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 host cells 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 restricting 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 as well as 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 . Specifically, *Salmonella* relies
57 on two distinct T3SSs encoded on *Salmonella* Pathogenicity Islands 1 and 2 (SPI-1 and
58 SPI-2) to invade and replicate within host cells, respectively (Mills et al., 1995; Shea et
59 al., 1996; Hensel et al., 1998; Galan & Zhou, 2000; Galán, 1999; Galán & Collmer,
60 1999; Galan & Curtiss, 1989; Ochman et al., 1996; Cirillo et al., 1998). The SPI-2 T3SS
61 translocates effectors to facilitate biogenesis and maintenance of the *Salmonella*-
62 containing vacuole (SCV), wherein *Salmonella* resides and replicates (Cirillo et al.,
63 1998; Takeuchi, 1967; Takeuchi & Sprinz, 1967; Kihlström & Latkovic, 1978; Garcia-del
64 Portillo et al., 1993; Garcia-del Portillo & Finlay, 1995; Steele-Mortimer et al., 1999;
65 Hansen-Wester et al., 2002; Steele-Mortimer, 2008; Jennings et al., 2017; Brumell,
66 Goosney, et al., 2002; Brumell, Tang, et al., 2002; Beuzón et al., 2000; Hensel et al.,
67 1998). In epithelial cells, *Salmonella* escapes its vacuolar niche and hyperreplicates in
68 the host cell cytosol (Knodler, Crowley, et al., 2014; Knodler et al., 2010; Knodler, Nair,
69 et al., 2014; Malik-Kale et al., 2012). While the activity of T3SSs are essential for

70 *Salmonella* to infect and replicate within host cells, they also inject bacterial ligands that
71 enable innate immune sensing of *Salmonella* (E. A. Miao, Mao, et al., 2010; Rayamajhi
72 et al., 2013; Sun et al., 2007; Yang et al., 2013).

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; Lamkanfi & Dixit, 2009; Medzhitov &
76 Janeway, 2002). A subset of these PRRs includes the nucleotide-binding domain,
77 leucine-rich repeat (NLR) family proteins. NLRs respond to their cognate stimuli by
78 oligomerizing and inducing the assembly of multiprotein complexes termed
79 inflammasomes, which activate inflammatory caspases (Broz & Dixit, 2016; Lamkanfi &
80 Dixit, 2009, 2014; Martinon et al., 2002). Canonical inflammasomes recruit and activate
81 caspase-1, which cleaves and activates pro-inflammatory interleukin-1 (IL-1) family
82 cytokines and the pore-forming protein gasdermin D (GSDMD) (Kuida et al., 1995; Li et
83 al., 1995; Agard et al., 2010; Thornberry et al., 1992; Shi et al., 2015; Kayagaki et al.,
84 2015). Alternatively, noncanonical inflammasomes are formed by caspase-11 in mice
85 and two orthologs in humans, caspase-4 and caspase-5, in response to cytosolic
86 lipopolysaccharide (LPS) (Casson et al., 2015; Hagar et al., 2013; Kayagaki et al., 2011,
87 2013; Lagrange et al., 2018; Schmid-Burgk et al., 2015; Shi et al., 2014). These
88 caspases directly process and activate GSDMD (Kayagaki et al., 2015; Shi et al., 2015).
89 Liberated 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; E. A. Miao, Mao, et al., 2010; Molofsky et al., 2006; Rauch et al.,
99 2016; Rayamajhi et al., 2013; Ren et al., 2006; Reyes Ruiz et al., 2017; Sun et al.,
100 2007; Yang et al., 2013; Zhao et al., 2011, 2016). Upon ligand recognition, NAIP
101 recruits NLRC4, which oligomerizes to form the active NAIP/NLRC4 inflammasome
102 (Diebolder et al., 2015; Z. Hu et al., 2015; Zhang et al., 2015). *Salmonella* also activates
103 the NLR pyrin domain-containing protein 3 (NLRP3) inflammasome and the
104 noncanonical caspase-4/5 inflammasome in human macrophages (Bierschenk et al.,
105 2019; Casson et al., 2015; Gram et al., 2021; Naseer, Egan, et al., 2022). NLRP3
106 responds to diverse stimuli, including ionic fluxes as a result of host plasma membrane
107 damage (Franchi et al., 2007; Hornung et al., 2008; Mariathasan et al., 2006; Muñoz-
108 Planillo et al., 2013; Perregaux & Gabel, 1994), and can be secondarily activated by the
109 noncanonical inflammasome (Baker et al., 2015; Casson et al., 2015; Kayagaki et al.,
110 2013; Pilla et al., 2014; Rathinam et al., 2012; Rühl & Broz, 2015; Schmid-Burgk et al.,
111 2015; Shi et 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; E. A. Miao et al., 2006; E. A.
115 Miao, Mao, et al., 2010; Rauch et al., 2016, 2017; Sellin et al., 2014; Zhao et al., 2016).

116 In murine intestinal epithelial cells (IECs), NAIP/NLRC4 inflammasome activation
117 triggers pyroptosis and expulsion of infected cells, and is both necessary and sufficient
118 in IECs to restrict *Salmonella* replication and prevent bacterial dissemination to distal
119 organ sites (Hausmann et al., 2020; Rauch et al., 2017; Sellin et al., 2014). Unlike
120 murine IECs, human epithelial cells do not rely on NAIP/NLRC4 or caspase-1, but
121 instead rely on caspase-4 to control *Salmonella* replication, in part through pyroptosis
122 and cell extrusion (Holly et al., 2020; Knodler, Crowley, et al., 2014; Knodler et al.,
123 2010; 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 (Man, Ekpenyong, et al., 2014; Thurston et al., 2016). In contrast, we found that human
127 macrophages rely on NAIP/NLRC4- and NLRP3-dependent inflammasome responses
128 to control intracellular WT *Salmonella* replication (Naseer, Egan, et al., 2022). However,
129 how inflammasome signaling restricts *Salmonella* replication in human macrophages
130 remains unknown.

131 In this study, we show that caspase-1 is required to restrict intracellular
132 *Salmonella* replication early during infection in human macrophages, and that caspase-
133 4 enables the restriction of *Salmonella* later during infection. We find that the cell lysis
134 mediators GSDMD and ninjurin-1 (NINJ1) also contribute to bacterial restriction.
135 Importantly, we observed that while human macrophages unable to undergo
136 inflammasome responses showed slightly elevated bacterial replication within SCVs,
137 they became permissive to hyperreplication of *Salmonella* within the cytosolic
138 compartment. Thus, inflammasome activation appears to preferentially restrict cytosolic

139 *Salmonella* replication. Our results offer insight into how inflammasomes control
140 *Salmonella* in human macrophages and restrict the distinct intracellular spatial niches
141 that *Salmonella* occupies in these cells.
142
143 Results
144 Caspase-1 promotes the control of *Salmonella* replication within human macrophages
145 Human macrophages undergo NAIP/NLRC4- and NLRP3-dependent
146 inflammasome activation during *Salmonella* infection (Bierschenk et al., 2019; Gram et
147 al., 2021; Naseer, Egan, et al., 2022), which restricts intracellular *Salmonella* replication
148 (Naseer, Egan, et al., 2022). Nonetheless, how inflammasome activation controls
149 *Salmonella* replication in human macrophages remains unclear. Inflammasomes recruit
150 and activate caspase-1 in both murine and human cells (Franchi et al., 2006; Man,
151 Hopkins, et al., 2014; Mariathasan et al., 2004; E. A. Miao et al., 2006; Ross et al.,
152 2022; Zamboni et al., 2006), and caspase-1 promotes the control of *Salmonella* both *in*
153 *vivo* and *in vitro* (Broz et al., 2010, 2012; Crowley et al., 2020; Hausmann et al., 2020;
154 Holly et al., 2020; Lara-Tejero et al., 2006; E. A. Miao, Leaf, et al., 2010; Rauch et al.,
155 2017; Raupach et al., 2006; Sellin et al., 2014; Thurston et al., 2016). Thus, we sought
156 to test whether caspase-1 restricts *Salmonella* replication specifically in human
157 macrophages.
158 To first interrogate the impact of caspase activity on *Salmonella* replication in
159 human macrophages, we primed macrophages derived from the human monocytic cell
160 line, THP-1, with the TLR1/2 agonist Pam3CSK4 to upregulate the expression of
161 inflammasome components. Then, we pretreated the cells with Ac-YVAD-cmk (YVAD),

162 a chemical inhibitor of caspase-1 activity, or Z-VAD-FMK (ZVAD), a pan-caspase
163 inhibitor. Upon infection with wild-type (WT) *Salmonella*, WT THP-1 cells pretreated with
164 either YVAD or ZVAD had significantly reduced levels of IL-1β release and cell death
165 compared to infected WT cells pretreated with the vehicle control (Figure 1 – figure
166 supplement 1A-B). Since pretreatment with YVAD largely phenocopies ZVAD, these
167 data suggest that caspase-1 is the primary caspase that responds to *Salmonella*
168 infection in THP-1 cells. We next assessed intracellular *Salmonella* burdens by
169 determining bacterial colony forming units (CFUs). The increase in bacterial CFUs was
170 significantly higher in WT cells pretreated either YVAD or ZVAD compared to cells
171 pretreated with DMSO (Figure 1 – figure supplement 1C). Microscopic analysis revealed
172 that WT cells treated with either YVAD or ZVAD prior to infection harbored significantly
173 higher intracellular *Salmonella* burdens compared to cells pretreated with DMSO
174 (Figure 1 – figure supplement 1D). Overall, these results suggest that caspase-1 activity
175 primarily controls *Salmonella* burdens in human macrophages.
176 Next, to genetically test the requirement of caspase-1, we used two independent
177 *CASP1*$^{-/-}$ THP-1 single cell clones generated through CRISPR/Cas9-mediated deletion
178 (Okondo et al., 2017). In agreement with previous reports (Bierschenk et al., 2019;
179 Gram et al., 2021; Naseer, Egan, et al., 2022), WT THP-1 cells primed with Pam3CSK4
180 and then infected with WT *Salmonella* exhibited high levels of IL-1β, IL-18, and IL-1α
181 release as well as cell death at 6 hpi (Figure 1A-B; Figure 1 – figure supplement 2A-B).
182 In contrast, Pam3CSK4-primed *CASP1*$^{-/-}$ THP-1 cells released negligible levels of
183 inflammasome-dependent cytokines and did not undergo substantial cell death upon
184 infection (Figure 1A-B; Figure 1 – figure supplement 2A-B). Infected WT and *CASP1*$^{-/-}$

185 THP-1 cells released similar levels of the inflammasome-independent cytokine TNF-α
186 (Figure 1 – figure supplement 2C). These results indicate that caspase-1 is required for
187 inflammasome responses to *Salmonella* infection in human macrophages.

188 We next tested whether caspase-1 is required to restrict *Salmonella* replication in
189 human macrophages. At 1 hour post-infection (hpi), we did not observe any significant
190 differences in bacterial uptake between Pam3CSK4-primed WT and *CASP1*$^{-/-}$ THP-1
191 cells (Figure 1 – figure supplement 2D). However, at 6 hpi, *CASP1*$^{-/-}$ cells harbored
192 significantly higher bacterial burdens and a significant fold-increase in bacterial CFUs
193 compared to WT cells (Figure 1C; Figure 1 – figure supplement 2E). To assess
194 intracellular bacterial burdens on a single-cell level, we quantified the number of WT
195 *Salmonella* per cell by microscopy. WT cells contained relatively low numbers of
196 *Salmonella* on average (~5 bacteria per cell), while *CASP1*$^{-/-}$ cells harbored significantly
197 higher numbers of *Salmonella* on average (~30 bacteria per cell) at 6 hpi (Figure 1D-E).
198 Overall, these data indicate that caspase-1 limits intracellular *Salmonella* replication in
199 human macrophages.

200 We next examined whether caspase-1-mediated control of *Salmonella* replication
201 in human macrophages requires TLR1/2 priming. At 1 hpi, we did not observe any
202 significant differences in bacterial uptake between unprimed WT and *CASP1*$^{-/-}$ THP-1
203 cells (Figure 1 – figure supplement 3A). However, at 6 hpi, unprimed *CASP1*$^{-/-}$ cells
204 harbored significantly higher bacterial burdens and a significant fold-increase in
205 bacterial CFUs compared to unprimed WT cells (Figure 1 – figure supplement 3B-C).
206 Since unprimed conditions phenocopy TLR1/2-primed conditions (Figure 1C; Figure 1 –

207 figure supplement 2D-E), these data indicate that caspase-1 promotes the restriction of
208 *Salmonella* replication within human macrophages independent of TLR1/2 priming.
209 In the previous experiments, we infected THP-1 cells with *Salmonella* grown
210 under SPI-1-inducing conditions. So, we subsequently sought to determine whether
211 *Salmonella* grown to stationary phase similarly replicated in human macrophages. At 1
212 hpi and at 6 hpi, we did not observe any significant differences in bacterial uptake
213 between WT and *CASP1*$^{-/-}$ THP-1 cells infected with stationary phase *Salmonella*
214 (Figure 1 – figure supplement 4A-B). Strikingly, we did not observe any fold-change in
215 the bacterial CFUs in both WT and *CASP1*$^{-/-}$ THP-1 cells (Figure 1 – figure supplement
216 4C). These data indicate that by 6 hours post-infection, *Salmonella* do not replicate
217 efficiently in human macrophages unless grown under SPI-1-inducing conditions (Figure
218 1C; Figure 1 – figure supplement 2D-E).
219 Next, we sought to address the possibility that the caspase-1-mediated control of
220 *Salmonella* replication we observed was due to an experimental artifact of gentamicin
221 entering WT macrophages through caspase-1-dependent gasdermin D pores and
222 subsequently killing intracellular bacteria. At 30 minutes post-infection, THP-1 cells were
223 treated with a lower dose of 25 μg/ml of gentamicin to kill extracellular bacteria. At 1
224 hour post-infection (hpi), the cells were washed to remove the gentamicin, and then the
225 media was replaced with fresh media containing no gentamicin. Alternatively, we
226 treated cells with our standard bactericidal concentration of gentamicin (100 μg/ml),
227 washed the cells to remove the gentamicin, and then replaced the media with fresh
228 media containing a bacteriostatic concentration of gentamicin (10 μg/ml). At 1 hpi, we
229 did not observe any significant differences in bacterial uptake between WT and *CASP1*$^{-}$

230 $^{/-}$ THP-1 cells treated with 25 µg/ml of gentamicin or treated with 100 µg/ml of
231 gentamicin (Figure 1 – figure supplement 5A). However, at 6 hpi, regardless of the
232 presence of absence of gentamicin, *CASP1*$^{-/-}$ cells harbored significantly higher
233 bacterial burdens and a significant fold-increase in bacterial CFUs compared to WT
234 cells (Figure 1 – figure supplement 5B-C). Altogether, these data indicate that
235 gentamicin does not contribute to tcaspase-1-mediated restriction of *Salmonella*
236 replication in human macrophages.
237
238 <u>Caspase-4 contributes to the control of *Salmonella* replication within human</u>
239 <u>macrophages later during infection</u>
240 Caspase-4 contributes to inflammasome responses during *Salmonella* infection
241 of THP-1 macrophages and primary human macrophages (Casson et al., 2015; Naseer,
242 Egan, et al., 2022), and caspases-4/5 are required for inflammasome responses to
243 *Salmonella* in THP-1 monocytes (Baker et al., 2015). In human intestinal epithelial cells
244 (IECs), caspase-4 drives inflammasome responses during *Salmonella* infection and
245 limits intracellular bacterial replication (Holly et al., 2020; Knodler, Crowley, et al., 2014;
246 Naseer, Zhang, et al., 2022). However, whether caspase-4 contributes to restriction of
247 intracellular *Salmonella* replication within human macrophages is unclear.
248 To genetically test the contribution of caspase-4 during *Salmonella* infection in
249 THP-1 macrophages, we used CRISPR/Cas9 to disrupt the *CASP4* gene. We selected
250 and sequence-validated two independent *CASP4*$^{-/-}$ THP-1 single cell clones (Figure 2 –
251 figure supplement 1). We observed a slight decrease in secreted IL-1β levels in *CASP4*$^{-}$
252 $^{/-}$ THP-1 macrophages infected with WT *Salmonella* compared to infected WT THP-1

253 macrophages at 6 hpi (Figure 2 – figure supplement 2A). We also observed a slight
254 decrease in cell death at 6 hpi in infected *CASP4*$^{-/-}$ clone #2 compared to infected WT
255 cells, whereas cytotoxicity levels were not significantly affected in infected *CASP4*$^{-/-}$
256 clone #6 (Figure 2 – figure supplement 2B). However, at 24 hpi, we observed a
257 significant decrease in IL-1 release and cell death in both *CASP4*$^{-/-}$ clones infected with
258 WT *Salmonella* compared to infected WT cells (Figure 2A-B; Figure 2 – figure
259 supplement 3A-B). WT and *CASP4*$^{-/-}$ cells also released similar levels of the
260 inflammasome-independent cytokine TNF-α upon infection with WT *Salmonella* at 24
261 hpi (Figure 2 – figure supplement 3C). Overall, these data indicate that caspase-4
262 contributes to inflammasome responses in THP-1 macrophages later during *Salmonella*
263 infection.

