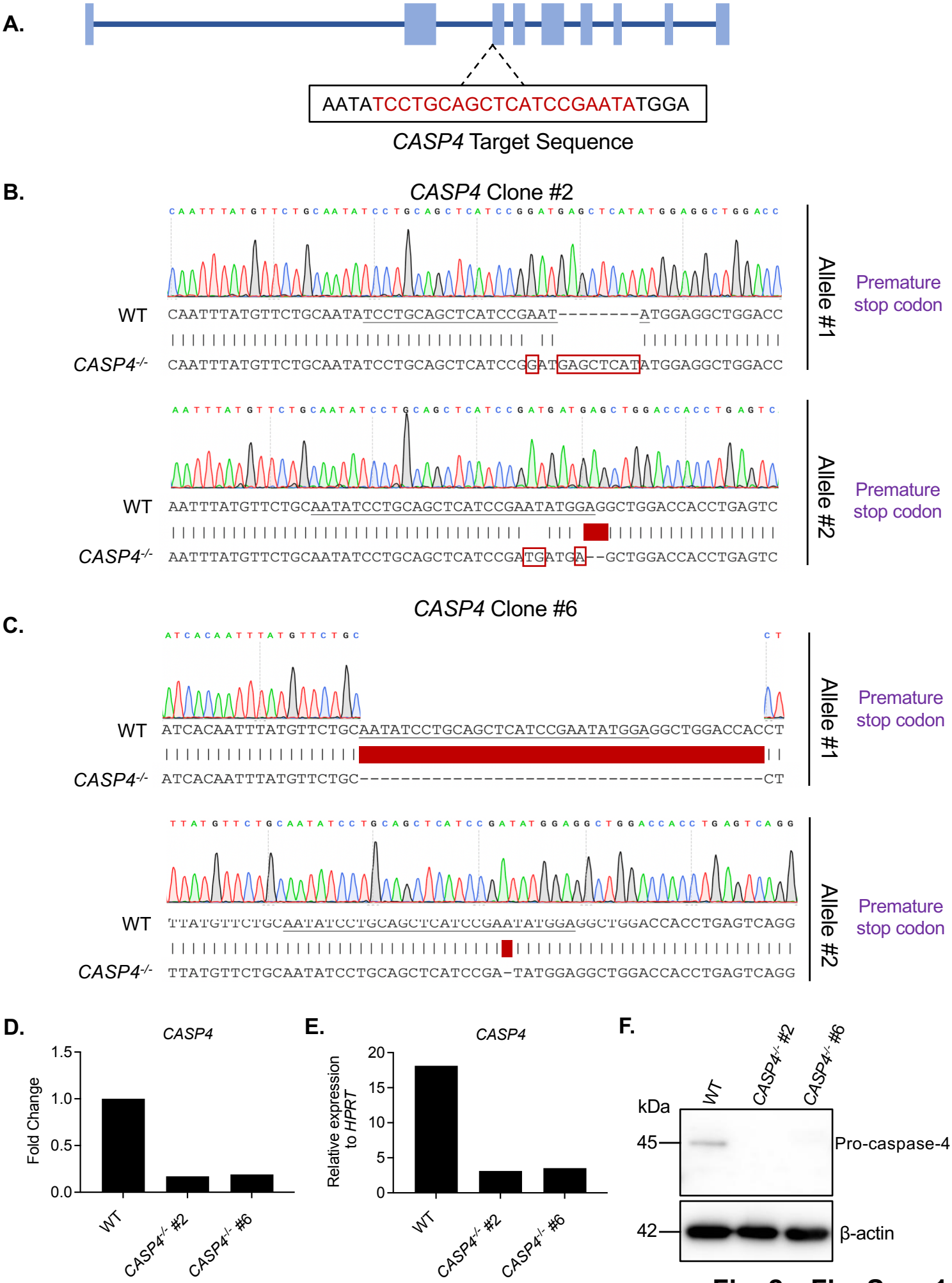

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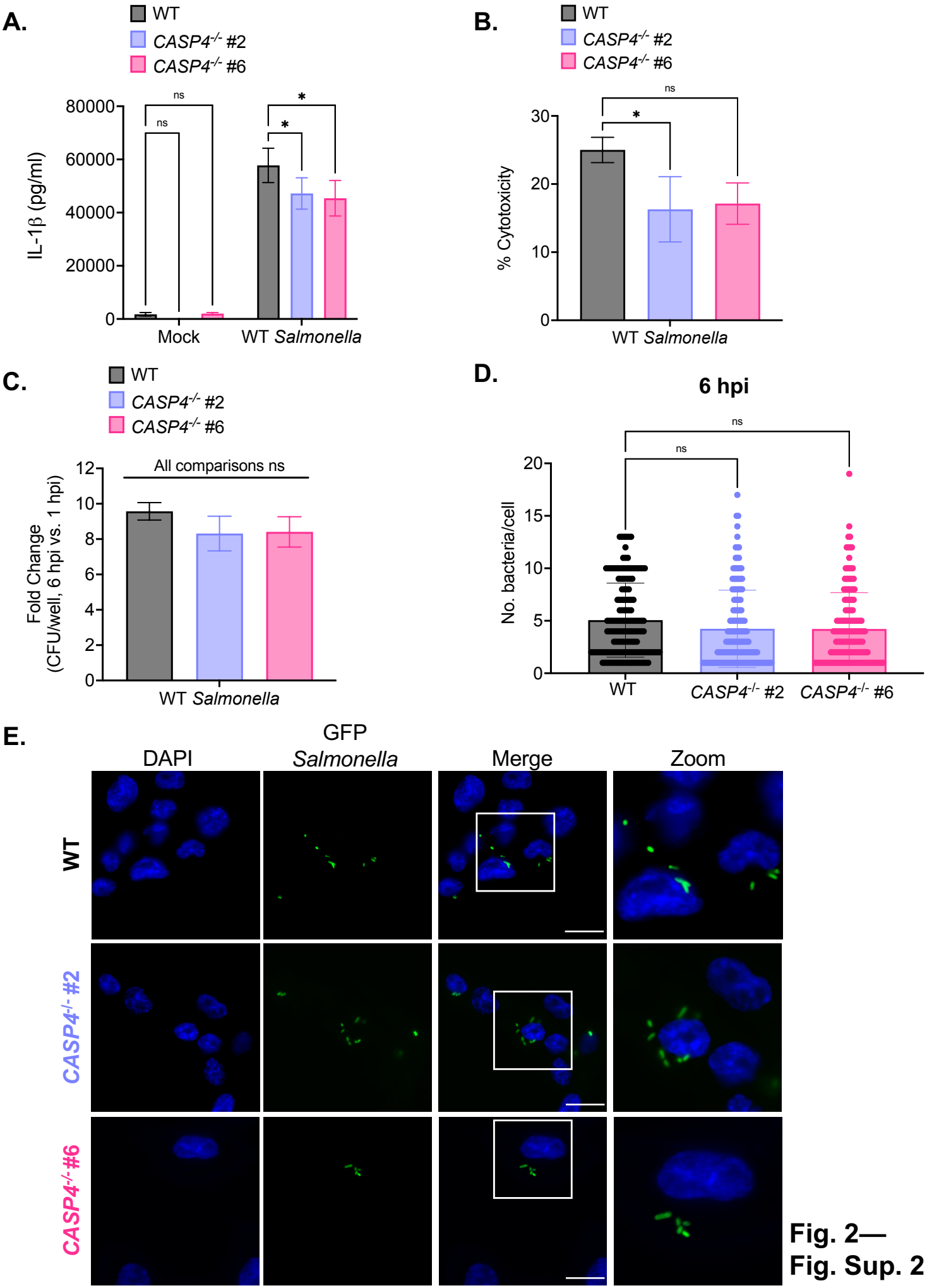