264 Since caspase-4 restricts *Salmonella* replication in human epithelial cells (Holly
265 et al., 2020; Knodler, Crowley, et al., 2014; Naseer, Zhang, et al., 2022), we asked
266 whether caspase-4 contributes to the control of *Salmonella* replication in human
267 macrophages as well. Upon examination of the fold-change in bacterial CFUs at 6 hpi,
268 we did not observe any significant differences between WT and *CASP4*$^{-/-}$ THP-1 cells
269 (Figure 2 – figure supplement 2C). However, at 24 hpi, *CASP4*$^{-/-}$ cells harbored higher
270 bacterial burdens and a significant fold-increase in bacterial CFUs compared to WT
271 cells (Figure 2C; Figure 2 – figure supplement 3D). To confirm these findings, we
272 enumerated the amount of WT *Salmonella* per cell by microscopy. At 6 hpi, WT and
273 *CASP4*$^{-/-}$ THP-1 cells contained comparable numbers of *Salmonella* per cell (Figure 2 –
274 figure supplement 2D-E). In contrast, at 24 hpi, *CASP4*$^{-/-}$ cells harbored significantly
275 higher burdens of *Salmonella* per cell on average (~20 bacteria per cell) compared to

276 WT cells (~10 bacteria per cell) (Figure 2D-E). Altogether, these results suggest that
277 caspase-4 plays a larger role in controlling *Salmonella* burdens later during infection.
278
279 GSDMD promotes the control of *Salmonella* replication within human macrophages
280 Inflammasome activation triggers a lytic form of cell death known as pyroptosis
281 (Lamkanfi & Dixit, 2014). Death of the infected host cell eliminates *Salmonella*'s
282 intracellular replicative niche and thus, may contribute to restricting intracellular bacterial
283 replication. Upon its cleavage by inflammatory caspases, GSDMD forms pores in the
284 host plasma membrane, resulting in pyroptosis (Ding et al., 2016; Kayagaki et al., 2015;
285 Shi et al., 2015). Whether GSDMD contributes to the control of *Salmonella* replication in
286 human macrophages is unknown.
287 First, we asked whether GSDMD pore formation plays a role in controlling
288 *Salmonella* replication in human macrophages. To do this, we pretreated THP-1
289 macrophages and primary human monocyte-derived macrophages (hMDMs) with the
290 chemical inhibitor disulfiram. Disulfiram prevents cleaved GSDMD from inserting into
291 the host plasma membrane, thereby limiting GSDMD-mediated pore formation (J. J. Hu
292 et al., 2020). Disulfiram treatment led to the loss of IL-1β release and cytotoxicity in
293 macrophages infected with WT *Salmonella*, compared to treatment with the vehicle
294 control, DMSO (Figure 3 – figure supplement 1A-B, D-E). Next, we examined the effect
295 of GSDMD-mediated pore formation on intracellular *Salmonella* burdens. The fold-
296 increase in bacterial CFUs at 6 hpi was significantly higher in cells treated with
297 disulfiram compared to cells treated with DMSO (Figure 3 – figure supplement 1C, F).

298 Collectively, these results suggest that GSDMD-mediated pore formation promotes the
299 restriction of intracellular *Salmonella* replication.

300 Consistent with our findings with disulfiram, we observed a significant decrease
301 in IL-1 and IL-18 release in *GSDMD*$^{-/-}$ THP-1 macrophages (Okondo et al., 2017;
302 Taabazuing et al., 2017) compared to WT THP-1 macrophages following *Salmonella*
303 infection (Figure 3A; Figure 3 – figure supplement 2A-B). Interestingly, loss of GSDMD
304 did not completely abrogate the release of IL-1 and IL-18, suggesting that there is also
305 GSDMD-independent IL-1 and IL-18 release (Figure 3A; Figure 3 – figure supplement
306 2A-B). Infected WT and *GSDMD*$^{-/-}$ THP-1 cells secreted similar levels of the
307 inflammasome-independent cytokine TNF-α (Figure 3 – figure supplement 2C).
308 Importantly, infected *GSDMD*$^{-/-}$ cells failed to undergo substantial cell death, in contrast
309 to infected WT cells, indicating that GSDMD facilitates cell death during *Salmonella*
310 infection (Figure 3B). Overall, these results indicate that GSDMD plays an important
311 role in mediating inflammasome responses during *Salmonella* infection of human
312 macrophages.

313 We next examined whether GSDMD controls intracellular *Salmonella* replication
314 in human macrophages. Notably, while there were no differences in bacterial uptake
315 between WT and *GSDMD*$^{-/-}$ cells at 1 hpi (Figure 3 – figure supplement 2D), we did
316 observe significantly higher bacterial CFUs in *GSDMD*$^{-/-}$ cells compared to WT cells at 6
317 hpi (Figure 3 – figure supplement 2E). Moreover, the fold-increase in bacterial CFUs at
318 6 hpi was significantly higher in *GSDMD*$^{-/-}$ cells than in WT cells (Figure 3C). Next, we
319 used microscopy to further interrogate intracellular *Salmonella* burdens. As expected,
320 WT cells contained low numbers of *Salmonella* per cell (~5 bacteria per cell) (Figure 3D-

321 E). However, *GSDMD*$^{-/-}$ cells harbored significantly higher numbers of *Salmonella* per
322 cell on average (~20 bacteria per cell) at 6 hpi (Figure 3D-E). Altogether, these data
323 indicate that GSDMD promotes the control of *Salmonella* replication in human
324 macrophages.
325
326 NINJ1 contributes to the control of *Salmonella* replication within human macrophages
327 While GSDMD pores release a subset of molecules, including IL-1 family
328 cytokines, the extensive release of cellular contents is mediated by plasma membrane
329 rupture (Ding et al., 2016; Ruan et al., 2018). Recently, NINJ1 was identified as an
330 executioner of terminal cell lysis (Bjanes et al., 2021; Borges et al., 2022; Degen et al.,
331 2023; Kayagaki et al., 2021, 2023). NINJ1 is a transmembrane protein that oligomerizes
332 in the host plasma membrane to induce lytic rupture of the cell after the initiation of
333 pyroptosis and other forms of regulated cell death (David et al., 2024; Degen et al.,
334 2023; Kayagaki et al., 2021). Cells can be protected from plasma membrane rupture
335 through treatment with the amino acid glycine (Fink & Cookson, 2006; Frank et al.,
336 2000; Heilig et al., 2018; Verhoef et al., 2005), which interferes with the clustering of
337 NINJ1 (Borges et al., 2022). Thus, upon incubation with glycine, cells can still form
338 GSDMD pores but cannot undergo terminal cell lysis (Fink & Cookson, 2006; Heilig et
339 al., 2018; Tsuchiya et al., 2021; Verhoef et al., 2005). Whether NINJ1-dependent cell
340 lysis contributes to control of *Salmonella* replication in human macrophages remains
341 unknown.
342 First, to determine whether glycine exerts a cytoprotective effect on THP-1
343 macrophages, we pretreated WT and *NAIP*$^{-/-}$ THP-1 cells with glycine prior to infection

344 with WT *Salmonella* and assayed for downstream inflammasome responses. Notably,
345 WT and *NAIP*$^{-/-}$ cells pretreated with glycine exhibited significantly decreased cell death
346 and a small defect in IL-1β release following infection compared to infected cells treated
347 with the vehicle control (Figure 4 – figure supplement 1A-B). Release of the
348 inflammasome-independent cytokine TNF-α was unaffected by glycine treatment
349 (Figure 4 – figure supplement 1C). Altogether, these results indicate that glycine
350 prevents cell lysis and limits IL-1 release in THP-1 macrophages upon *Salmonella*
351 infection.

352 Given glycine's cytoprotective effect on infected THP-1 macrophages, we next
353 sought to determine whether glycine impacts intracellular *Salmonella* burdens in human
354 macrophages. THP-1 macrophages pretreated with glycine retained significantly higher
355 intracellular bacterial burdens at 6 hpi compared to cells given the vehicle control
356 (Figure 4 – figure supplement 1D). We did not detect any significant differences in
357 bacterial uptake (Figure 4 – figure supplement 1E). Moreover, WT and *NAIP*$^{-/-}$ THP-1
358 cells pretreated with glycine exhibited a greater increase in *Salmonella* CFUs compared
359 to cells treated with the vehicle control (Figure 4 – figure supplement 1F), suggesting
360 that cytoprotection by glycine interferes with the intracellular control of *Salmonella* in
361 human macrophages.

362 Next, to genetically test the role of NINJ1, we transfected WT THP-1
363 macrophages with small interfering RNA (siRNA) targeting *NINJ1*, which led to robust
364 knockdown of *NINJ1* ranging from 77% to 85%, or control scrambled siRNA.
365 Knockdown of *NINJ1* resulted in a significant decrease, yet not complete abrogation, of
366 IL-1β secretion and cytotoxicity in infected cells, in contrast to control siRNA treatment

367 (Figure 4A-B). Collectively, these data imply a critical role for NINJ1 in contributing to IL-
368 1 release and cell death during *Salmonella* infection in human macrophages.
369 Notably, while *NINJ1* knockdown did not affect bacterial uptake at 1 hpi (Figure
370 4C), cells treated with *NINJ1* siRNA contained higher intracellular bacterial burdens
371 than cells treated with control siRNA at 6 hpi (Figure 4D). Moreover, there was a greater
372 fold-increase in bacterial CFUs at 6 hpi in *NINJ1* siRNA-treated cells compared to
373 control siRNA-treated cells (Figure 4E). Together, these findings indicate that NINJ1
374 contributes to intracellular bacterial control.
375
376 Inflammasome activation primarily controls cytosolic *Salmonella* replication in human
377 macrophages
378 In epithelial cells, WT *Salmonella* can enter the cytosol and replicate in both
379 vacuolar and cytosolic compartments, specifically hyperreplicating in the cytosol
380 (Knodler, Crowley, et al., 2014; Knodler et al., 2010; Knodler, Nair, et al., 2014; Malik-
381 Kale et al., 2012). However, in murine macrophages, WT *Salmonella* appears to
382 replicate exclusively in vacuoles (Beuzón et al., 2002; Thurston et al., 2016). *Salmonella*
383 lacking the SPI-2 effector SifA (Δ*sifA*), which is required for SCV membrane stability,
384 frequently enter the cytosol and can replicate within epithelial cells, but cannot replicate
385 efficiently in murine macrophages (Beuzón et al., 2000, 2002; Brumell, Tang, et al.,
386 2002; Thurston et al., 2016). Whether *Salmonella* replicates within vacuoles or the
387 cytosol of human macrophages remains largely unknown. One study suggested that a
388 small but significant proportion of *Salmonella* are exposed to the cytosol in THP-1
389 macrophages (Fisch et al., 2020). Our recently published data and current findings

390 indicate that when human macrophages fail to undergo robust inflammasome
391 responses, *Salmonella* hyperreplicates to large numbers within these cells (Naseer,
392 Egan, et al., 2022). The hyperreplication that we observed in human macrophages was
393 reminiscent of previous reports describing *Salmonella* hyperreplication within the cytosol
394 of IECs (Knodler, Crowley, et al., 2014; Knodler et al., 2010; Knodler, Nair, et al., 2014;
395 Malik-Kale et al., 2012). Thus, we hypothesized that inflammasome activation limits
396 *Salmonella* replication in both SCVs and the host cell cytosol, and that in the absence of
397 inflammasome responses, *Salmonella* hyperreplicates to large numbers within the host
398 cell cytosol and also exhibits increased replication within SCVs.

399 First, to test whether inflammasome activation restricts the ability of *Salmonella*
400 to replicate in vacuolar and cytosolic compartments of human macrophages, we used a
401 chloroquine (CHQ) resistance assay. As a weak base, CHQ accumulates in endosomal
402 compartments, including the SCV, without entering the cytosol of host cells (Klein,
403 Powers, et al., 2017; Knodler, Nair, et al., 2014; Steinberg, 1994). Thus, vacuolar
404 bacteria are CHQ-sensitive, while cytosolic bacteria are CHQ-resistant (Knodler, Nair, et
405 al., 2014). We infected THP-1 macrophages with WT *Salmonella* and treated the
406 infected cells with CHQ. Then, we determined the bacterial CFUs at 2 hpi and 6 hpi,
407 quantifying the numbers of vacuolar bacteria (CHQ-sensitive) and cytosolic bacteria
408 (CHQ-resistant). At 2 hpi, we did not observe any significant differences in vacuolar or
409 cytosolic *Salmonella* between WT and *CASP1*$^{-/-}$ THP-1 cells (Figure 5A). We found that
410 WT THP-1 cells contained mostly vacuolar *Salmonella* while also retaining some
411 cytosolic *Salmonella* at 6 hpi (Figure 5B), suggesting that a subset of *Salmonella* dwells
412 in the cytosol of THP-1 macrophages, in agreement with a previous study (Fisch et al.,

413 2020). In *NAIP*$^{-/-}$ and *CASP1*$^{-/-}$ THP-1 cells, we observed slightly higher burdens of
414 vacuolar *Salmonella* compared to WT cells (Figure 5B). Strikingly, we also observed
415 significantly larger numbers of cytosolic *Salmonella* in *NAIP*$^{-/-}$ and *CASP1*$^{-/-}$ cells
416 compared to WT cells (Figure 5B). These data indicate that inflammasome signaling
417 restricts *Salmonella* primarily within the host cell cytosol but also within SCVs in human
418 macrophages.

419 The SPI-1 T3SS can damage the SCV (Roy et al., 2004), and the SPI-1 T3SS
420 and its effectors contribute to *Salmonella* escape from the SCV and replication in the
421 cytosol of epithelial cells (Chong et al., 2019; Klein, Grenz, et al., 2017; Knodler, Nair, et
422 al., 2014; Röder & Hensel, 2020). So, we next assessed whether the SPI-1 T3SS was
423 required for the cytosolic exposure of *Salmonella* in human macrophages. We infected
424 WT and *CASP1*$^{-/-}$ THP-1 macrophages with *Salmonella* lacking the SPI-1 T3SS
425 translocon protein SipB (Δ*sipB*), thus preventing SPI-1 T3SS effector translocation into
426 host cells. While we observed a significant increase in the amount of vacuolar and
427 cytosolic Δ*sipB Salmonella* in *CASP1*$^{-/-}$ cells compared to WT cells at 6 hpi, the
428 numbers of cytosolic Δ*sipB* were significantly lower than vacuolar Δ*sipB* in both WT and
429 *CASP1*$^{-/-}$ cells (Figure 5C). We also observed lower cytosolic Δ*sipB Salmonella* burdens
430 compared to WT *Salmonella* in both WT and *CASP1*$^{-/-}$ THP-1 cells at 6 hpi (Figure 5B-
431 C). Therefore, these results suggest that the SPI-1 T3SS contributes to *Salmonella*'s
432 cytosolic access in human macrophages.

433 Since CFU assays are population-based, we next used single-cell microscopy-
434 based methods to interrogate the subcellular localization of WT *Salmonella* in human
435 macrophages. We employed a strain of WT *Salmonella* that constitutively expresses

436 mCherry and maintains a reporter plasmid, pNF101, that expresses *gfp-ova* under the
437 control of a promoter responsive to the host cytosolic metabolite glucose-6-phosphate
438 (Lau et al., 2019; Spinnenhirn et al., 2014). We scored the number of GFP-
439 positive/mCherry-positive bacteria (cytosolic) and GFP-negative/mCherry-positive
440 bacteria (vacuolar) in WT, *NAIP*$^{-/-}$ and *CASP1*$^{-/-}$ THP-1 macrophages at 8 hpi by
441 microscopy. We observed low numbers of both vacuolar and cytosolic populations of
442 *Salmonella* in WT THP-1 cells (Figure 5D-E). In contrast, *NAIP*$^{-/-}$ and *CASP1*$^{-/-}$ THP-1
443 cells maintained significantly larger numbers of vacuolar *Salmonella*, with a substantial
444 increase in the numbers of cytosolic *Salmonella* (Figure 5D-E). Taken together, these
445 results suggest that inflammasome responses primarily restrict *Salmonella* replication
446 within the host cell cytosol and also control bacterial replication within SCVs in human
447 macrophages.

448 We next asked whether inflammasome responses also restrict the replication of
449 *Salmonella* within the cytosol and SCV in primary human monocyte-derived
450 macrophages (hMDMs). We pretreated hMDMs with either ZVAD, to inhibit
451 inflammasome responses mediated by caspase activity, or the vehicle control DMSO,
452 and then infected the cells with WT *Salmonella* constitutively expressing mCherry and
453 harboring pNF101. We observed that hMDMs treated with ZVAD contained significantly
454 more cytosolic *Salmonella* than hMDMs treated with DMSO at 8 hpi (Figure 5 – figure
455 supplement 1A-B). We observed no significant differences between the vacuolar
456 burdens of *Salmonella* in hMDMs pretreated with ZVAD or DMSO (Figure 5 – figure
457 supplement 1A-B). Thus, inflammasome responses appear to primarily control
458 *Salmonella* replication within the cytosol of primary human macrophages.

459

460 Inflammasome activation modulates the cytosolic exposure of *Salmonella* in human
461 macrophages

462 Finally, to characterize the effect of inflammasomes on the subcellular niches of
463 *Salmonella* in human macrophages at higher resolution, we used transmission electron
464 microscopy (TEM). In WT THP-1 cells, TEM analysis revealed that the majority of
465 bacteria resided in a membrane-bound compartment (vacuolar, white arrows) (Figure
466 6A; Figure 6 – figure supplement 1B). In *CASP1*$^{-/-}$ THP-1 cells, we observed a more
467 mixed population of vacuolar bacteria and bacteria that were exposed to the host cell
468 cytosol to varying extents (Figure 6B; Figure 6 – figure supplement 1B). We observed
469 *Salmonella* free-living in the cytosol without a vacuolar membrane (fully cytosolic, black
470 asterisk), as well as *Salmonella* exposed to the cytosol within discontinuous vacuolar
471 membranes (partially cytosolic, cyan arrows) (Figure 6B; Figure 6 – figure supplement
472 1B). We observed a greater proportion of cytosol-exposed *Salmonella* in *CASP1*$^{-/-}$ cells
473 compared to WT cells (Figure 6 – figure supplement 1A). Strikingly, these TEM results
474 revealed distinct intracellular populations of *Salmonella* in human macrophages,
475 providing further evidence that inflammasome signaling controls the replicative niches
476 that *Salmonella* occupies in human macrophages.

477 We next sought to further characterize the cytosolic exposure of *Salmonella* in
478 *CASP1*$^{-/-}$ THP-1 cells using electron tomography (ET), a technique that can reveal three-
479 dimensional (3D) detail about SCV membranes. Indeed, we confirmed the presence of
480 *Salmonella* in SCVs with varying degrees of vacuolar membrane discontinuities,
481 yielding various extents of cytosolic exposure (Figure 6C; Figure 6 – figure supplement

482 1C; Video 1). These discontinuities were often numerous and found in clusters.
483 Altogether, our TEM and ET data reveal the full spectrum of cytosolic exposure for
484 *Salmonella* in *CASP1*$^{-/-}$ cells as a result of vacuolar membrane discontinuities. Overall,
485 our findings indicate that inflammasome responses modulate the subcellular
486 populations of *Salmonella*, thereby controlling the number of *Salmonella* able to
487 replicate within the cytosol and SCV in human macrophages.
488
489 Discussion
490 Our data reveal that inflammatory caspases and downstream cell lysis mediators
491 are required to restrict *Salmonella* replication in human macrophages. Furthermore, our
492 findings indicate that inflammasomes restrict *Salmonella* hyperreplication within the
493 cytosol of human macrophages. Caspase-1 is required for inflammasome responses
494 and control of intracellular *Salmonella* replication early during infection. In contrast,
495 caspase-4 contributed minimally early during infection and instead played a larger role
496 in inflammasome responses and restriction of *Salmonella* replication at later timepoints.
497 We also found that GSDMD and NINJ1 were required for cell death, IL-1 cytokine
498 release, and control of *Salmonella* replication. Finally, in the absence of these
499 inflammasome components and effectors in human macrophages, we observed a
500 hyperreplicating population of *Salmonella* within the cytosol, as well as increased
501 bacterial loads within the SCV.
502 There are multiple downstream consequences of inflammasome activation,
503 including pyroptosis, cytokine secretion, phagolysosomal fusion, the formation of pore-
504 induced intracellular traps (PITs), and the generation of reactive oxygen species (ROS),

505 which could be singly or collectively responsible for the control of intracellular
506 *Salmonella* replication in human macrophages. Previous studies reported that
507 inflammasome activation and inflammatory caspases limit intracellular replication of WT
508 *Salmonella* in human epithelial cells and murine macrophages through ROS, actin
509 polymerization, and host cell death (Aachoui et al., 2013; Holly et al., 2020; Knodler,
510 Crowley, et al., 2014; Man, Ekpenyong, et al., 2014). Interestingly, in murine
511 macrophages, the restriction of a mutant strain of *Salmonella* that frequently enters the
512 cytosol is dependent on caspase-1/11 but independent of IL-1 signaling and host cell
513 death (Thurston et al., 2016). Moreover, caspase-1/11-dependent production of
514 mitochondrial ROS and hydrogen peroxide contributes to the control of WT *Salmonella*
515 replication in SCVs in murine macrophages (Man, Ekpenyong, et al., 2014). Future
516 studies investigating the downstream consequences of inflammasome activation will
517 help elucidate how inflammatory caspases and cell lysis mediators control intracellular
518 *Salmonella* replication in human macrophages.
519 Whereas caspase-1 is the primary caspase that mediates inflammasome
520 responses and control of *Salmonella* burdens in human macrophages, our data indicate
521 that caspase-4 also plays a role at a later stage of infection. Whether caspase-4 is
522 activated by vacuolar *Salmonella* or hyperreplicating cytosolic *Salmonella* remains an
523 open question. Other host immune factors may also contribute to caspase-4-dependent
524 inflammasome responses to *Salmonella*. For example, guanylate binding proteins
525 (GBPs) can modulate inflammasome responses to intracellular LPS and impact
526 intracellular bacterial replication (Degrandi et al., 2007; Fisch et al., 2020; Kim et al.,

527 2011; Kutsch et al., 2020; Meunier et al., 2014; Pilla et al., 2014; J. C. Santos et al.,
528 2020; Tietzel et al., 2009; Wandel et al., 2020).

529 The impact of GSDMD on the restriction of intracellular *Salmonella* replication
530 may be due to downstream responses, such as IL-1 release and pyroptosis. Moreover,
531 pyroptosis can induce PIT formation, which traps and damages intracellular bacteria,
532 rendering them more susceptible to immune defenses, such as neutrophil-mediated
533 killing (Jorgensen et al., 2016). It is possible that pyroptosis leads to the development of
534 PITs in human macrophages during *Salmonella* infection, thereby limiting *Salmonella*'s
535 intracellular replication. In addition, the cleaved N-terminal fragment of GSDMD can
536 directly kill bacteria (Ding et al., 2016; Liu et al., 2016; Wang et al., 2019), raising the
537 possibility that GSDMD directly targets cytosolic *Salmonella* to mediate restriction. A
538 recent study revealed that GSDMD binds and permeabilizes mitochondrial membranes
539 to mediate pyroptosis (R. Miao et al., 2023). Still, it remains unknown whether GSDMD
540 binds or permeabilizes the SCV, thereby affecting vacuolar integrity and influencing
541 intracellular *Salmonella* replication.

542 Our data indicate that inflammasome responses primarily control *Salmonella*
543 replication within the cytosol of human macrophages and also within SCVs. How
544 inflammasome activation inhibits *Salmonella* replication in the cytosol of human
545 macrophages remains unknown. *Salmonella* hyperreplicates in the cytosol of human
546 epithelial cells, which are unable to undergo caspase-1-dependent inflammasome
547 responses (Holly et al., 2020; Knodler, Crowley, et al., 2014; Knodler et al., 2010;
548 Knodler, Nair, et al., 2014; Naseer, Zhang, et al., 2022). Our study suggests that
549 *CASP1*$^{-/-}$ THP-1 macrophages behave similarly to human IECs, as they support

550 *Salmonella* hyperreplication in the cytosol in the absence of caspase-1. Inflammasome
551 activation could curtail cytosolic replication of *Salmonella* through host cell death, direct
552 GSDMD targeting of cytosolic *Salmonella,* and/or another effector mechanism.
553 Alternatively, inflammasome activation could regulate the cytosolic access of
554 *Salmonella* through host factors that directly damage the SCV, such as pore-forming
555 proteins like GSDMD.

556 The bacterial factors that facilitate *Salmonella*'s cytosolic lifestyle in human
557 macrophages remain largely unknown. Our findings indicate that the SPI-1 T3SS is
558 partially required for *Salmonella* to access the cytosol in THP-1 macrophages. In
559 epithelial cells, the SPI-1 T3SS SopE, SopB, and SipA enable *Salmonella* to escape the
560 SCV and efficiently colonize and replicate in the cytosol (Chong et al., 2019; Klein,
561 Grenz, et al., 2017; Röder & Hensel, 2020). Future studies will elucidate whether these
562 same effectors also support *Salmonella*'s escape into the cytosol and cytosolic
563 replication in human macrophages.

564 Our data also indicate that there is considerable single-cell heterogeneity in
565 terms of total intracellular bacterial burdens as well as proportions of vacuolar and
566 cytosolic *Salmonella* in human macrophages using single-cell microscopic analysis.
567 Several factors could account for this phenotypic heterogeneity, including expression
568 levels of inflammasome components, the amount of translocated *Salmonella* ligands, or
569 the extent of inflammasome activation, all of which might differ in individual cells and are
570 masked in bulk-population assays. Notably, heterogeneity in *Salmonella* gene
571 expression, intracellular *Salmonella* populations, and intracellular *Salmonella*
572 proliferation and viability has been previously observed in human epithelial cells and

573 murine macrophages (Helaine et al., 2010, 2014; Knodler, Nair, et al., 2014; Malik-Kale
574 et al., 2012; Powers et al., 2021).
575 In this study, we utilized tissue culture models to examine intracellular
576 *Salmonella* replication in human macrophages. These *in vitro* systems allow for precise
577 control of experimental conditions and, therefore, serve as powerful tools to interrogate
578 the molecular mechanisms underlying inflammasome responses and *Salmonella*
579 replication in both immortalized and primary human cells. Still, there are limitations of
580 tissue culture models, as they lack the inherent complexity of tissues and organs *in vivo*.
581 To assess whether our findings reflect *Salmonella* dynamics in the mammalian host, it
582 will be important to complement our studies and extend the implications of our work
583 using approaches that model more complex systems, such as organoids or organ
584 explant models co-cultured with immune cells, and *in vivo* techniques, such as
585 humanized mouse models.
586 Collectively, our results reveal that inflammasome responses restrict intracellular
587 *Salmonella* replication, particularly within the cytosol of human macrophages. These
588 findings are in contrast to mouse macrophages, where inflammasomes are activated
589 but only marginally restrict the intracellular replication of WT *Salmonella*. Our findings
590 provide insight into how human macrophages leverage inflammasomes to restrict
591 *Salmonella* intracellular replication. Moreover, our work offers a basis for future studies
592 to investigate how inflammasome activation modulates the subcellular localization of
593 bacterial replicative niches within host cells.
594
595 <u>Materials and Methods</u>

596 Ethics statement

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

604

605 Cell culture of THP-1 cells

606 THP-1 cells (TIB-202; American Type Culture Collection) were maintained in RPMI
607 supplemented with 10% (vol/vol) heat-inactivated FBS, 0.05 nM β-mercaptoethanol, 100
608 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified incubator. Two
609 days prior to experimentation, the cells were replated in media without antibiotics in a
610 48-well plate at a concentration of 2×10^5 cells/well or in a 24-well plate at a
611 concentration of 3.5×10^5 cells per well and incubated with phorbol 12-myristate 13-
612 acetate (PMA) for 24 hours to allow differentiation into macrophages. Then,
613 macrophages were either left unprimed or primed with 100 ng/mL Pam3CSK4
614 (Invivogen) for 16 to 20 hours prior to bacterial infections. For fluorescence microscopy
615 experiments, cells were plated on glass coverslips in a 24-well plate.

616

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

618 Purified human monocytes from deidentified healthy human donors were obtained from
619 the University of Pennsylvania Human Immunology Core. The monocytes were cultured
620 in RPMI supplemented with 10% (vol/vol) heat-inactivated FBS, 2 mM L-glutamine, 100
621 IU/mL penicillin, 100 µg/ml streptomycin, and 50 ng/ml recombinant human M-CSF
622 (Gemini Bio-Products). Cells were cultured for 4 days in 10 mL of media in at a
623 concentration of 4-5 × 10^5 cells/mL in 10 cm-dishes. Then, 10 mL of fresh growth media
624 was added for an additional 2 days to promote complete differentiation into
625 macrophages. One day prior to infection, the cells were rinsed with cold PBS, gently
626 detached with trypsin-EDTA (0.05%) and replated in media without antibiotics and with
627 25 ng/mL human M-CSF in a 48-well plate at a concentration of 1 × 10^5 cells per well or
628 in a 24-well plate at a concentration of 2 × 10^5 cells per well. For fluorescence
629 microscopy, cells were replated on glass coverslips in a 24-well plate. For experiments
630 involving LPS, cells were primed with 500 ng/mL LPS (Sigma-Aldrich) for 3 hours prior
631 to bacterial infections.
632
633 <u>Inhibitor experiments</u>
634 Human macrophages were treated 1 hour prior to infection at the indicated
635 concentrations with the following inhibitors: 20 µM of the pan-caspase inhibitor Z-
636 VAD(OMe)-FMK (SM Biochemicals; SMFMK001), 25 µM of the caspase-1 inhibitor Ac-
637 YVAD-cmk (Sigma-Aldrich; SML0429), and 40 µM of the GSDMD inhibitor disulfiram
638 (Sigma). DMSO treatment was used as a vehicle control with these inhibitors. To
639 prevent cell lysis, cells were treated with 20 mM glycine (Fisher Scientific) for 30
640 minutes prior to infection. Distilled water was used as a vehicle control.

641

642 Bacterial strains and growth conditions

643 *Salmonella enterica* serovar Typhimurium SL1344 WT was routinely grown shaking
644 overnight at 37°C in Luria-Bertani (LB) broth with streptomycin (100 µg/mL). For
645 infection of cultured cells, the overnight culture was diluted in LB with streptomycin
646 (100 µg/mL) containing 300 mM NaCl and grown standing for 3 hours at 37°C to induce
647 SPI-1 expression (Lee & Falkow, 1990), unless otherwise noted, when the overnight
648 culture of *Salmonella* was grown to stationary phase and subseuqnelty used to infect
649 cultured cells.

650

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

661

662 Bacterial infections

663 Overnight cultures of *Salmonella* were diluted into LB broth with streptomycin
664 (100 µg/mL) containing 300 mM NaCl and grown for 3 hours standing at 37°C to induce
665 SPI-1 gene expression (Lee & Falkow, 1990). Bacterial cultures were then pelleted at
666 6,010 × g for 3 minutes, washed once with PBS, and resuspended in PBS. Human
667 macrophages were infected with *Salmonella* at a multiplicity of infection (MOI) of 20.
668 Infected cells were centrifuged at 290 × g for 10 minutes and incubated at 37°C. At 30
669 minutes post-infection, the cells were treated with 100 µg/ml of gentamicin to kill any
670 extracellular *Salmonella*, unless otherwise noted when the cells were treated with 25
671 µg/ml of gentamicin. Then, the infection proceeded at 37°C for 6 to 8 hours, as
672 indicated. For all experiments, control cells were mock-infected with PBS.
673
674 Bacterial intracellular burden assay
675 Cells were infected with WT *Salmonella* as described above at an MOI of 20. Then, 30
676 minutes post-infection, cells were treated with 100 µg/ml of gentamicin to kill any
677 extracellular bacteria. 1 hour post-infection, the media was replaced with fresh media
678 containing 10 µg/ml of gentamicin or no gentamicin, as indicated. At the indicated time
679 points, the infected cells were lysed with PBS containing 0.5% Triton to collect all
680 intracellular *Salmonella*. Harvested bacteria were serially diluted in PBS and plated on
681 LB agar plates containing streptomycin (100 µg/ml) to enumerate colony forming units
682 (CFUs). Plates were incubated overnight at 37°C and CFUs were subsequently
683 counted.
684
685 Chloroquine (CHQ) Resistance Assay

686 THP-1 macrophages were infected in 48-well plates as described above. For each
687 timepoint, triplicate wells were incubated in the presence of CHQ (500 μM) and
688 gentamicin (100 ng/mL) for 1 hour to quantify the CHQ-resistant bacteria (Bârzu et al.,
689 1997; Fernandez et al., 2001; Klein, Powers, et al., 2017; Knodler, Nair, et al., 2014;
690 Zychlinsky et al., 1994). Another triplicate wells were incubated with gentamicin (100
691 ng/mL) only to quantify the total intracellular bacteria. At the indicated time points, the
692 infected cells were lysed with PBS containing 0.5% Triton to collect intracellular
693 *Salmonella*. Harvested bacteria were serially diluted in PBS and plated on LB agar
694 plates containing streptomycin (100 μg/ml) to enumerate colony forming units (CFUs).
695 Plates were incubated overnight at 37°C and CFUs were subsequently counted.
696
697 ELISAs
698 Harvested supernatants from infected human macrophages were assayed for cytokine
699 levels using ELISA kits for human IL-1α (R&D Systems), IL-18 (R&D Systems), IL-1β
700 (BD Biosciences), and TNF-α (R&D Systems).
701
702 LDH cytotoxicity assays
703 Harvested supernatants from infected human macrophages were assayed for
704 cytotoxicity by quantifying the loss of cellular membrane integrity via lactate
705 dehydrogenase (LDH) activity. LDH release was measured using an LDH Cytotoxicity
706 Detection Kit (Clontech) according to the manufacturer's instructions and normalized to
707 mock-infected cells.
708

709 siRNA-mediated knockdown of genes

710 All Silencer Select siRNA oligos were purchased from Ambion (Life Technologies).
711 Individual siRNA targeting *NINJ1* (ID# s9556) was used. The two Silencer Select
712 negative control siRNAs (Silencer Select Negative Control No. 1 siRNA and Silencer
713 Select Negative Control No. 2 siRNA) were used as a control. Three days prior to the
714 infection, 30 nM of siRNA was transfected into the human macrophages using
715 Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) following the
716 manufacturer's protocol. Then, 24 hours after transfection, the media was replaced with
717 fresh media containing antibiotics. Finally, 16 hours before infection, the media was
718 replaced with fresh antibiotic-free media containing 100 ng/ml Pam3CSK4.