Fig. 2—
Fig. Sup. 2

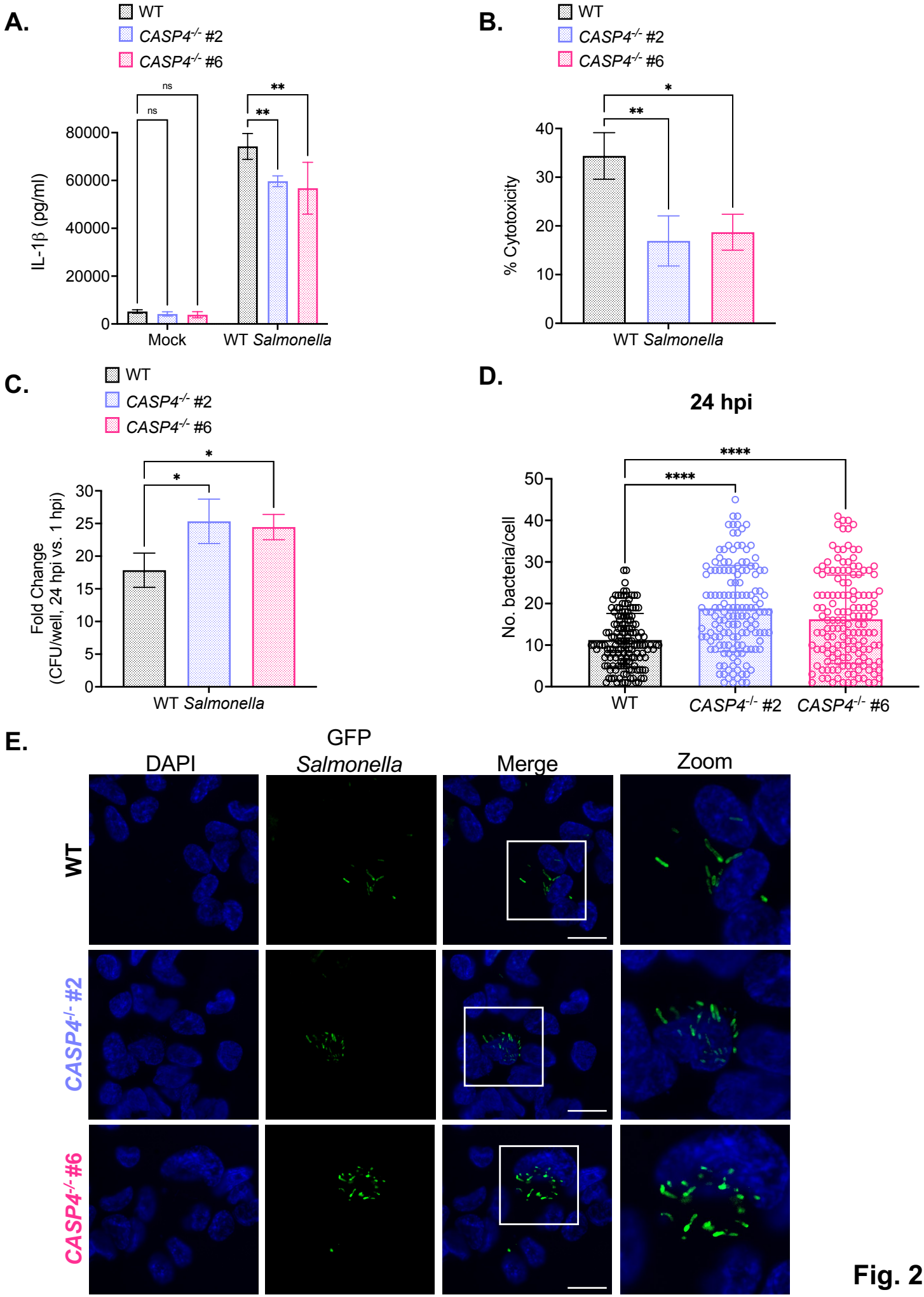
A.
WT
CASP4-/- #2
CASP4-/- #6
IL-1β (pg/ml)
80000
60000
40000
20000
0
ns
ns
**
**
Mock
WT Salmonella
B.
WT
CASP4-/- #2
CASP4-/- #6
% Cytotoxicity
40
30
20
10
0
*
**
WT Salmonella
C.
WT
CASP4-/- #2
CASP4-/- #6
Fold Change
(CFU/well, 24 hpi vs. 1 hpi)
30
25
20
15
10
5
0
*
*
WT Salmonella
D.
24 hpi
No. bacteria/cell
50
40
30
20
10
0
****
****
WT
CASP4-/- #2
CASP4-/- #6
E.
DAPI
GFP
Salmonella
Merge
Zoom
WT
CASP4-/- #2
CASP4-/- #6