719

720 Quantitative RT-PCR Analysis

721 RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) following the manufacturer's
722 protocol. Human macrophages were lysed in 350 μL RLT buffer with β-mercaptoethanol
723 and centrifuged through a QIAshredder spin column (Qiagen). cDNA was synthesized
724 from isolated RNA using SuperScript II Reverse Transcriptase (Invitrogen) following the
725 manufacturer's protocol. Quantitative PCR was conducted with the CFX96 real-time
726 system from Bio-Rad using the SsoFast EvaGreen Supermix with Low ROX (Bio-Rad).
727 To calculate knockdown efficiency, mRNA levels of siRNA-treated cells were
728 normalized to the housekeeping gene *HPRT* and control siRNA-treated cells using the
729 $2^{-\Delta\Delta CT}$ (cycle threshold) method (Livak & Schmittgen, 2001). The following primers from
730 PrimerBank were used. The PrimerBank identifications are *NINJ1* (148922910c1),
731 *CASP4* (73622124c2) and *HPRT* (164518913c1); all primers listed as 5′–3′:

732 *NINJ1* forward: TCAAGTACGACCTTAACAACCCG

733 *NINJ1* reverse: TGAAGATGTTGACTACCACGATG

734 *CASP4* forward: TCTGCGGAACTGTGCATGATG

735 *CASP4* reverse: TGTGTGATGAAGATAGAGCCCAT

736 *HPRT* forward: CCTGGCGTCGTGATTAGTGAT

737 *HPRT* reverse: AGACGTTCAGTCCTGTCCATAA

738

739 Immunoblot analysis

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

748

749 Fluorescent microscopy of intracellular *Salmonella*

750 Primary hMDMs or THP-1 cells were plated on glass coverslips in a 24-well plate as
751 described above. Cells were either infected with WT *Salmonella* constitutively
752 expressing GFP [SI1344 harboring pFPV25.1 (Valdivia & Falkow, 1996)], or WT
753 *Salmonella* constitutively expressing mCherry with a cytosolic GFP reporter [SL1344
754 *glmS::Ptrc-mCherryST::FRT* pNF101 (Lau et al., 2019)] at an MOI of 20 as described

755 above. At the indicated timepoints following infection, cells were washed 2 times with
756 PBS and then fixed with 4% paraformaldehyde for 10 minutes. Following fixation, cells
757 were mounted on glass slides with DAPI mounting medium (Sigma Fluoroshield).
758 Coverslips were imaged on an inverted fluorescence microscope (IX81; Olympus), and
759 the images were collected using a high-resolution charge-coupled-device camera
760 (FAST1394; QImaging) at a magnification of 100×. All images were analyzed and
761 presented using SlideBook (version 5.0) software (Intelligent Imaging Innovations, Inc.)
762 and ImageJ software. For experiments with WT *Salmonella* constitutively expressing
763 GFP, the proportion of infected cells containing GFP-expressing *Salmonella* (green)
764 were scored by counting 50 infected cells per coverslip. 150 total infected cells were
765 scored for each condition. For experiments with WT *Salmonella* constitutively
766 expressing mCherry with a cytosolic GFP reporter, the proportion of infected cells
767 containing GFP-positive *Salmonella* (cytosolic) and GFP-negative, mCherry-positive
768 *Salmonella* (vacuolar) were scored by counting 50 infected cells per coverslip. 150 total
769 infected cells were scored for each condition.
770
771 Transmission electron microscopy
772 Two days prior to experimentation, 2×10^6 cells THP-1 cells were replated in 10-cm
773 dishes in media without antibiotics and incubated with phorbol 12-myristate 13-acetate
774 (PMA) for 24 hours to allow differentiation into macrophages. Then, macrophages were
775 primed with 100 ng/mL Pam3CSK4 (Invivogen) for 16 hours prior to bacterial infection.
776 Cells were infected with WT *Salmonella* at an MOI of 20 as described above. At 8 hpi,
777 the media was aspirated, and the cells were fixed with 2.5% glutaraldehyde, 2.0%

778 paraformaldehyde in 0.1M sodium cacodylate buffer, pH 7.4. Then, at the Electron
779 Microscopy Resource Laboratory in the Perelman School of Medicine, after subsequent
780 buffer washes, the samples were post-fixed in 2.0% osmium tetroxide with 1.5%
781 K3Fe(CN)6 for 1 hour at room temperature, and rinsed in dH2O. After dehydration
782 through a graded ethanol series, the tissue was infiltrated and embedded in EMbed-812
783 (Electron Microscopy Sciences, Fort Washington, PA). Thin sections were stained with
784 uranyl acetate and SATO lead and examined with a JEOL 1010 electron microscope
785 fitted with a Hamamatsu digital camera and AMT Advantage NanoSprint500 software.
786

787 Electron tomography

788 Electron tomography was performed at room temperature on a ThermoFisher Krios G3i
789 TEM equipped with a 300 keV field emission gun. Imaging was performed using the
790 SerialEM software (Mastronarde, 2005) on a K3 direct electron detector (Xuong et al.,
791 2007) (Gatan Inc., Pleasanton, CA, USA) operated in the electron-counted mode. We
792 additionally used the Gatan Imaging Filter (Gatan Inc., Pleasanton, CA, USA) with a slit
793 width of 20 eV to increase contrast by removing inelastically scattered electrons
794 (Krivanek et al., 1995). After initially assessing the infected macrophages at lower
795 magnifications, tilt series were collected at a magnification of 26,000X (with a
796 corresponding pixel size of 3.38 Å) and a defocus of −6 µm. Precooking the target areas
797 with a total dosage of 1000-1500 e/Å^2 was required to minimize sample shrinking and
798 drifting during tilt series collection. A bi-directional tilt scheme was employed with 2°
799 increments and 120° span (−60° to +60°). The cumulative dose of each tilt-series was in
800 the order of ~500 e−/Å2. Once acquired, tilt series were aligned (using the patch

801 tracking function) and reconstructed into tomograms, both using the IMOD software
802 package (Kremer et al., 1996). After careful assessment of the 3-dimensional (3-D)
803 tomograms, optimal 2-D slices were chosen for figure presentations. Color overlays
804 provide additional assistance with interpretation.
805
806 Statistical analysis
807 Prism 9.5.0 (GraphPad Software) was utilized for the graphing of data and all statistical
808 analyses. Statistical significance for experiments with THP-1 macrophages and hMDMs
809 was determined using the appropriate test and are indicated in each figure legend.
810 Differences were considered statistically significant if the *p* value was <0.05.
811
812
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1446 Figure Legends

1447 **Figure 1. Caspase-1 promotes the control of *Salmonella* replication within human**
1448 **macrophages**. WT and two independent clones of *CASP1*$^{-/-}$ THP-1 monocyte-derived
1449 macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were then
1450 infected with PBS (Mock), WT *S*. Typhimurium (A, B, C), or WT *S.* Typhimurium
1451 constitutively expressing GFP (D,E) at an MOI = 20. (A) Release of IL-1β into the
1452 supernatant was measured by ELISA at 6 hpi. (B) Cell death (% cytotoxicity) was
1453 measured by LDH release assay and normalized to mock-infected cells at 6 hpi. (C)
1454 Cells were lysed at 1 hpi and 6 hpi, and bacteria were subsequently plated to calculate
1455 CFU. Fold-change in CFU/well was calculated. (D, E) Cells were fixed at 6 hpi and
1456 stained for DAPI to label DNA (blue). (D) The number of bacteria per cell at 6 hpi was
1457 scored by fluorescence microscopy. Each dot represents one infected cell. 150 infected
1458 cells were scored for each genotype. (E) Representative images are shown. Scale bar
1459 represents 10 µm. Bars represent the mean for each genotype, and error bars represent
1460 the standard deviation of triplicate wells from one experiment (A, B, C, D). ***$p < 0.001$,
1461 ****$p < 0.0001$ by Dunnett's multiple comparisons test (A, B, C, D). Data shown are
1462 representative of at least three independent experiments.

1463

1464 **Figure 2. Caspase-4 contributes to the control of *Salmonella* replication within**
1465 **human macrophages later during infection.** WT and two independent clones of
1466 *CASP4*$^{-/-}$ THP-1 monocyte-derived macrophages were primed with 100 ng/mL
1467 Pam3CSK4 for 16 hours. Cells were then infected with PBS (Mock), WT *S*.
1468 Typhimurium (A, B, C), or WT *S.* Typhimurium constitutively expressing GFP (D, E) at

1469 an MOI = 20. (A) Release of IL-1β into the supernatant was measured by ELISA at 24
1470 hpi. (B) Cell death (% cytotoxicity) was measured by LDH release assay and normalized
1471 to mock-infected cells at 24 hpi. (C) Cells were lysed at 1 hpi and 24 hpi, and bacteria
1472 were subsequently plated to calculate CFU. Fold-change in CFU/well was calculated.
1473 (D, E) Cells were fixed at 24 hpi and stained for DAPI to label DNA (blue). (D) The
1474 number of bacteria per cell at 24 hpi was scored by fluorescence microscopy. Each dot
1475 represents one infected cell. 150 infected cells were scored for each genotype. (E)
1476 Representative images are shown. Scale bar represents 10 μm. Bars represent the
1477 mean for each genotype, and error bars represent the standard deviation of triplicate
1478 wells from one experiment (A, B, C, D). ns – not significant, *$p < 0.05$ by Dunnett's
1479 multiple comparisons test (A, B, C, D). Data shown are representative of at least three
1480 independent experiments.
1481
1482 **Figure 3. GSDMD promotes the control of *Salmonella* replication within human**
1483 **macrophages.** WT and *GSDMD*$^{-/-}$ THP-1 monocyte-derived macrophages were primed
1484 with 100 ng/mL Pam3CSK4 for 16 hours. Cells were then infected with PBS (Mock), WT
1485 *S*. Typhimurium (A, B, C), or WT *S.* Typhimurium constitutively expressing GFP (D,E) at
1486 an MOI = 20. (A) Release of IL-1β into the supernatant was measured by ELISA at 6
1487 hpi. (B) Cell death (% cytotoxicity) was measured by LDH release assay and normalized
1488 to mock-infected cells at 6 hpi. (C) Cells were lysed at 1 hpi and 6 hpi, and bacteria
1489 were subsequently plated to calculate CFU. Fold-change in CFU/well was calculated.
1490 (D, E) Cells were fixed at 6 hpi and stained for DAPI to label DNA (blue). (D) The
1491 number of bacteria per cell at 6 hpi was scored by fluorescence microscopy. Each dot

1492 represents one infected cell. 150 infected cells were scored for each genotype. (E)
1493 Representative images are shown. Scale bar represents 10 μm. Bars represent the
1494 mean for each genotype, and error bars represent the standard deviation of triplicate
1495 wells from one experiment (A, B, C, D). **p< 0.01, ***p < 0.001, ****p < 0.0001 by
1496 Šídák's multiple comparisons test (A) or by unpaired t-test (B, C, D). Data shown are
1497 representative of at least three independent experiments.
1498
1499 **Figure 4. NINJ1 contributes to the control of *Salmonella* replication within human**
1500 **macrophages.** WT THP-1 monocyte-derived macrophages were treated with siRNA
1501 targeting a control scrambled siRNA or siRNA targeting *NINJ1* for 72 hours prior to
1502 infection. Cells were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were then
1503 infected with PBS (Mock) or WT *S*. Typhimurium at an MOI = 20. (A) Release of IL-1β
1504 into the supernatant was measured by ELISA at 6 hpi. (B) Cell death (% cytotoxicity)
1505 was measured by LDH release assay and normalized to mock-infected cells at 6 hpi.
1506 (C, D, E) Cells were lysed at 1 hpi and 6 hpi, and bacteria were subsequently plated to
1507 calculate CFU. (C) CFU/well at 1 hpi. (D) CFU/well at 6 hpi. (E) Fold-change in
1508 CFU/well was calculated. Bars represent the mean for each condition. Error bars
1509 represent the standard deviation of triplicate wells from one experiment. ns – not
1510 significant, *p< 0.05, **p< 0.01 by unpaired t-test. Data shown are representative of at
1511 least three independent experiments.
1512
1513 **Figure 5. Inflammasome activation primarily controls cytosolic *Salmonella***
1514 **replication in human macrophages.** WT, *NAIP*$^{-/-}$ (A, B), and *CASP1*$^{-/-}$ (A, B, C) THP-1

1515 monocyte-derived macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours.
1516 (A) Cells were then infected WT *S*. Typhimurium (A) at an MOI = 20. At 1 hpi, cells were
1517 left untreated or treated with 500 μM CHQ for 1 hour. Then, at 2 hpi, cells were lysed,
1518 and bacteria were subsequently plated to calculate CFU. CFU/well of vacuolar (CHQ-
1519 sensitive) and cytosolic (CHQ-resistant) bacteria were calculated from the total bacteria.
1520 (B, C) Cells were then infected WT *S*. Typhimurium (B) or Δ*sipB S*. Typhimurium (C) at
1521 an MOI = 20. At 5 hpi, cells were left untreated or treated with 500 μM CHQ for 1 hour.
1522 Then, at 6 hpi, cells were lysed, and bacteria were subsequently plated to calculate
1523 CFU. CFU/well of vacuolar (CHQ-sensitive) and cytosolic (CHQ-resistant) bacteria were
1524 calculated from the total bacteria. (D,E) WT, *NAIP*$^{-/-}$, and *CASP1*$^{-/-}$ THP-1 monocyte-
1525 derived macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were
1526 then infected with PBS (Mock) or WT *S.* Typhimurium constitutively expressing mCherry
1527 and harboring the GFP cytosolic reporter plasmid, pNF101, at an MOI = 20. Cells were
1528 fixed at 8 hpi and stained for DAPI to label DNA (blue). (D) The number of GFP-positive,
1529 mCherry-positive bacteria (cytosolic) per cell and the number of GFP-negative,
1530 mCherry-positive bacteria (vacuolar) per cell were scored by fluorescence microscopy.
1531 Each dot represents one infected cell. 150 total infected cells were scored for each
1532 genotype. (E) Representative images from 8 hpi are shown. Scale bar represents 10
1533 μm. White arrows indicate cytosolic bacteria (GFP-positive, mCherry-positive). Bars
1534 represent the mean for each genotype (A, B). Error bars represent the standard
1535 deviation of triplicate wells from one experiment (A, B). ns – not significant, *$p < 0.05$,
1536 ****$p < 0.0001$ by Dunnett's multiple comparisons test (A) or by Tukey's multiple

1537 comparisons test (B). Data shown are representative of at least three independent
1538 experiments (A, B, C).
1539
1540 **Figure 6. Inflammasome activation modulates the cytosolic exposure of**
1541 ***Salmonella* in human macrophages.** (A, B) WT and *CASP1*$^{-/-}$ THP-1 monocyte-
1542 derived macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were
1543 then infected with WT *S*. Typhimurium at an MOI = 20. At 8 hpi, cells were fixed and
1544 collected to be processed for transmission electron microscopy. Representative
1545 transmission electron micrographs are shown. *Salmonella* (green) in an SCV (maroon)
1546 with cytosolic or vacuolar clearance (yellow). Scale bar represents 1 µm [A(i), B(i)] or
1547 400 nm [A(ii), B(ii)]. (A) WT THP-1s, (ii) is an inset from (i). White arrows indicate
1548 vacuolar bacteria. (B) *CASP1*$^{-/-}$ THP-1s, (ii) is an inset from (i). White arrows indicate
1549 vacuolar bacteria, cyan arrows indicate partially cytosolic bacteria, and black asterisk
1550 indicates a fully cytosolic bacterium. (C) *CASP1*$^{-/-}$ THP-1 monocyte-derived
1551 macrophages were primed with 100 ng/mL Pam3CSK4 for 16 hours. Cells were then
1552 infected with WT *S*. Typhimurium at an MOI = 20. At 8 hpi, cells were fixed and
1553 collected to be processed for transmission electron microscopy. Representative
1554 tomogram slices are shown, depicting *Salmonella* (green) in an SCV with a
1555 discontinuous vacuolar membrane (maroon).
1556
1557 **Video 1. Characterization of the cytosolic exposure of *Salmonella* in *CASP1*$^{-/-}$**
1558 **human macrophages.** *CASP1*$^{-/-}$ THP-1 monocyte-derived macrophages were primed
1559 with 100 ng/mL Pam3CSK4 for 16 hours. Cells were then infected with WT *S*.

1560 Typhimurium at an MOI = 20. At 8 hpi, cells were fixed and collected to be processed

1561 for transmission electron microscopy. Representative tomogram shown, depicting

1562 *Salmonella* in an SCV with a discontinuous vacuolar membrane.