Fig. 2

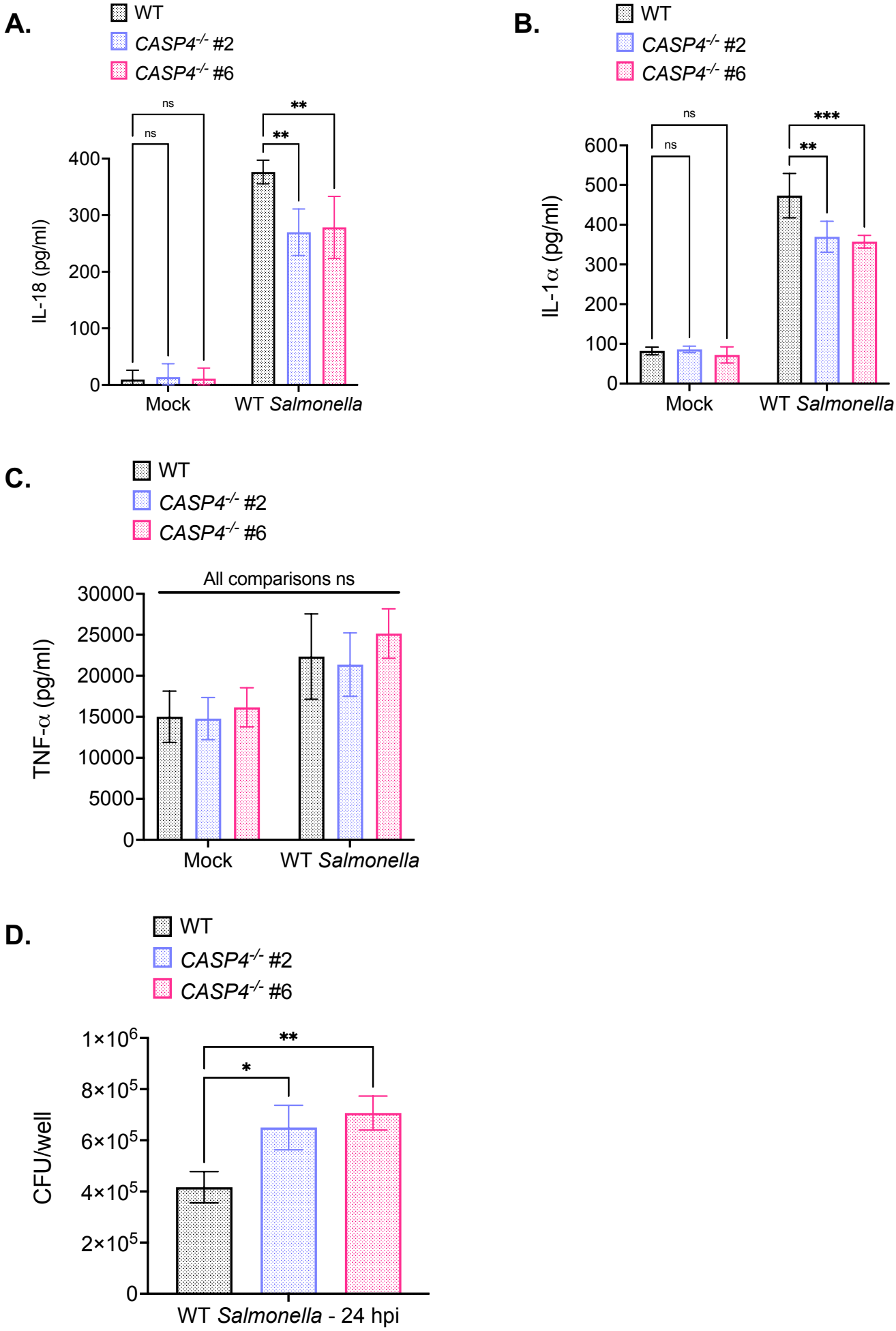

Fig. 2—Fig. Sup. 3

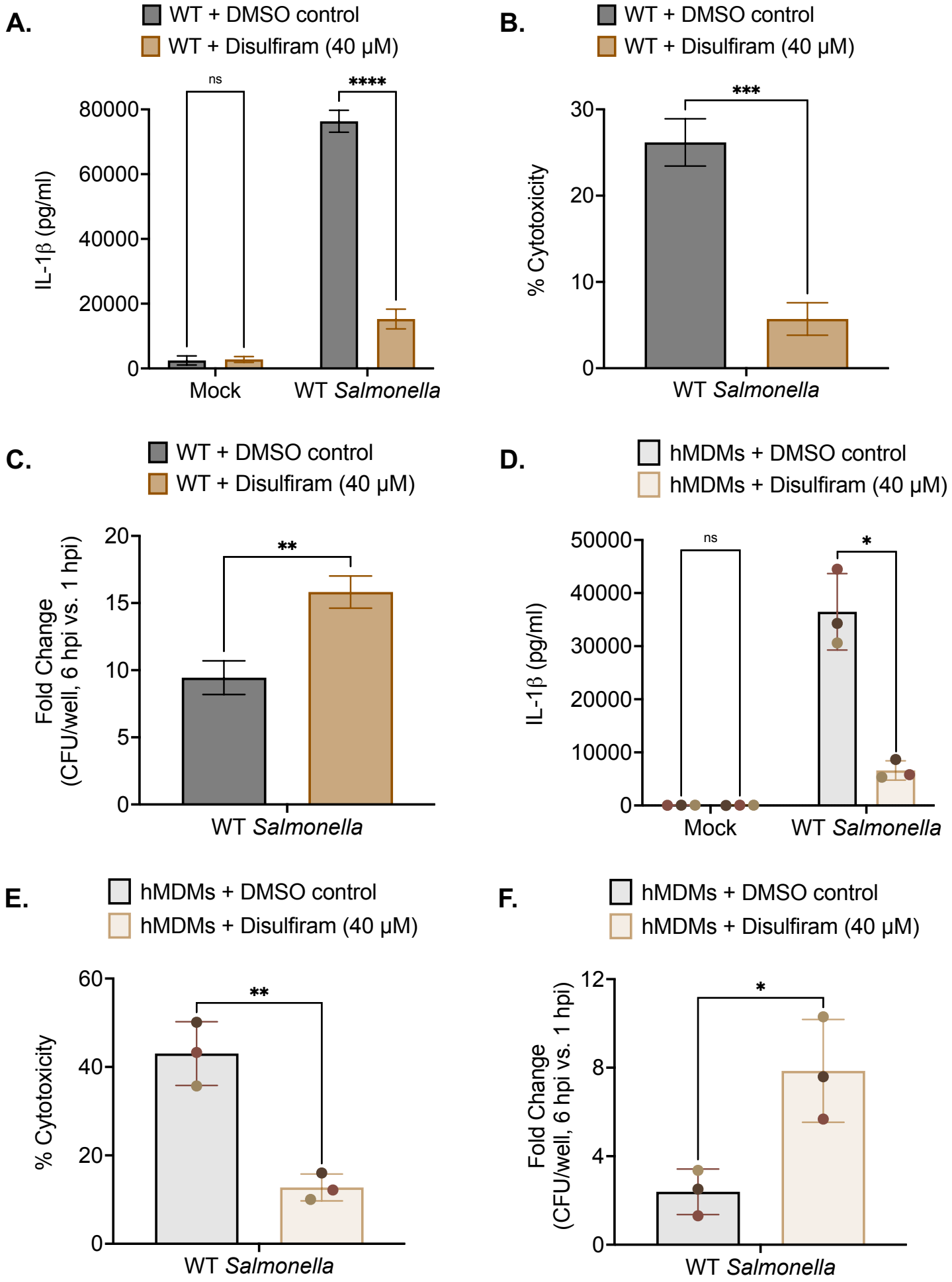

Fig. 3—Fig. Sup. 1

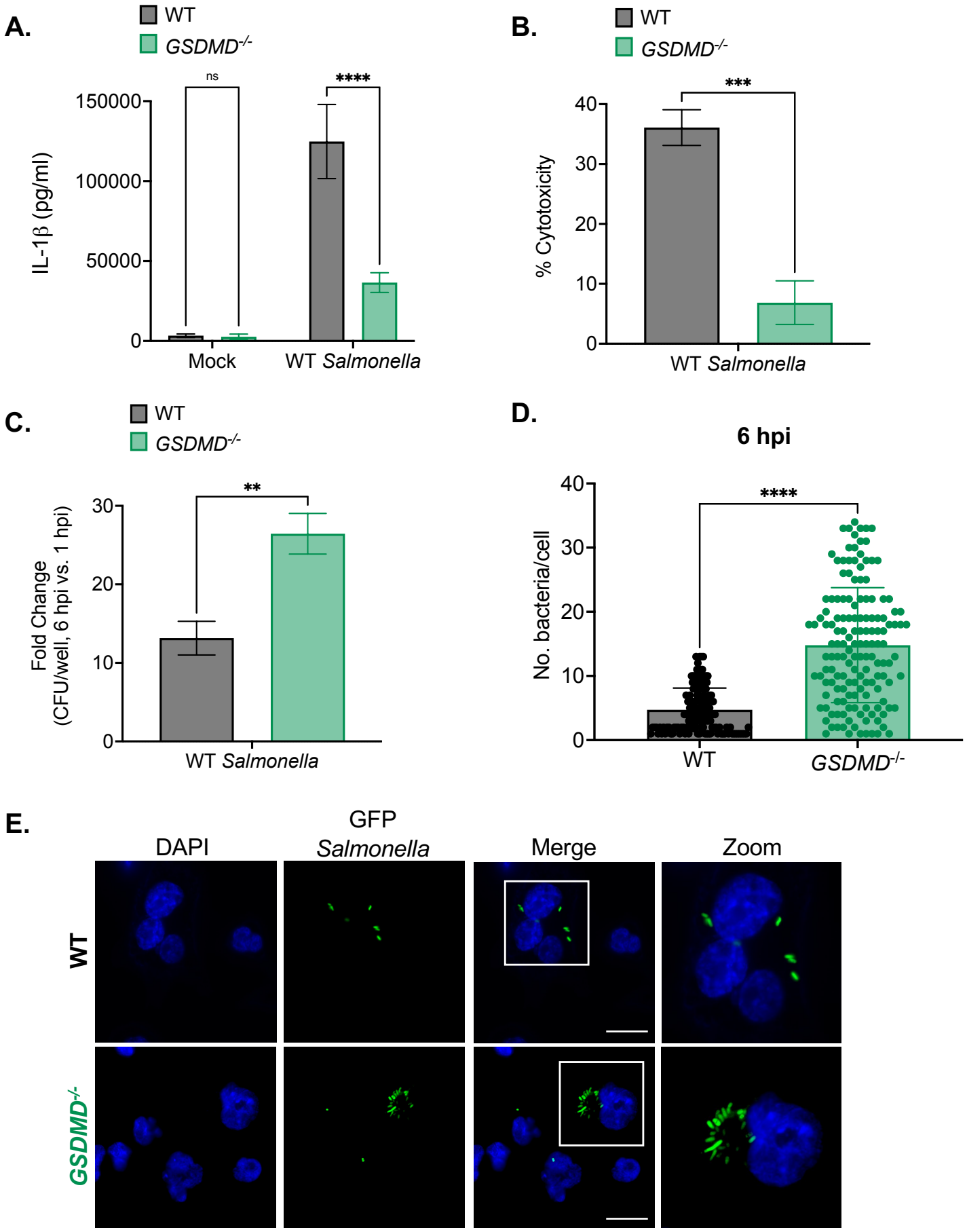
A.
WT
GSDMD-/-
ns
****
150000
100000
50000
0
IL-1β (pg/ml)
Mock
WT Salmonella
B.
WT
GSDMD-/-
***
40
30
20
10
0
% Cytotoxicity
WT Salmonella
C.
WT
GSDMD-/-
**
30
20
10
0
Fold Change
(CFU/well, 6 hpi vs. 1 hpi)
WT Salmonella
D.
6 hpi
****
40
30
20
10
0
No. bacteria/cell
WT
GSDMD-/-
E.
DAPI
GFP
Salmonella
Merge
Zoom
WT
GSDMD-/-

Fig. 3

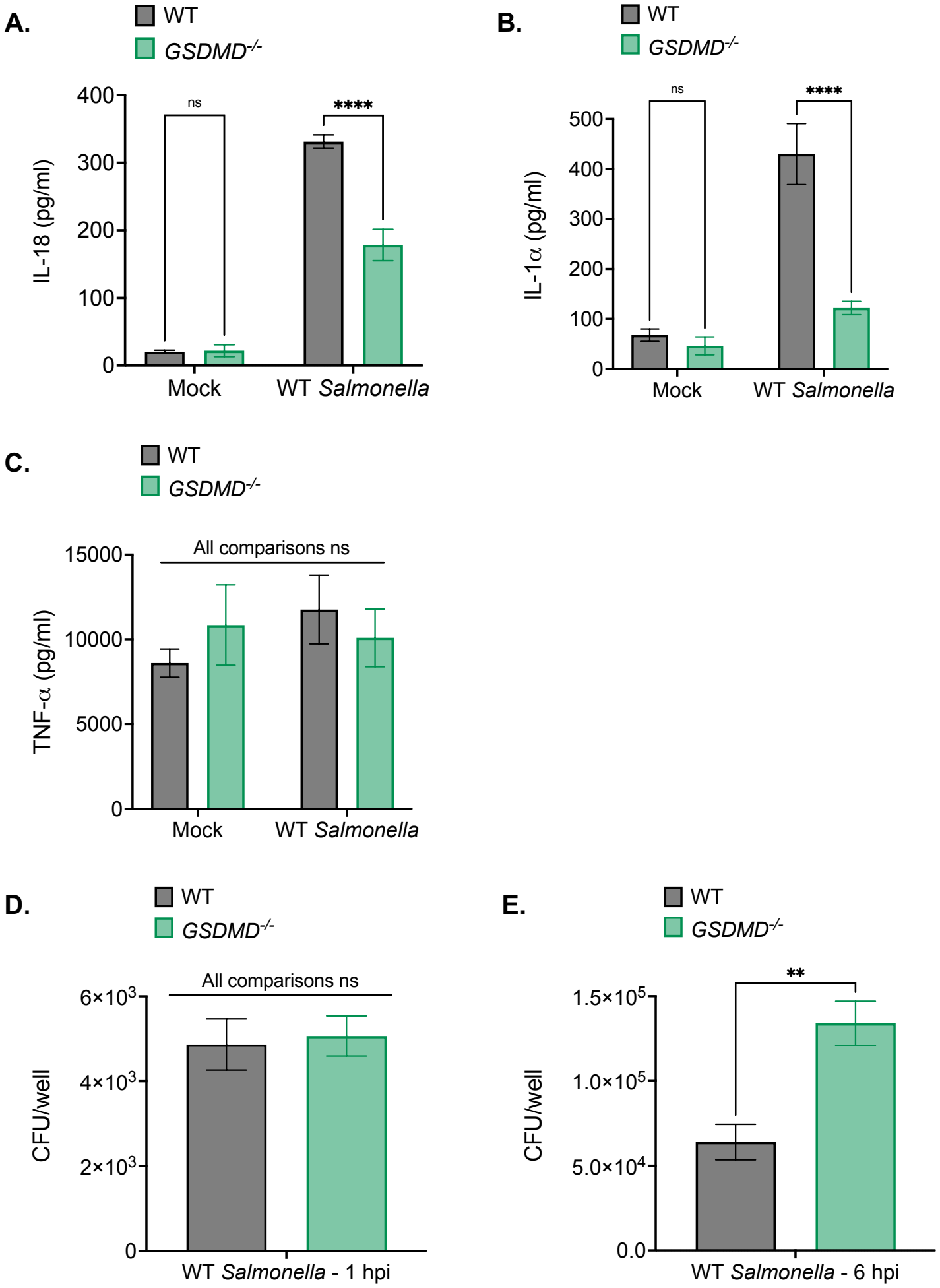

Fig. 3—Fig. Sup. 2

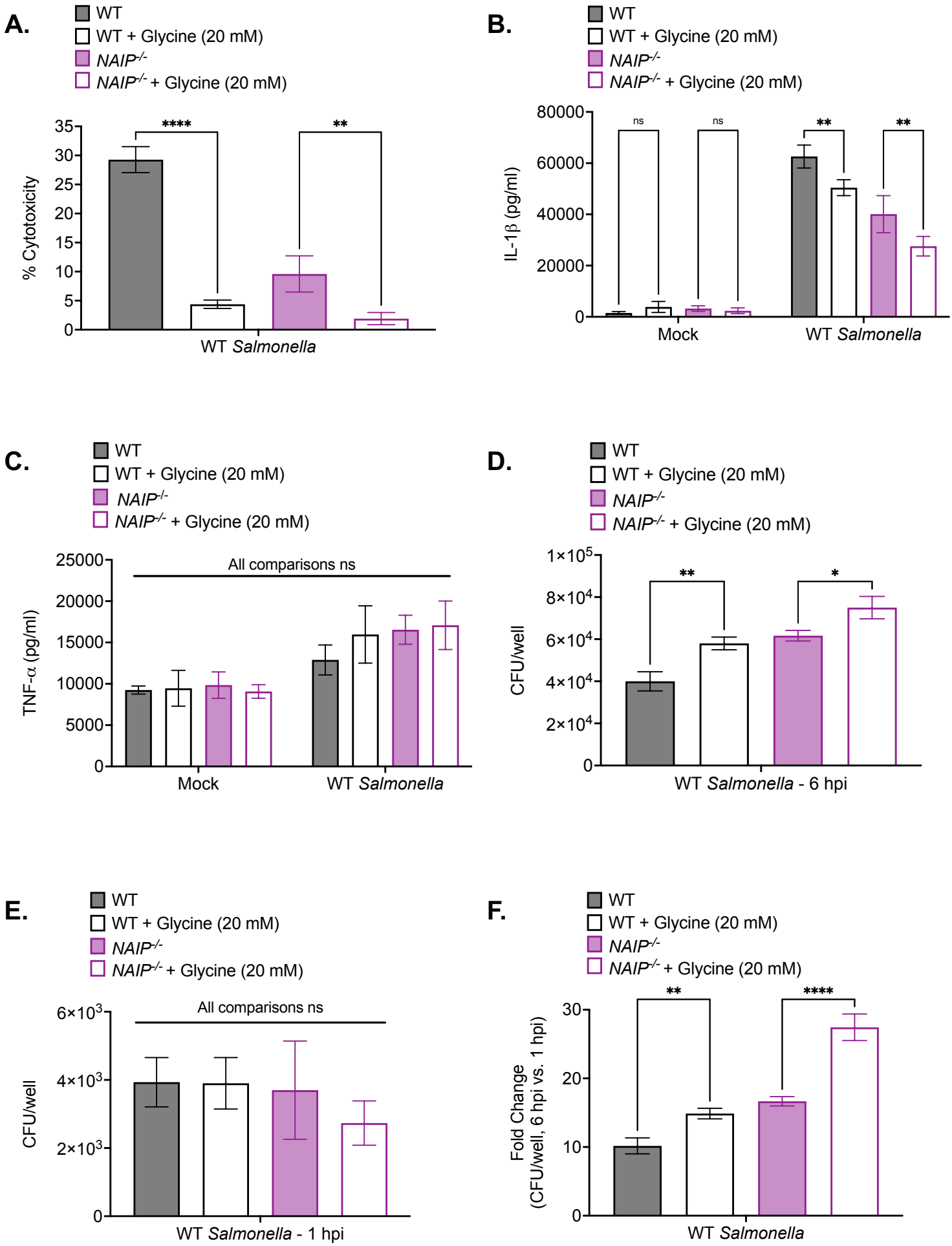

Fig. 4—Fig. Sup. 1

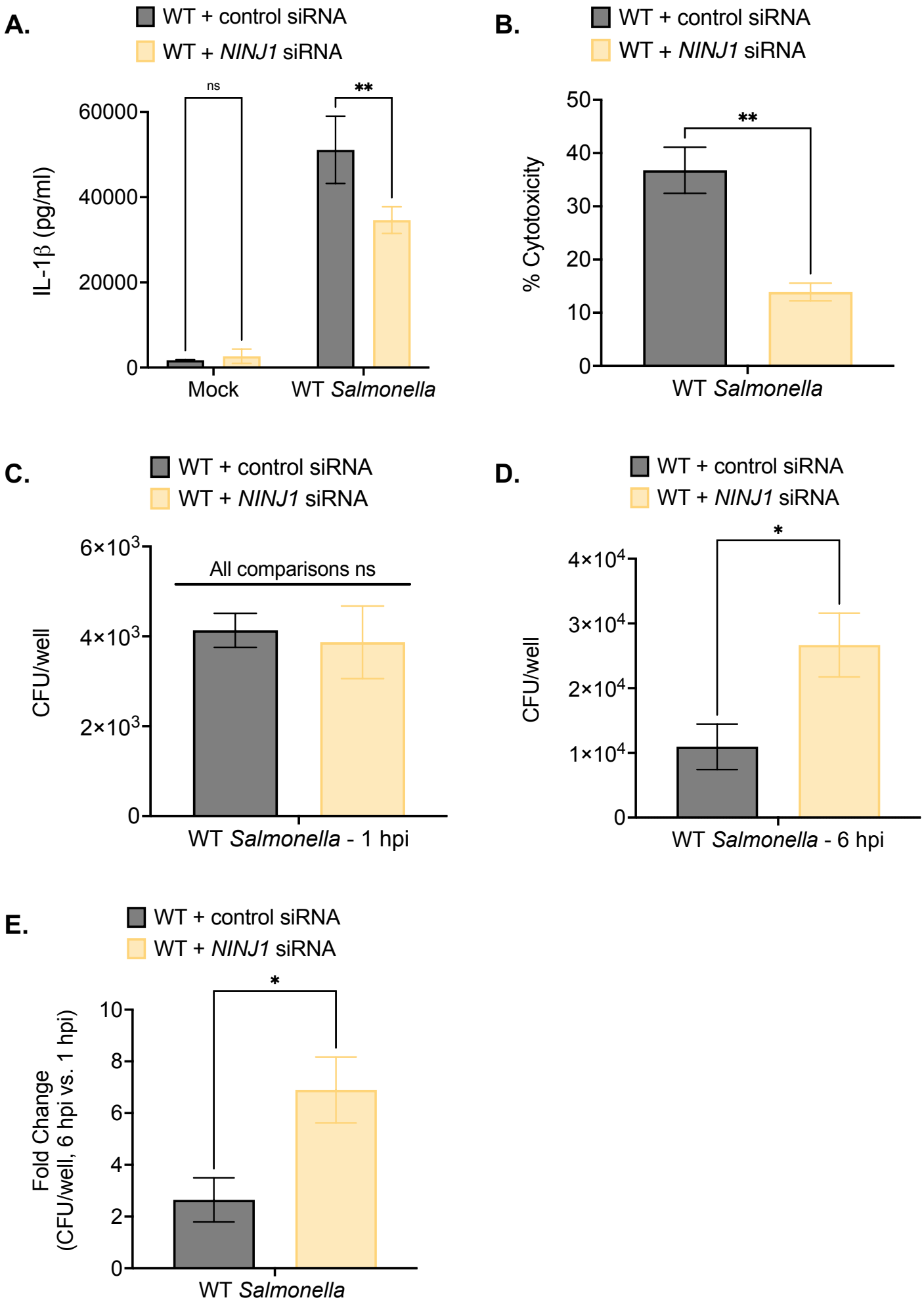
A.
WT + control siRNA
WT + NINJ1 siRNA
ns
**
IL-1β (pg/ml)
60000
40000
20000
0
Mock
WT Salmonella
B.
WT + control siRNA
WT + NINJ1 siRNA
**
% Cytotoxicity
50
40
30
20
10
0
WT Salmonella
C.
WT + control siRNA
WT + NINJ1 siRNA
All comparisons ns
CFU/well
6×10^3
4×10^3
2×10^3
0
WT Salmonella - 1 hpi
D.
WT + control siRNA
WT + NINJ1 siRNA
*
CFU/well
4×10^4
3×10^4
2×10^4
1×10^4
0
WT Salmonella - 6 hpi
E.
WT + control siRNA
WT + NINJ1 siRNA
*
Fold Change