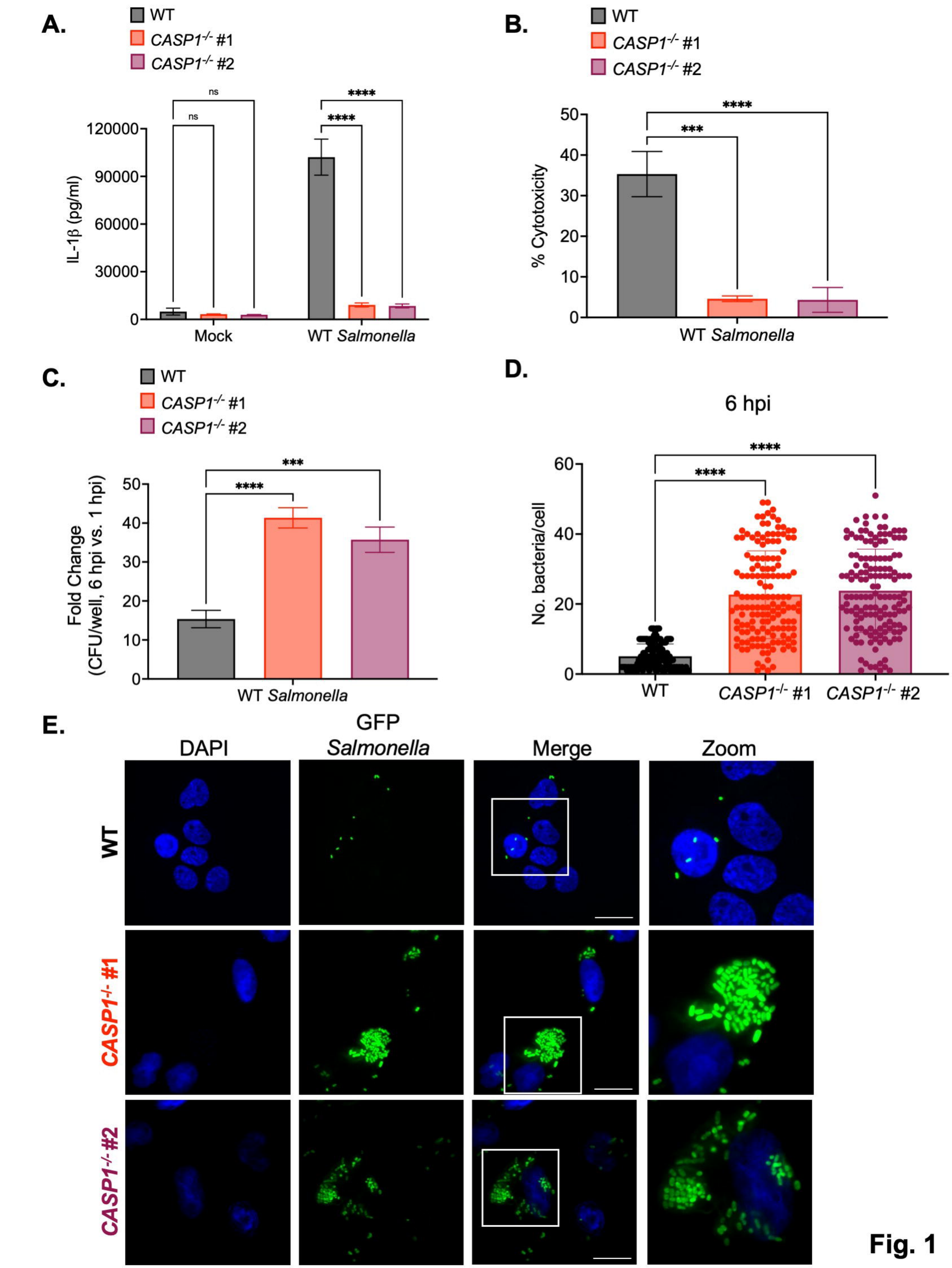
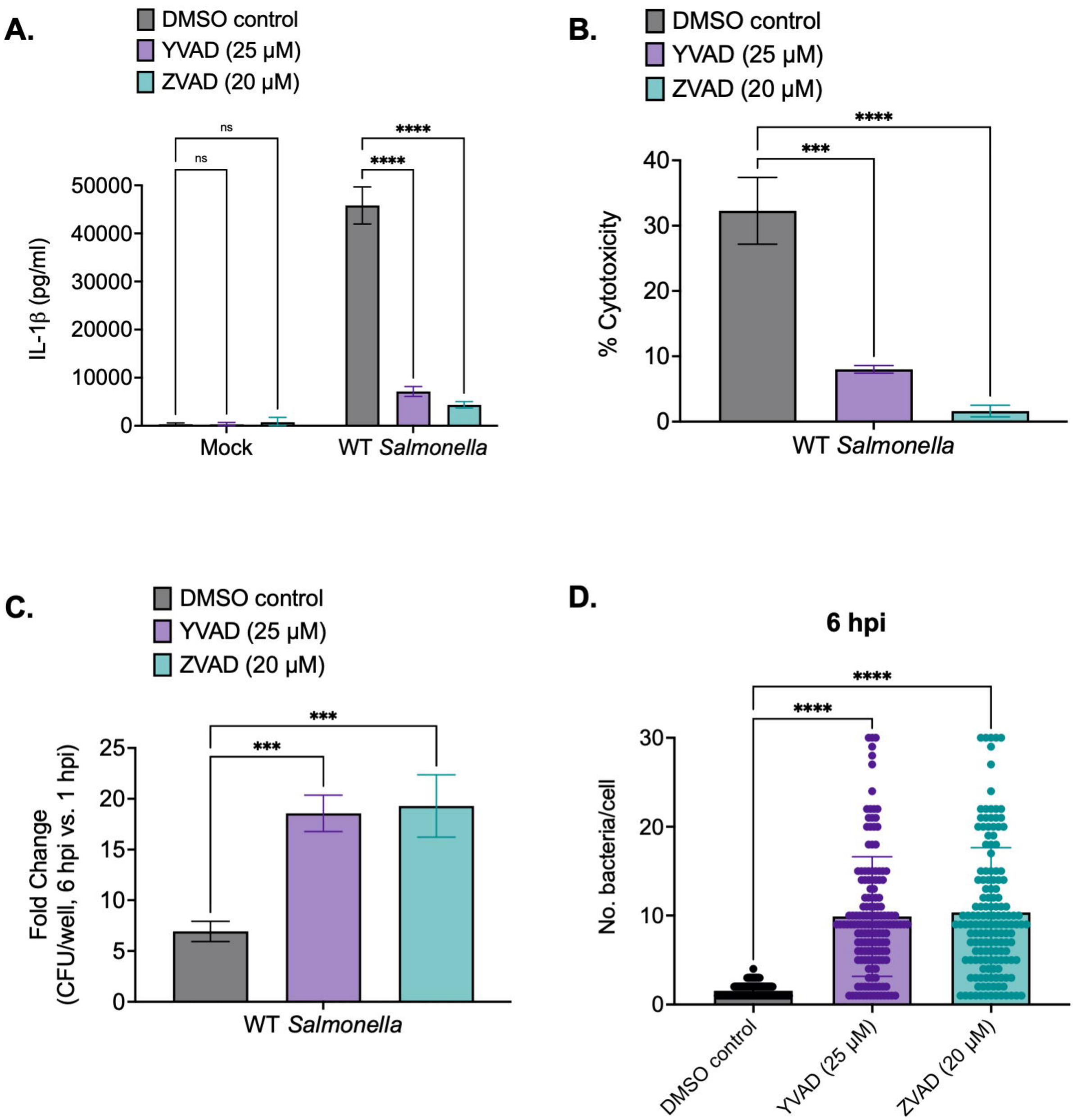

A.
WT
CASP1-/- #1
CASP1-/- #2
ns
ns
****
****
IL-1β (pg/ml)
120000
90000
60000
30000
0
Mock
WT Salmonella
B.
WT
CASP1-/- #1
CASP1-/- #2
****
***
% Cytotoxicity
50
40
30
20
10
0
WT Salmonella
C.
WT
CASP1-/- #1
CASP1-/- #2
***
****
Fold Change
(CFU/well, 6 hpi vs. 1 hpi)
50
40
30
20
10
0
WT Salmonella
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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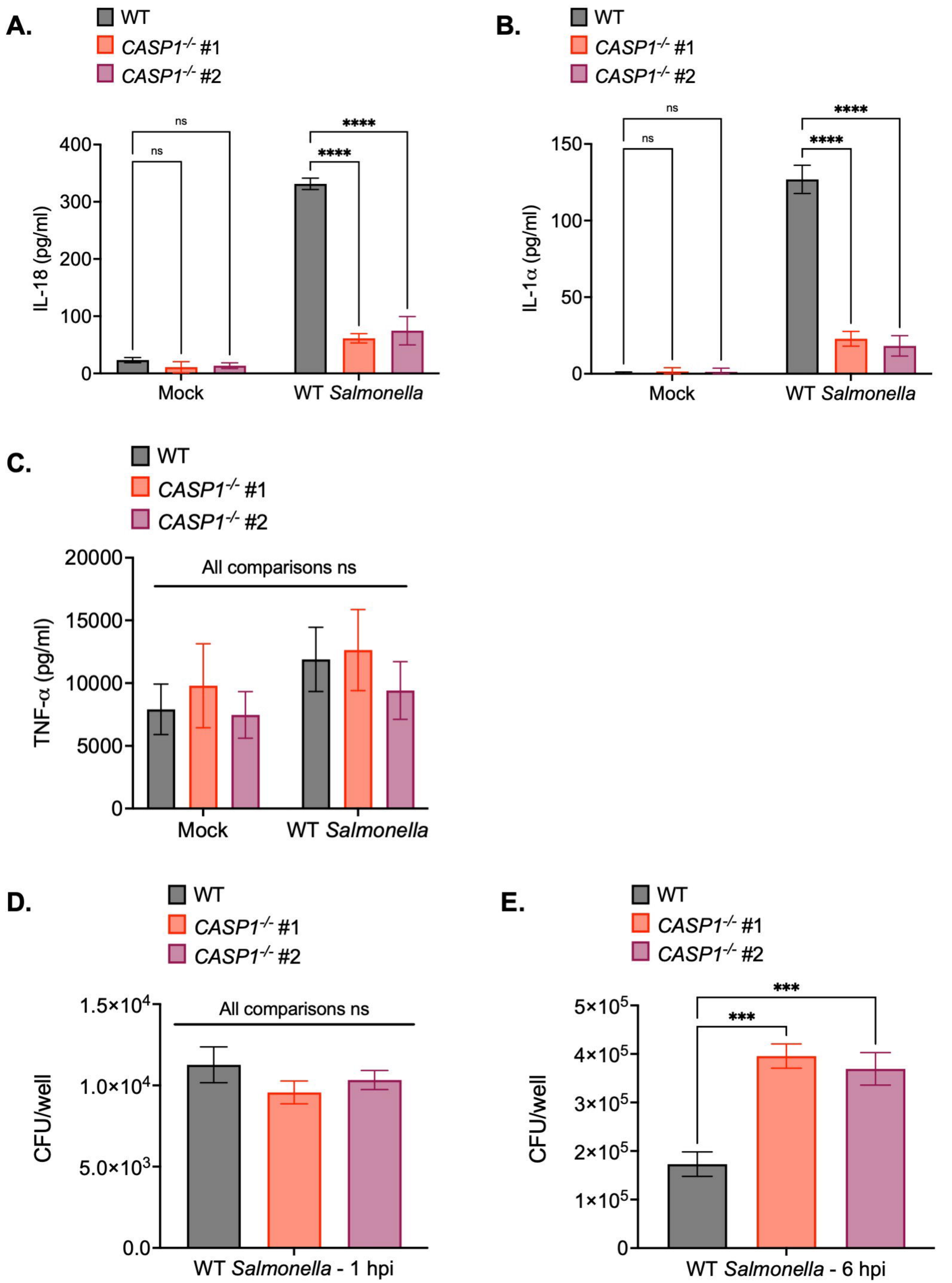

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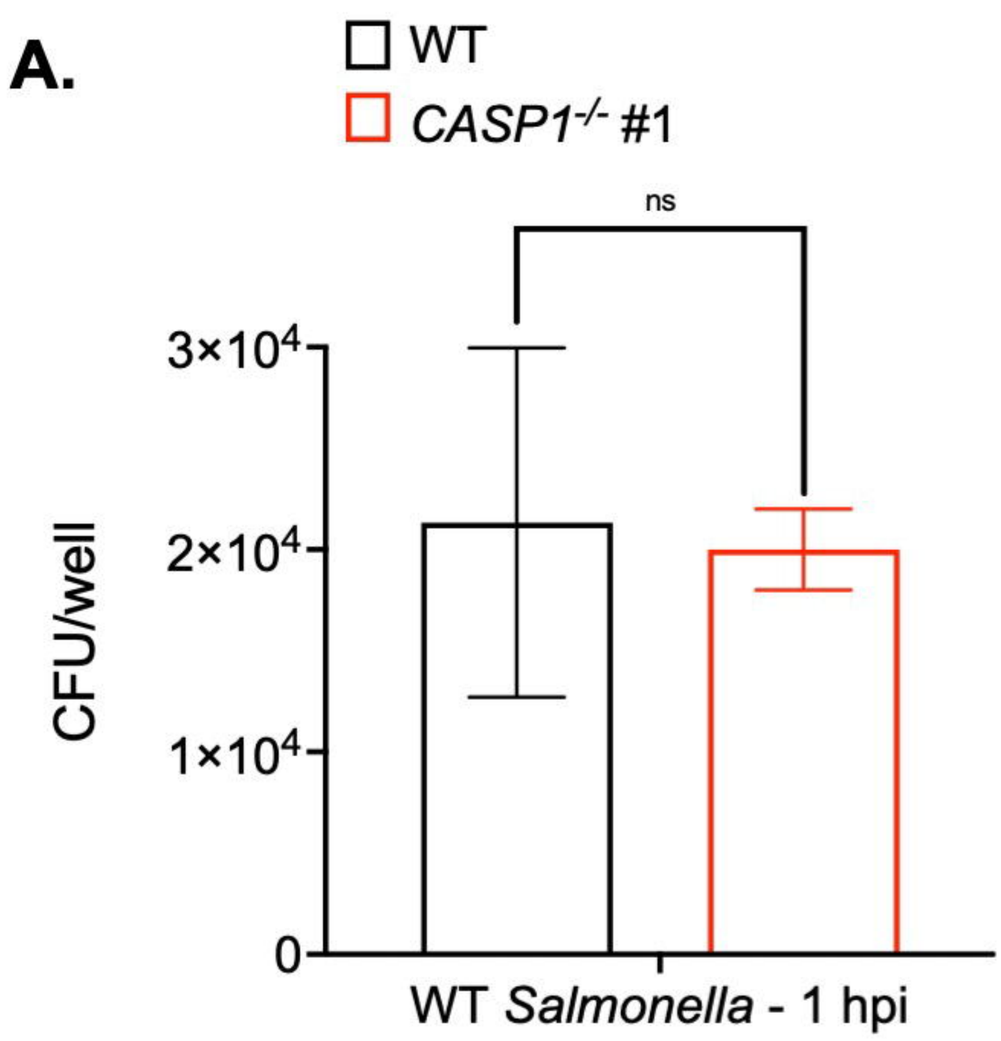