(CFU/well, 6 hpi vs. 1 hpi)
10
8
6
4
2
0
WT Salmonella

Fig. 4

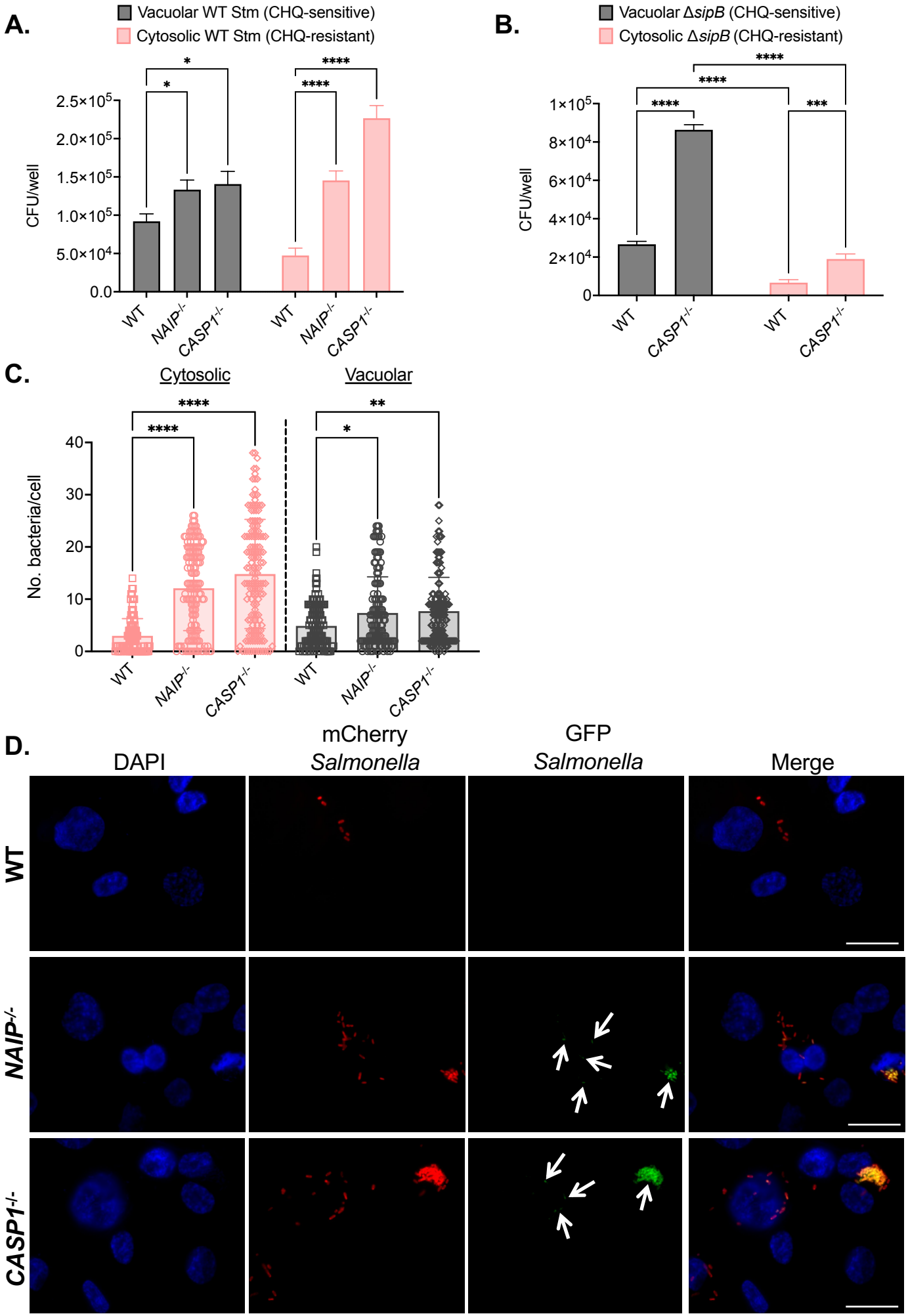
A.
Vacuolar WT Stm (CHQ-sensitive)
Cytosolic WT Stm (CHQ-resistant)
CFU/well
2.5×10^5
2.0×10^5
1.5×10^5
1.0×10^5
5.0×10^4
0.0
*
*
****
****
WT
NAIP-/-
CASP1-/-
WT
NAIP-/-
CASP1-/-
B.
Vacuolar ΔsipB (CHQ-sensitive)
Cytosolic ΔsipB (CHQ-resistant)
CFU/well
1×10^5
8×10^4
6×10^4
4×10^4
2×10^4
0
****
****
****
***
WT
CASP1-/-
WT
CASP1-/-
C.
Cytosolic
Vacuolar
No. bacteria/cell
40
30
20
10
0
****
****
**
*
WT
NAIP-/-
CASP1-/-
WT
NAIP-/-
CASP1-/-
D.
DAPI
mCherry Salmonella
GFP Salmonella
Merge
WT
NAIP-/-
CASP1-/-

Fig. 5

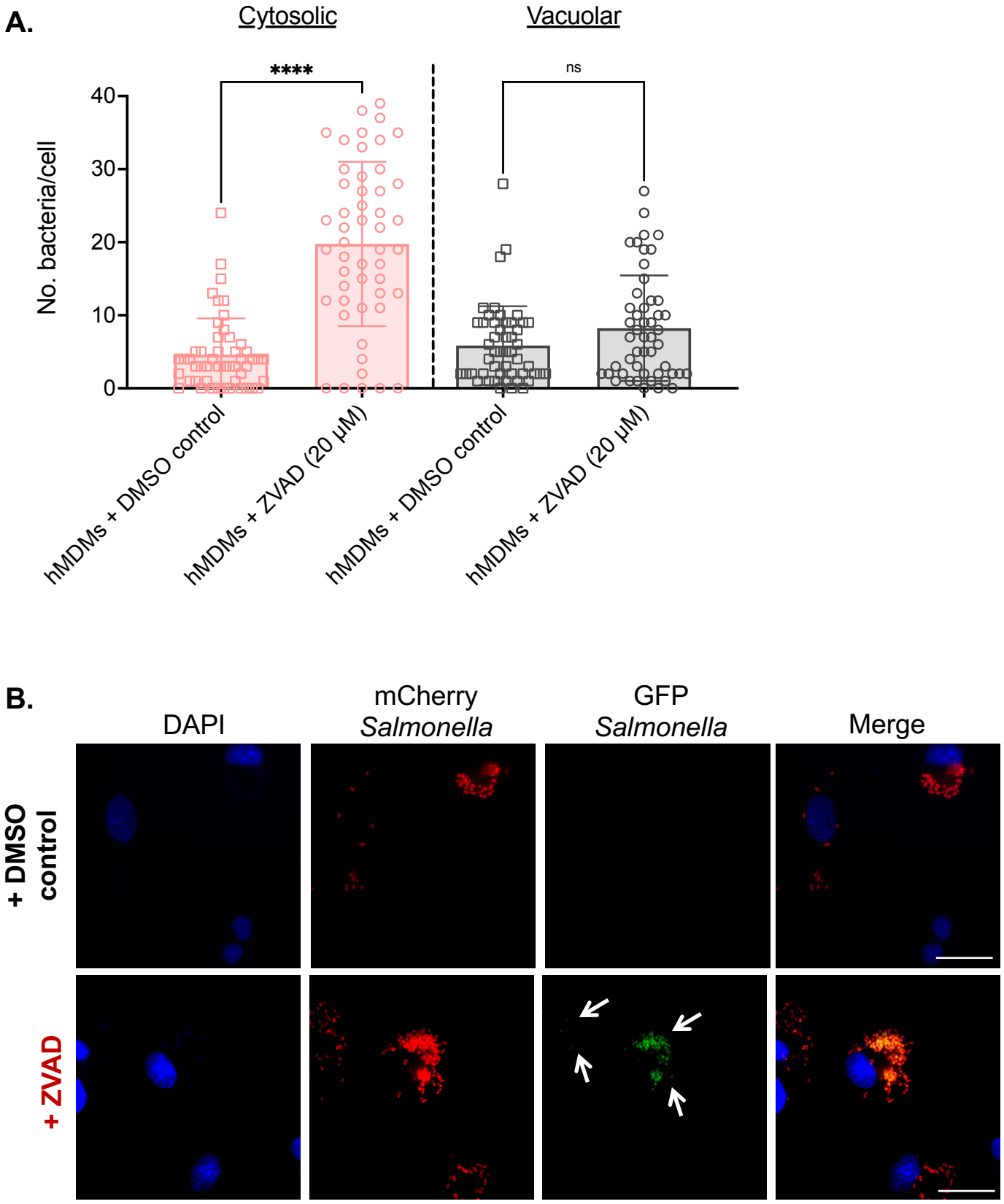

Fig. 5—Fig. Sup. 1

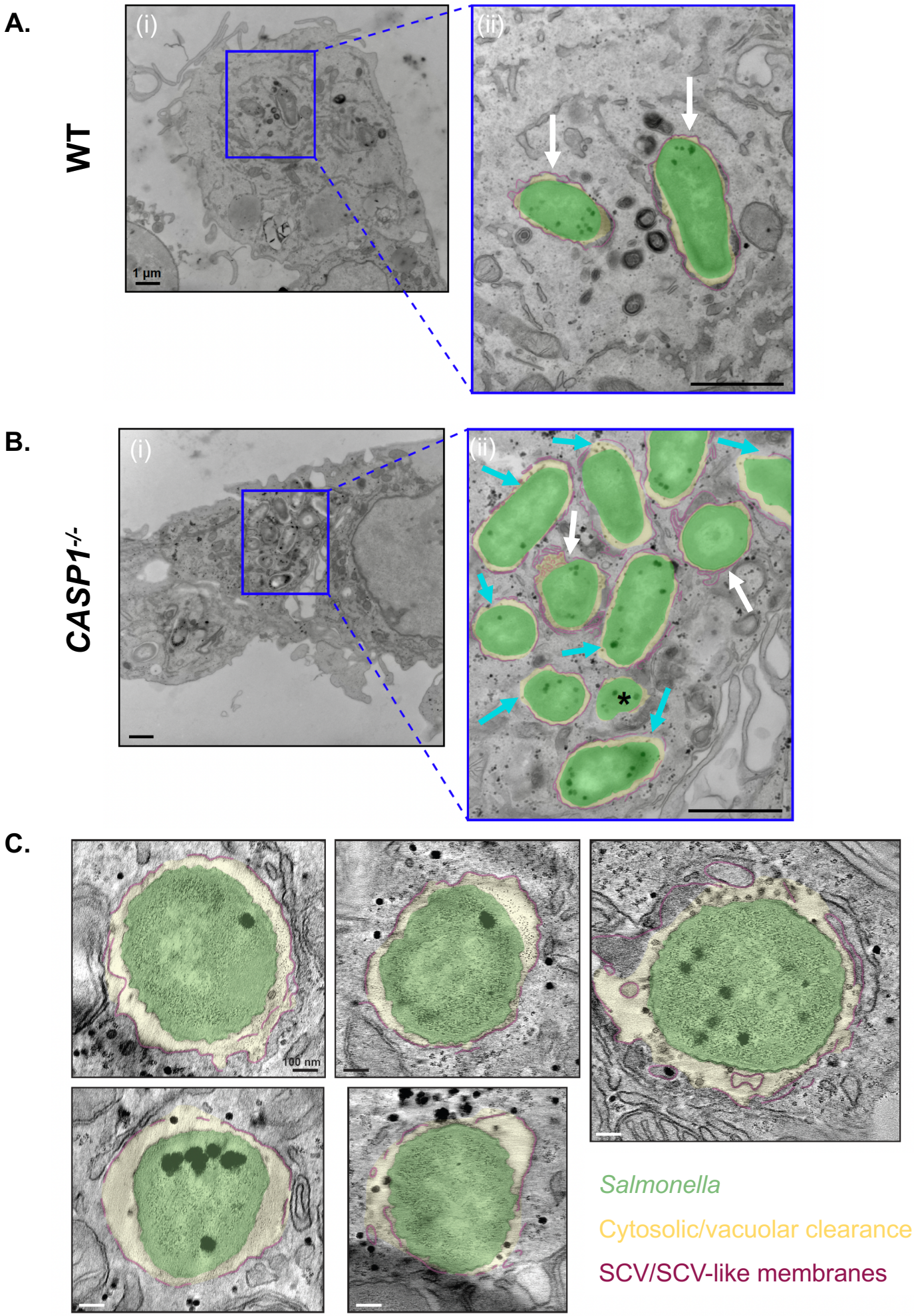
A.
WT
(i)
(ii)
1 µm
B.
CASP1-/-
(i)