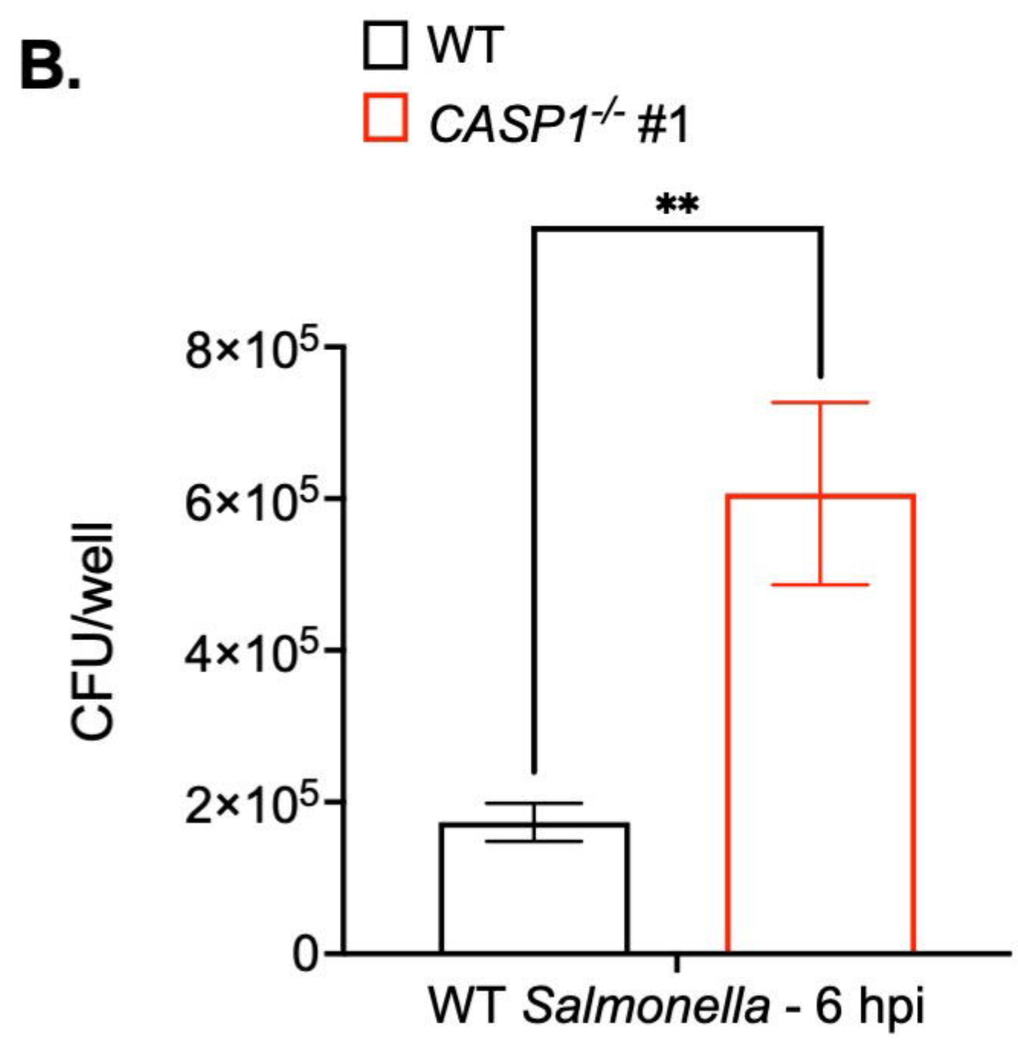

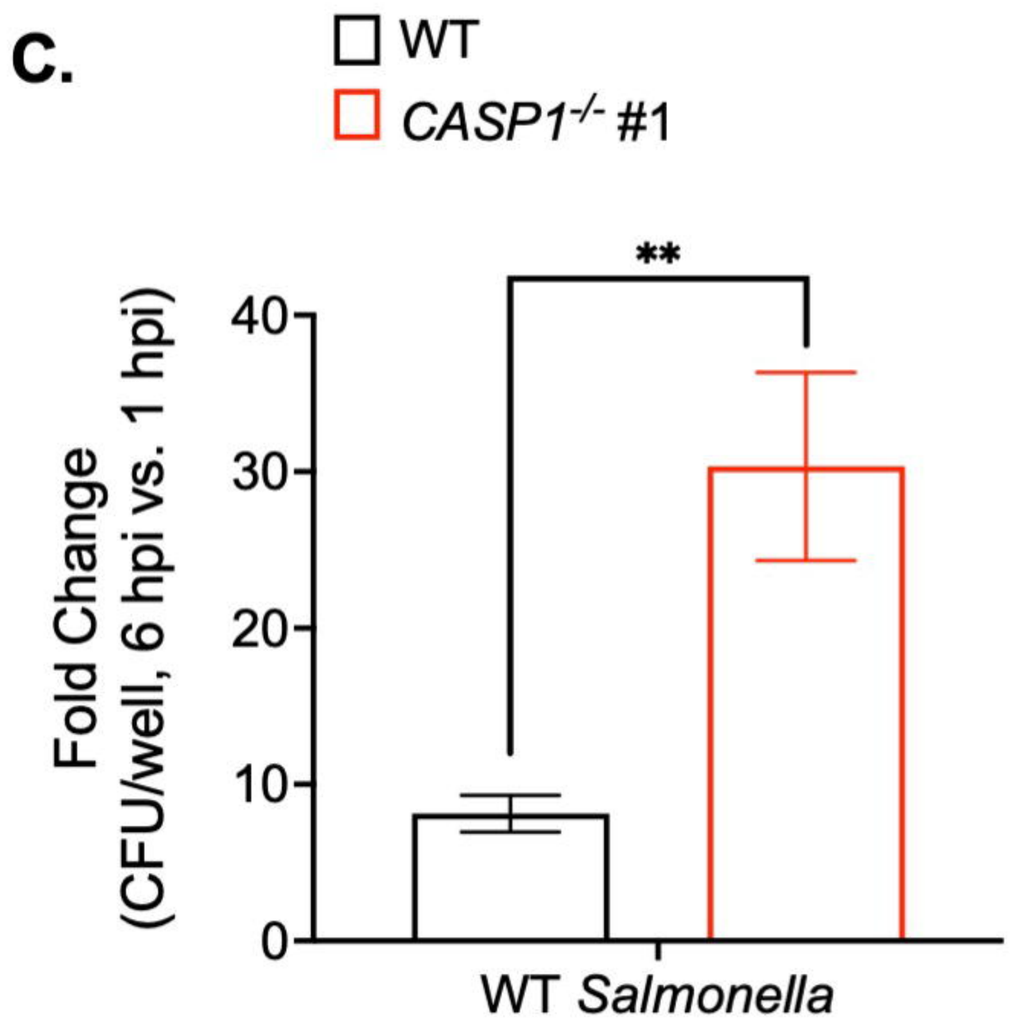

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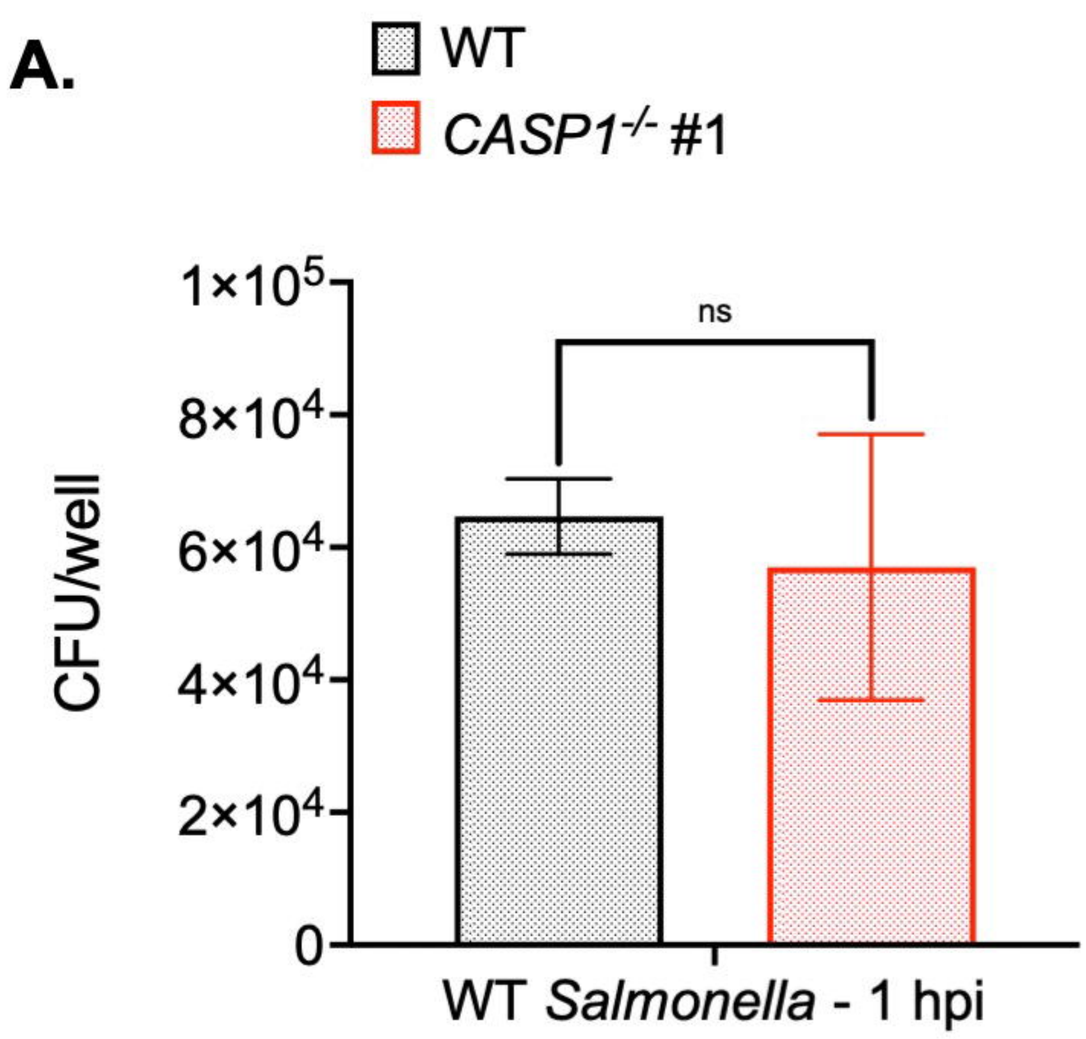

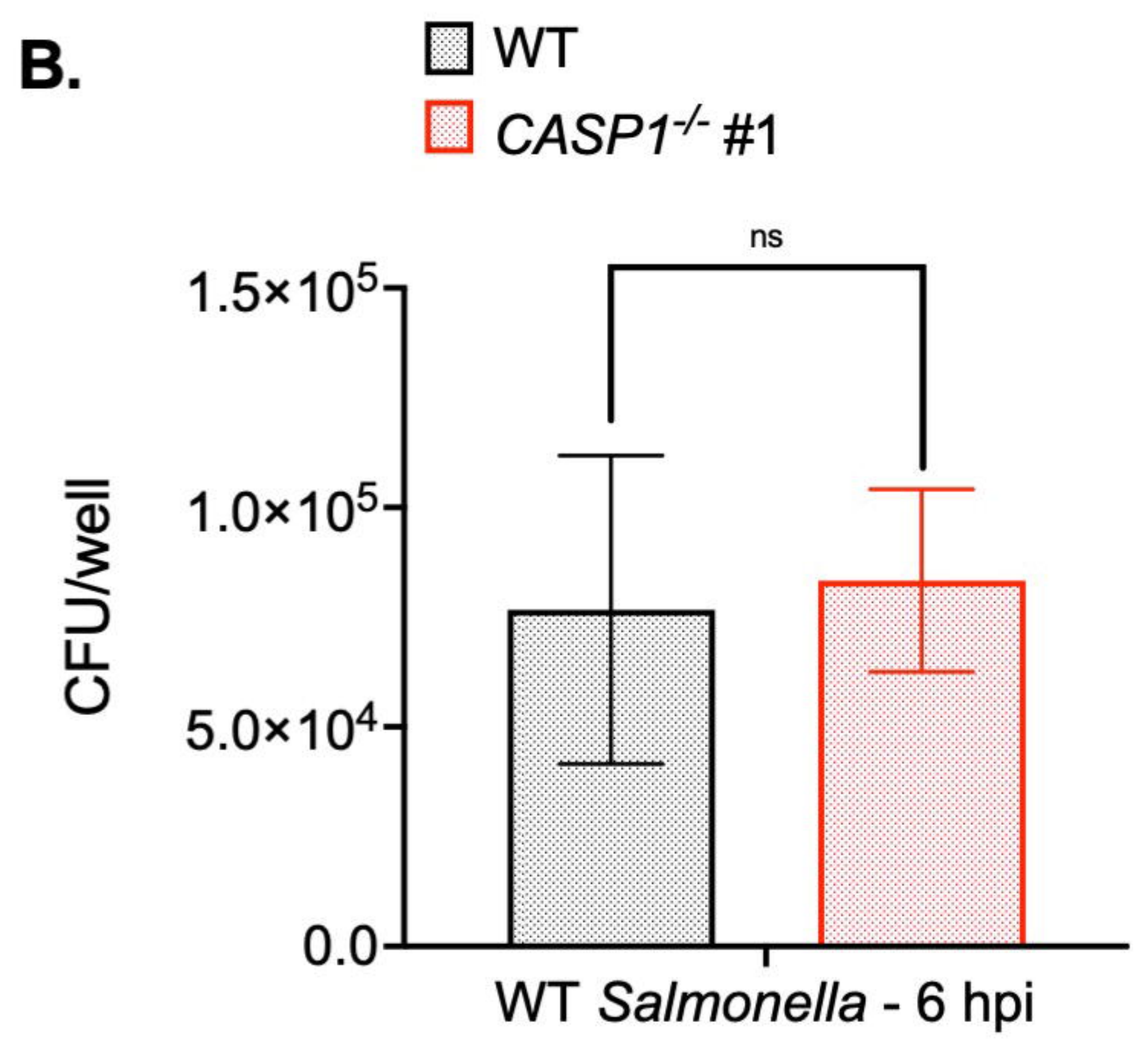

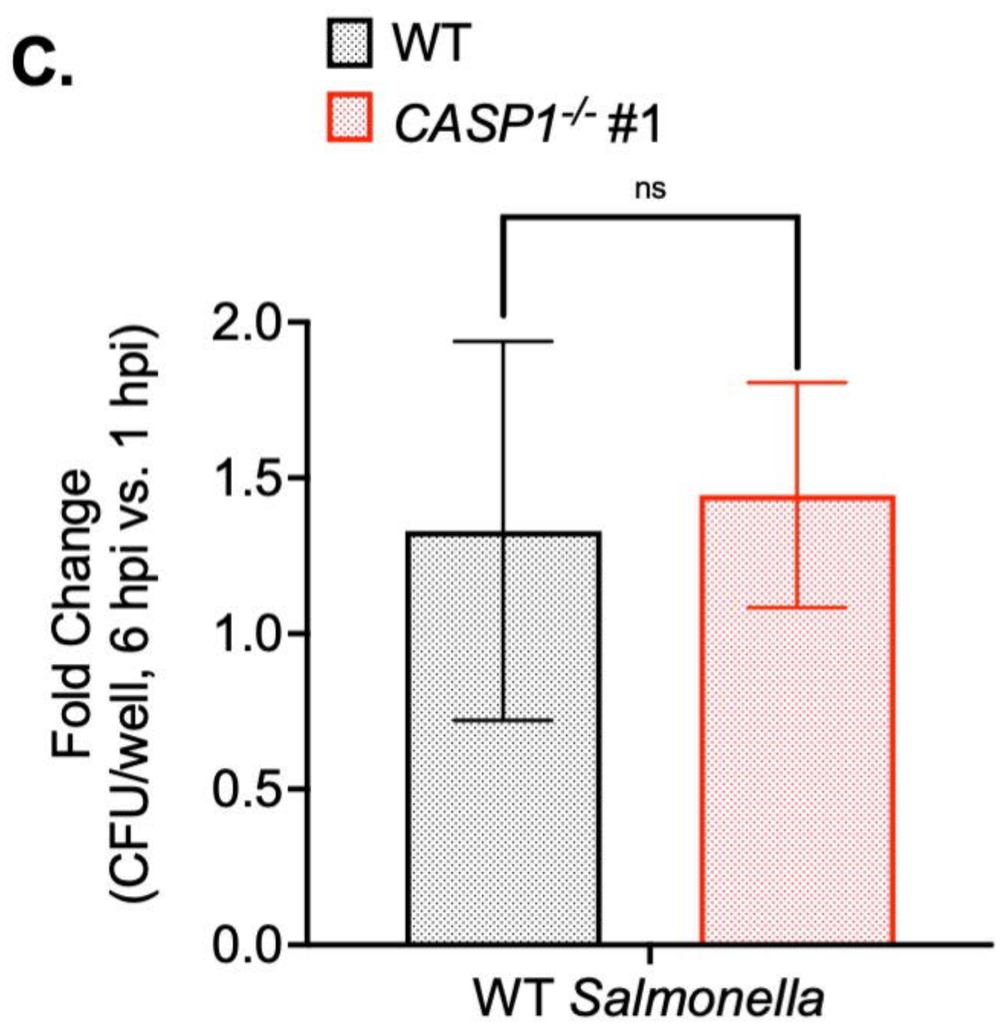

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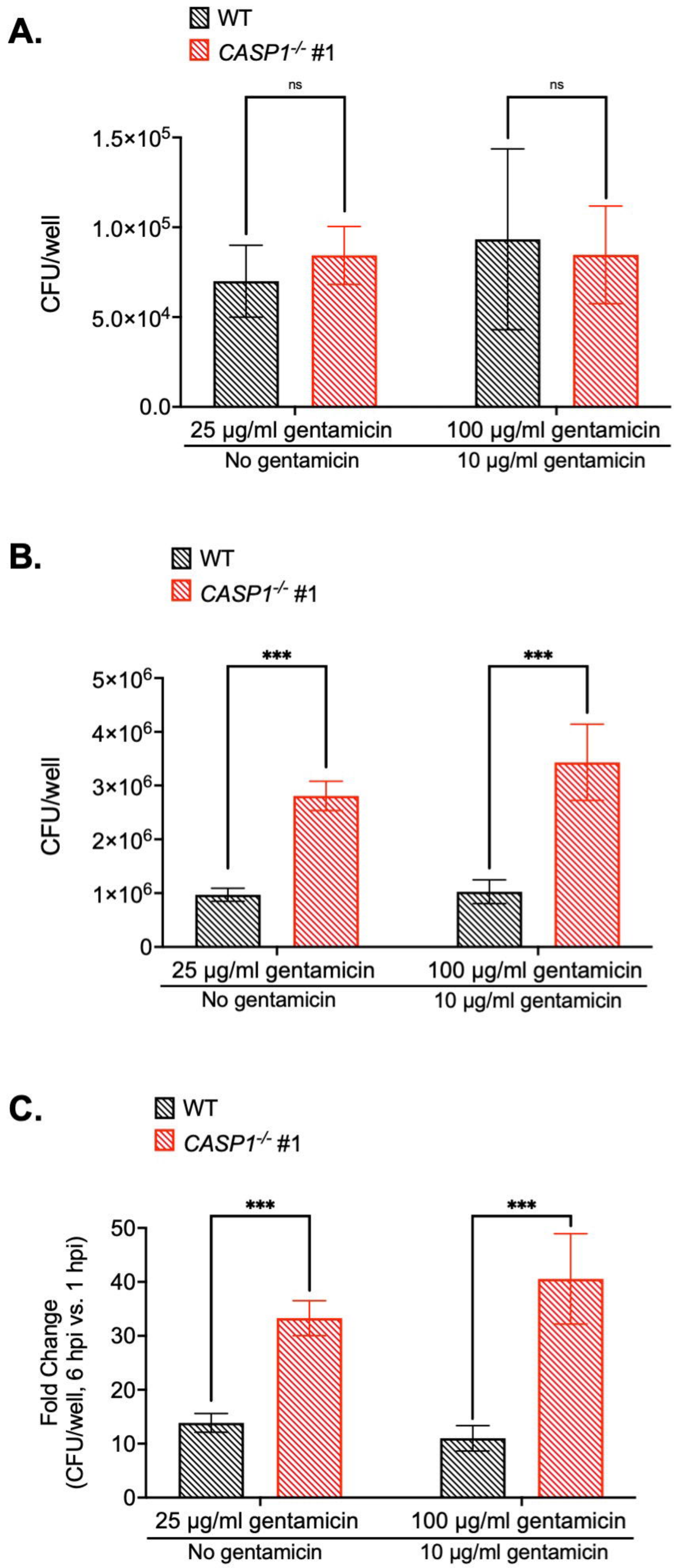