(ii)
*
C.
100 nm
Salmonella
Cytosolic/vacuolar clearance
SCV/SCV-like membranes

Fig. 6

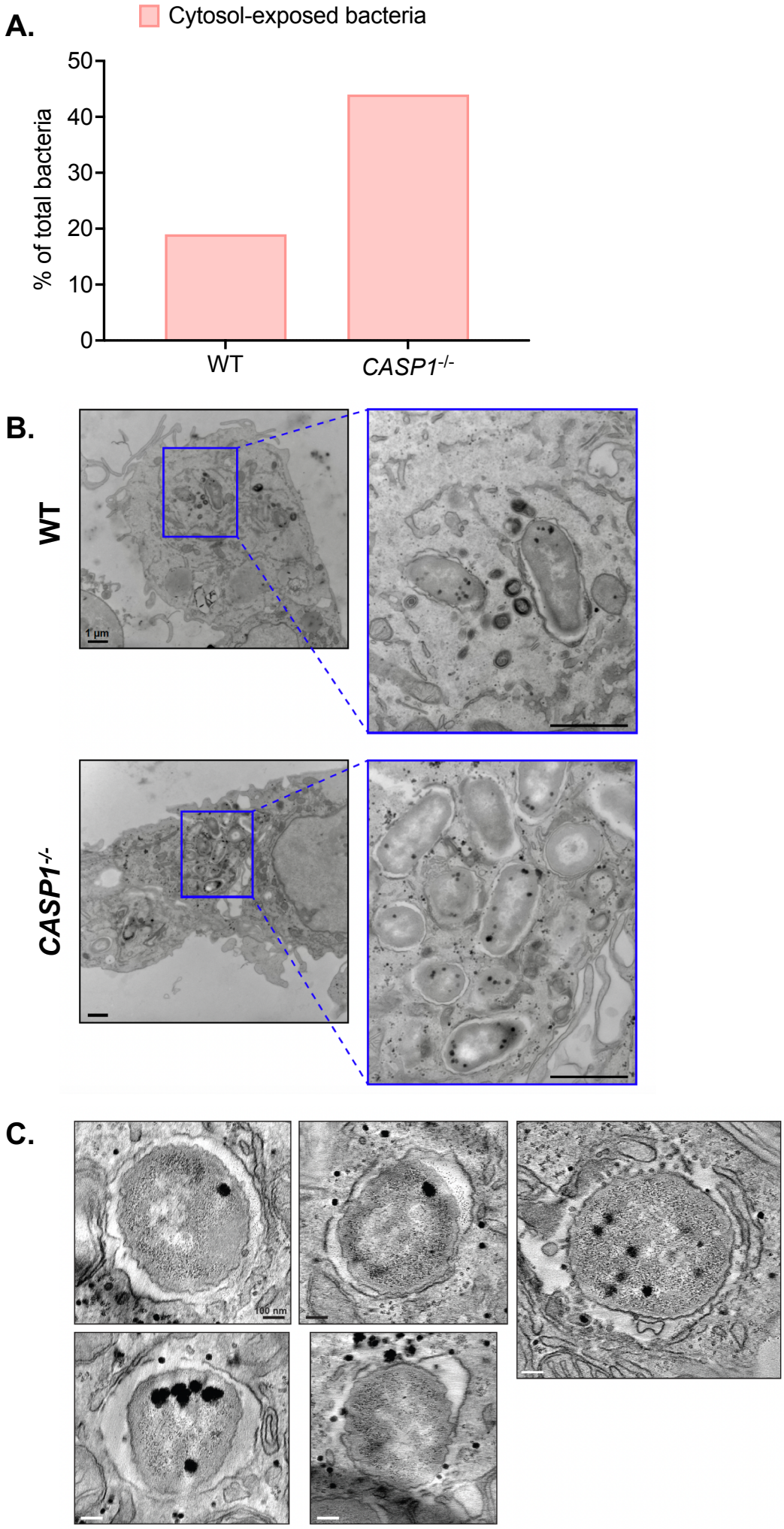

Fig. 6—Fig. Sup. 1