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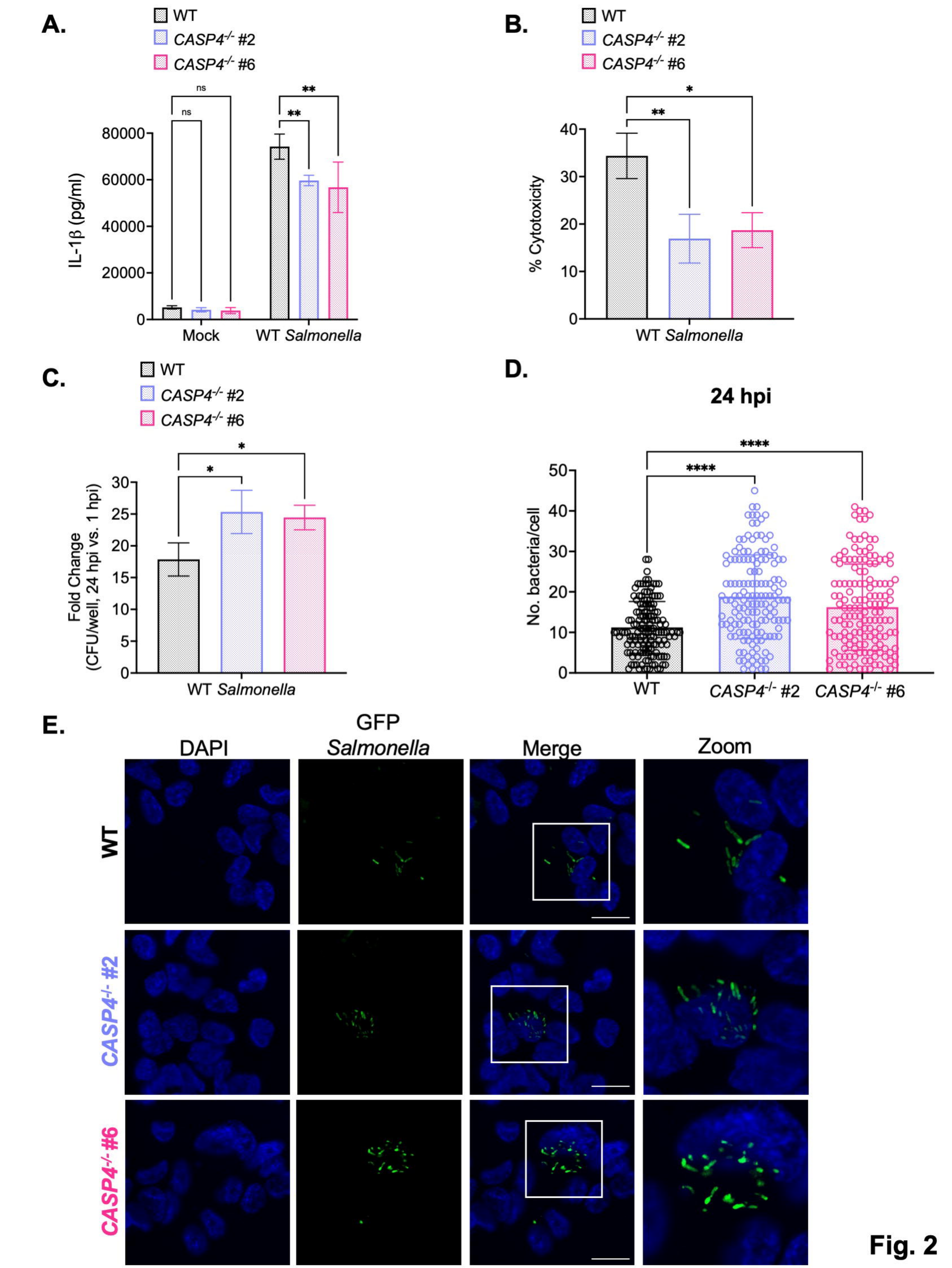
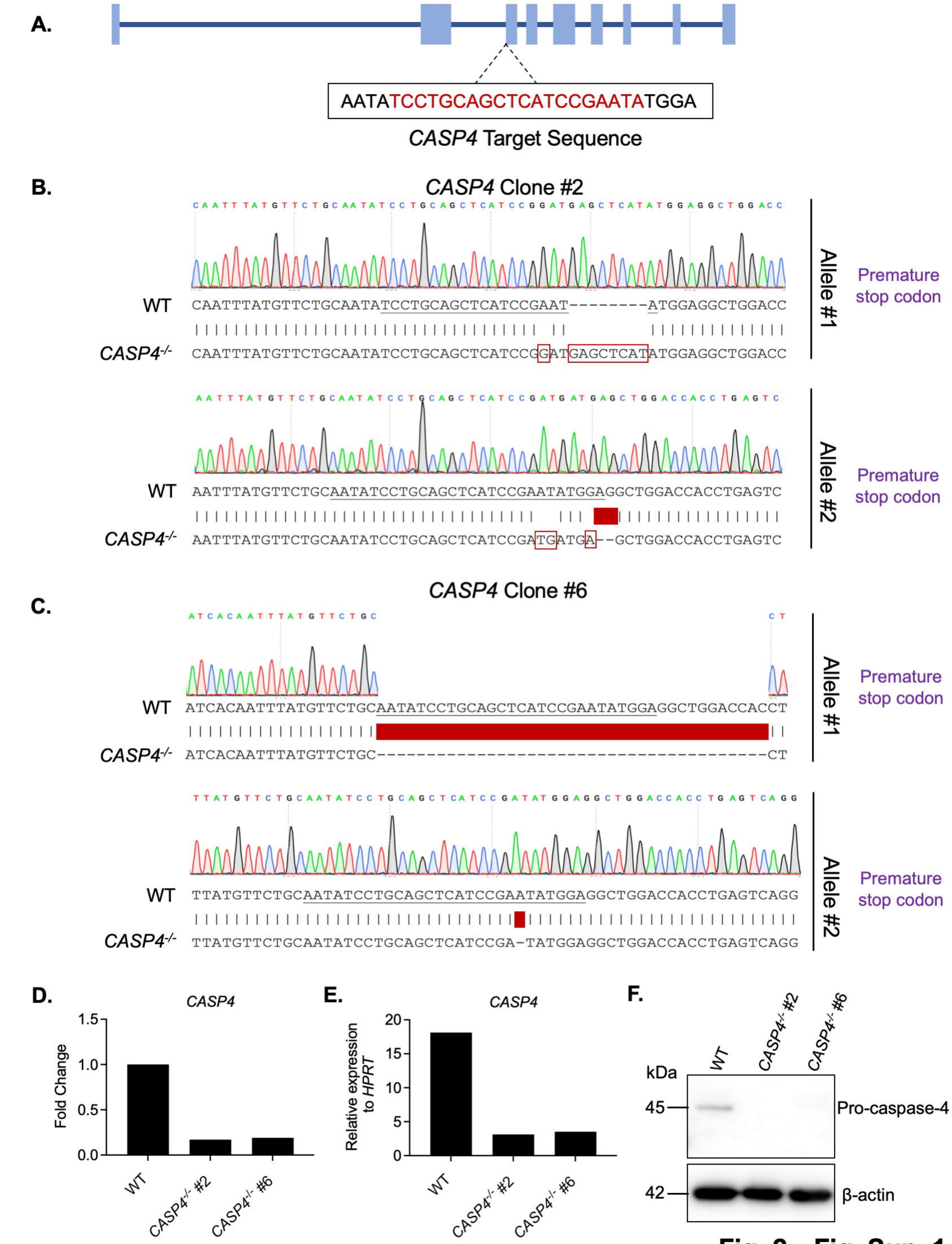

A.
WT
CASP4-/- #2
CASP4-/- #6
ns
ns
**
**
IL-1β (pg/ml)
80000
60000
40000
20000
0
Mock
WT Salmonella
B.
WT
CASP4-/- #2
CASP4-/- #6
*
**
% Cytotoxicity
40
30
20
10
0
WT Salmonella
C.
WT
CASP4-/- #2
CASP4-/- #6
*
*
Fold Change
(CFU/well, 24 hpi vs. 1 hpi)
30
25
20
15
10
5
0
WT Salmonella
D.
24 hpi
****
****
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



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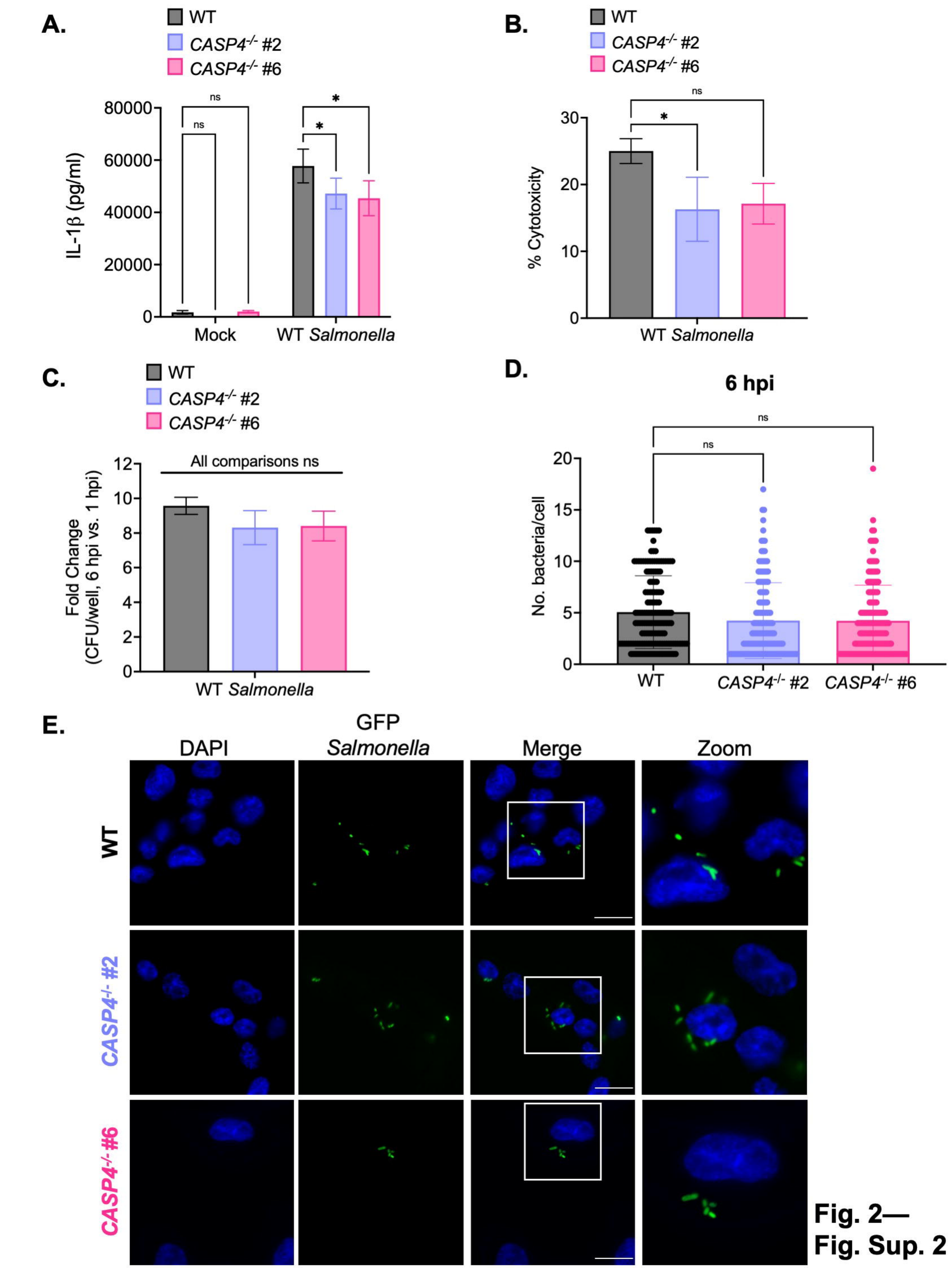
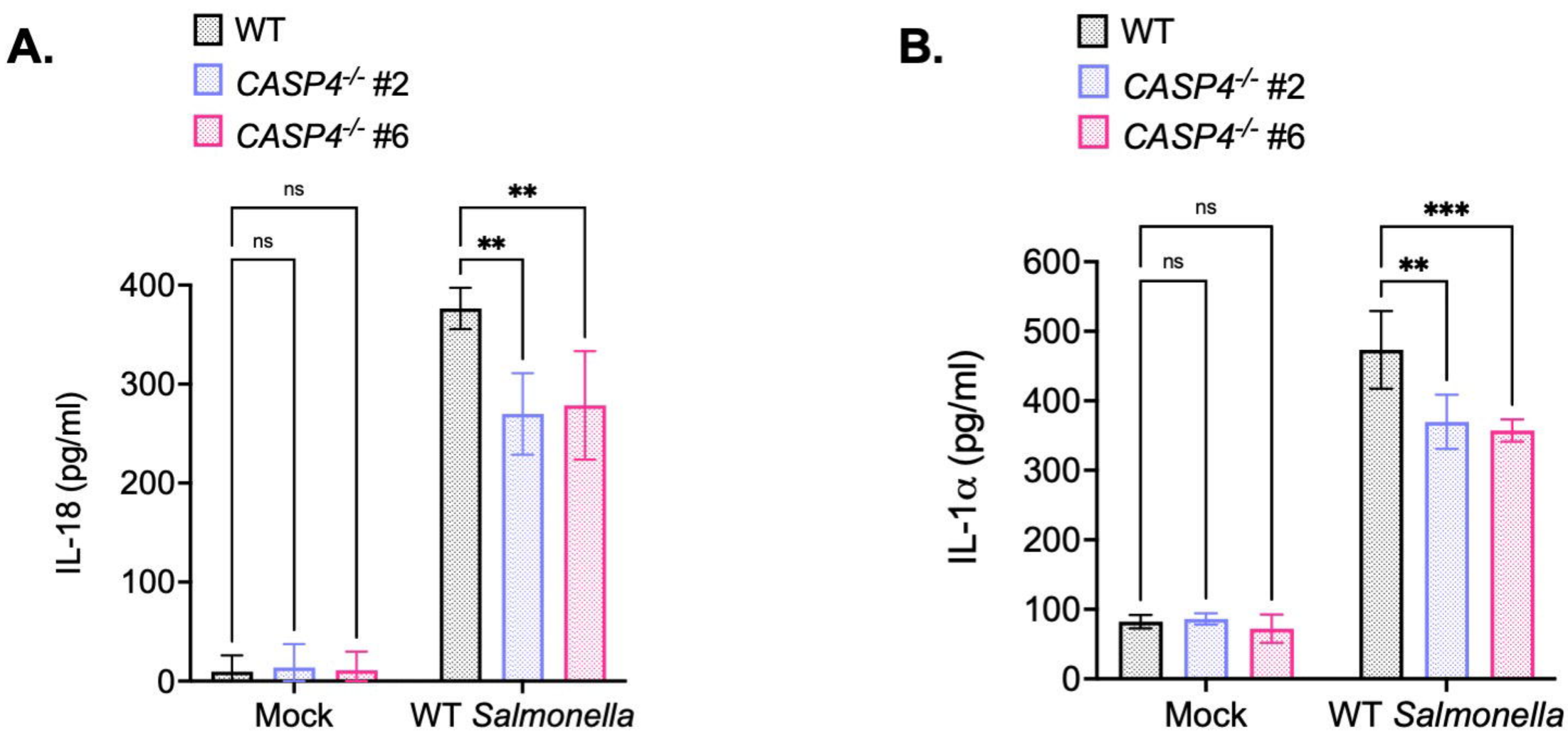
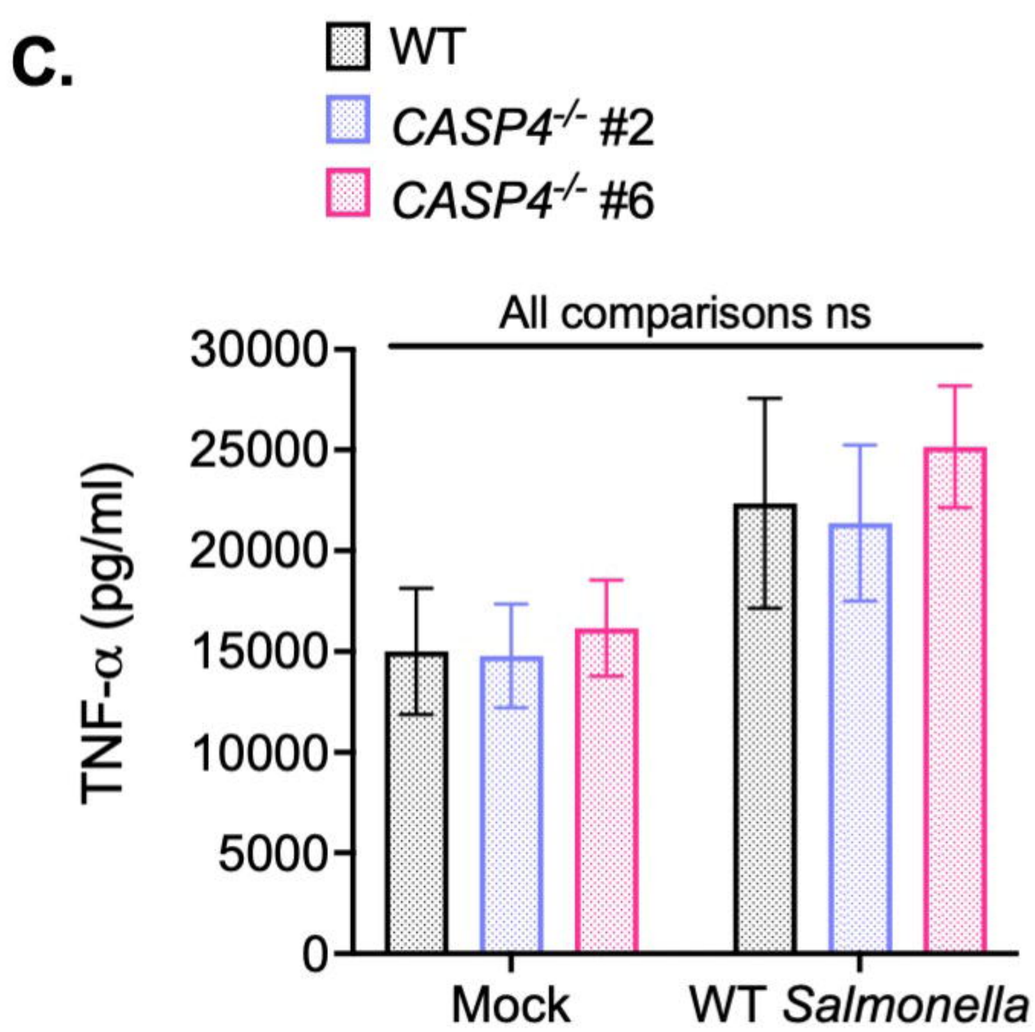
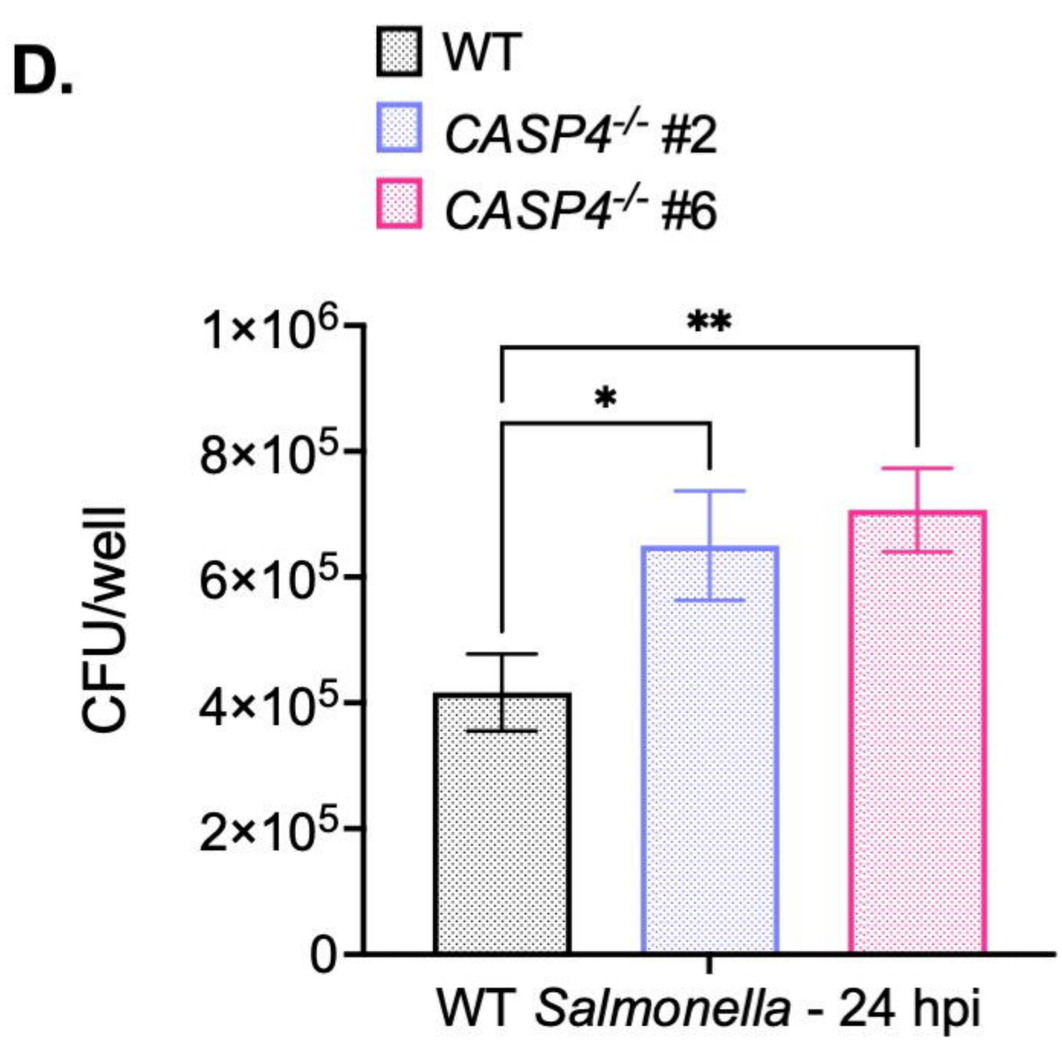


Fig. 2—
Fig. Sup. 2







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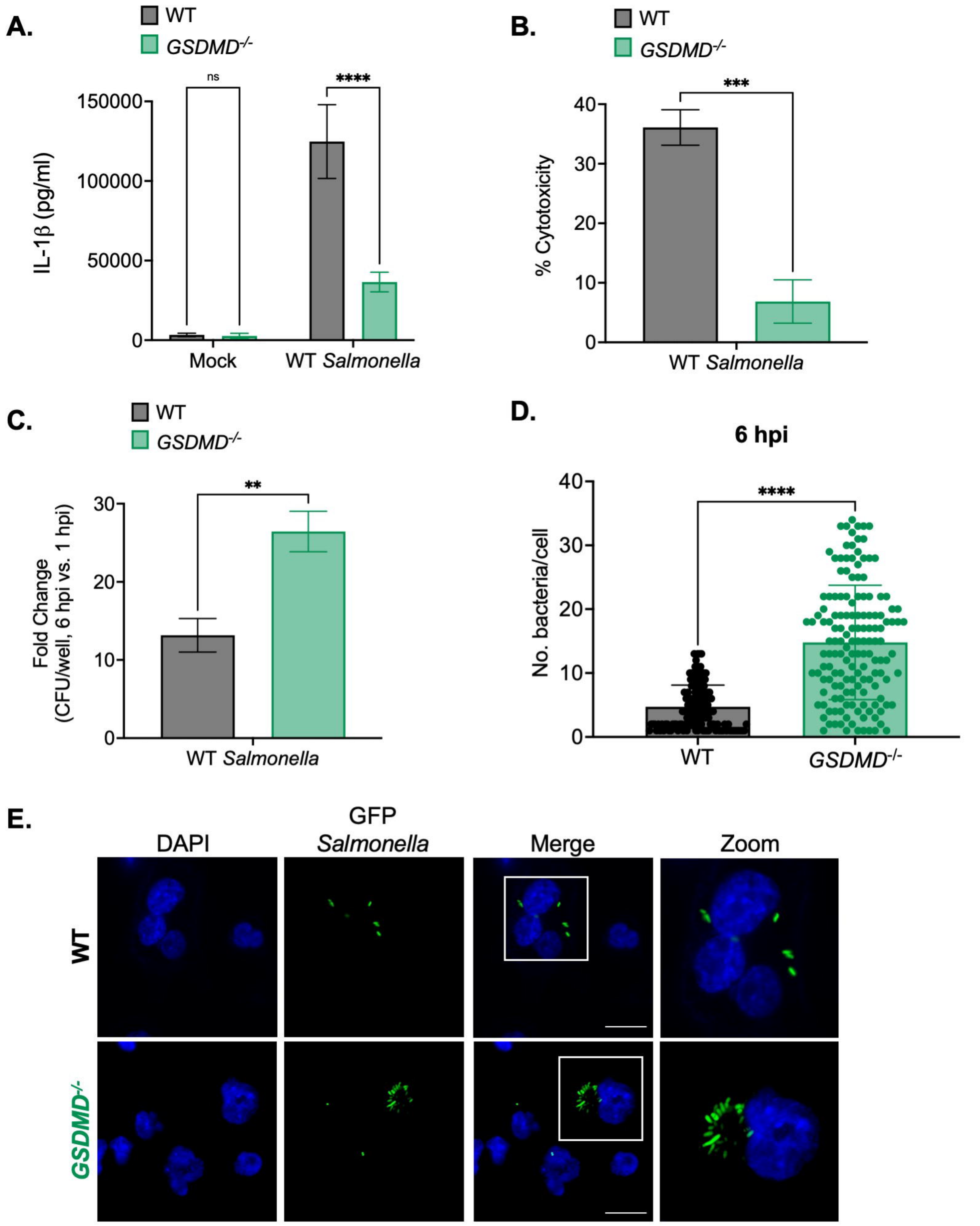
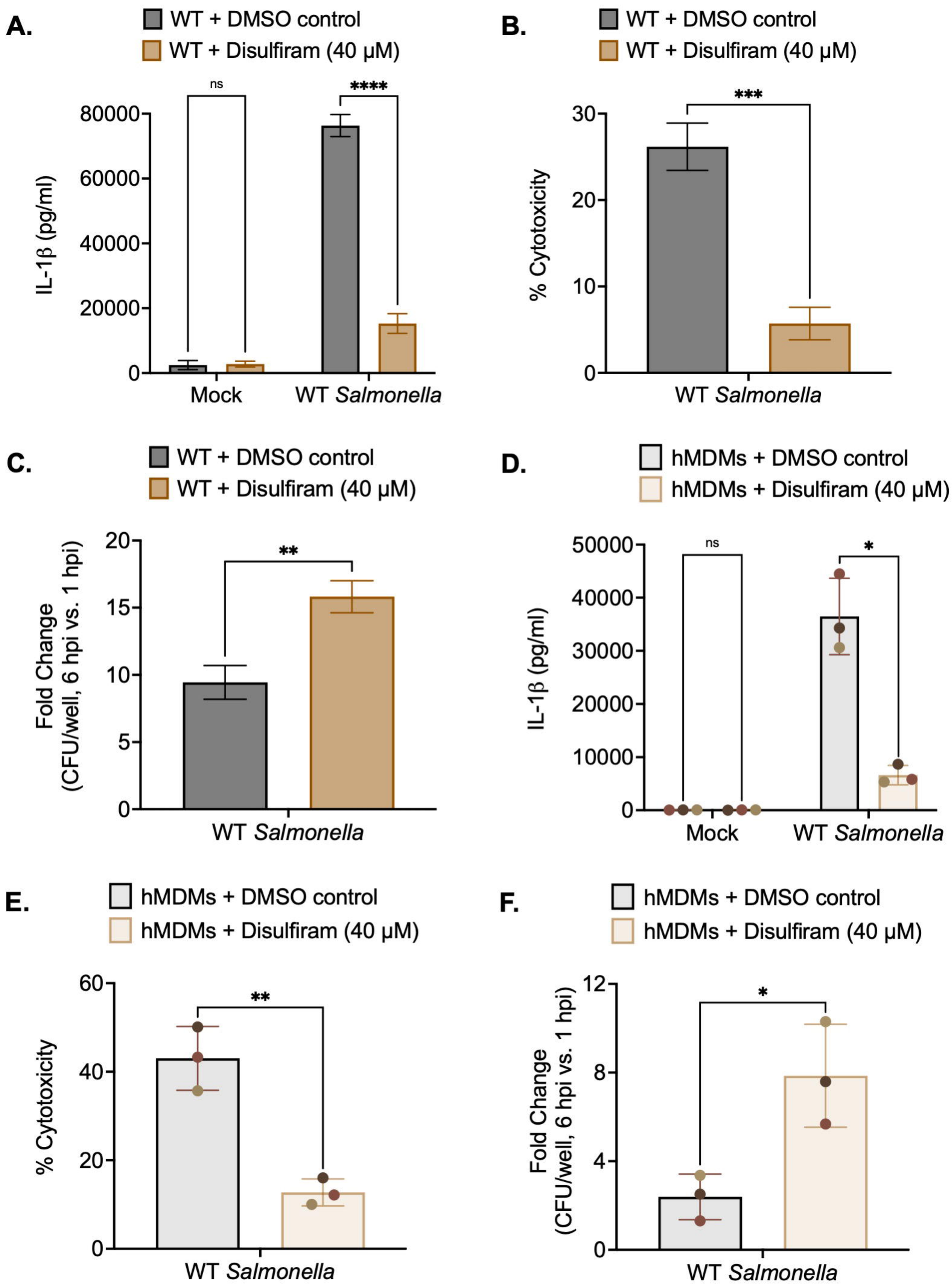

A.
WT
GSDMD-/-
ns
****
IL-1β (pg/ml)
150000
100000
50000
0
Mock
WT Salmonella
B.
WT
GSDMD-/-
***
% Cytotoxicity
40
30
20
10
0
WT Salmonella
C.
WT
GSDMD-/-
**
Fold Change
(CFU/well, 6 hpi vs. 1 hpi)
30
20
10
0
WT Salmonella
D.
6 hpi
****
No. bacteria/cell
40
30
20
10
0
WT
GSDMD-/-
E.
DAPI
GFP
Salmonella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

Fig. 3



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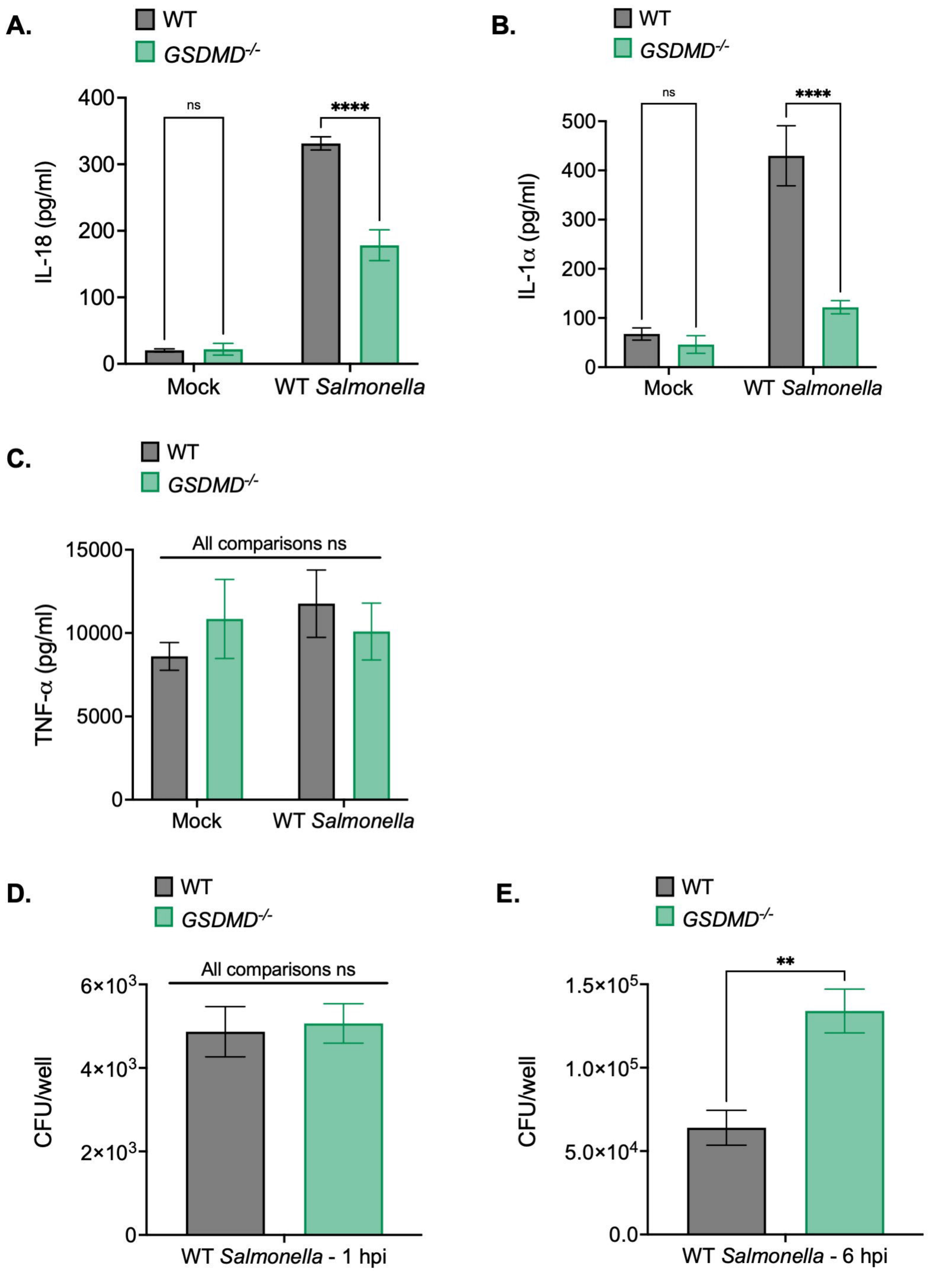

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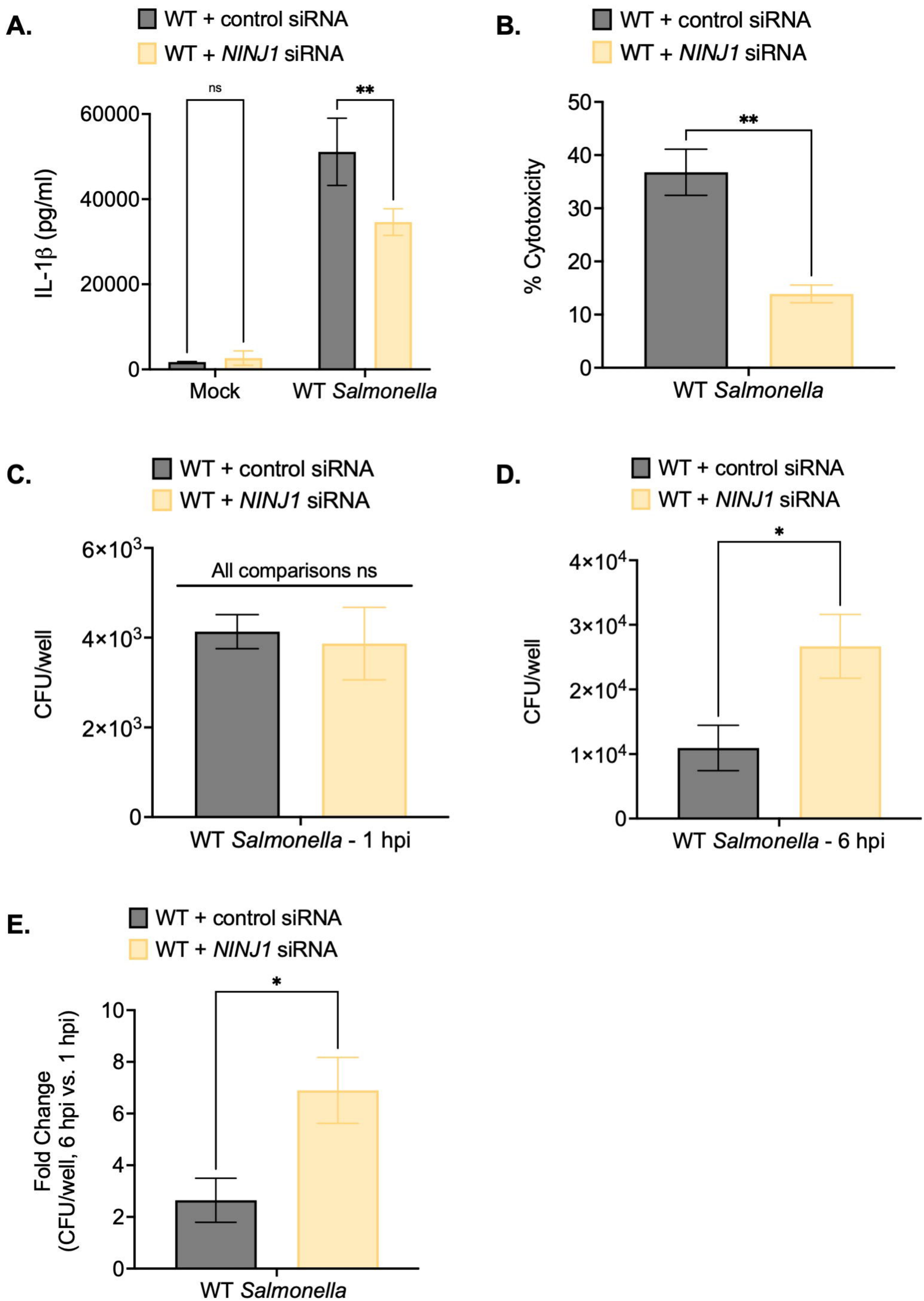
A.
WT + control siRNA
WT + NINJ1 siRNA
ns
**
IL-1β (pg/ml)
60000
40000
20000
0
Mock
WT Salmonella
B.
WT + control siRNA
WT + NINJ1 siRNA
**
% Cytotoxicity
50
40
30
20
10
0
WT Salmonella
C.
WT + control siRNA
WT + NINJ1 siRNA
All comparisons ns
CFU/well
6×10^3
4×10^3
2×10^3
0
WT Salmonella - 1 hpi
D.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

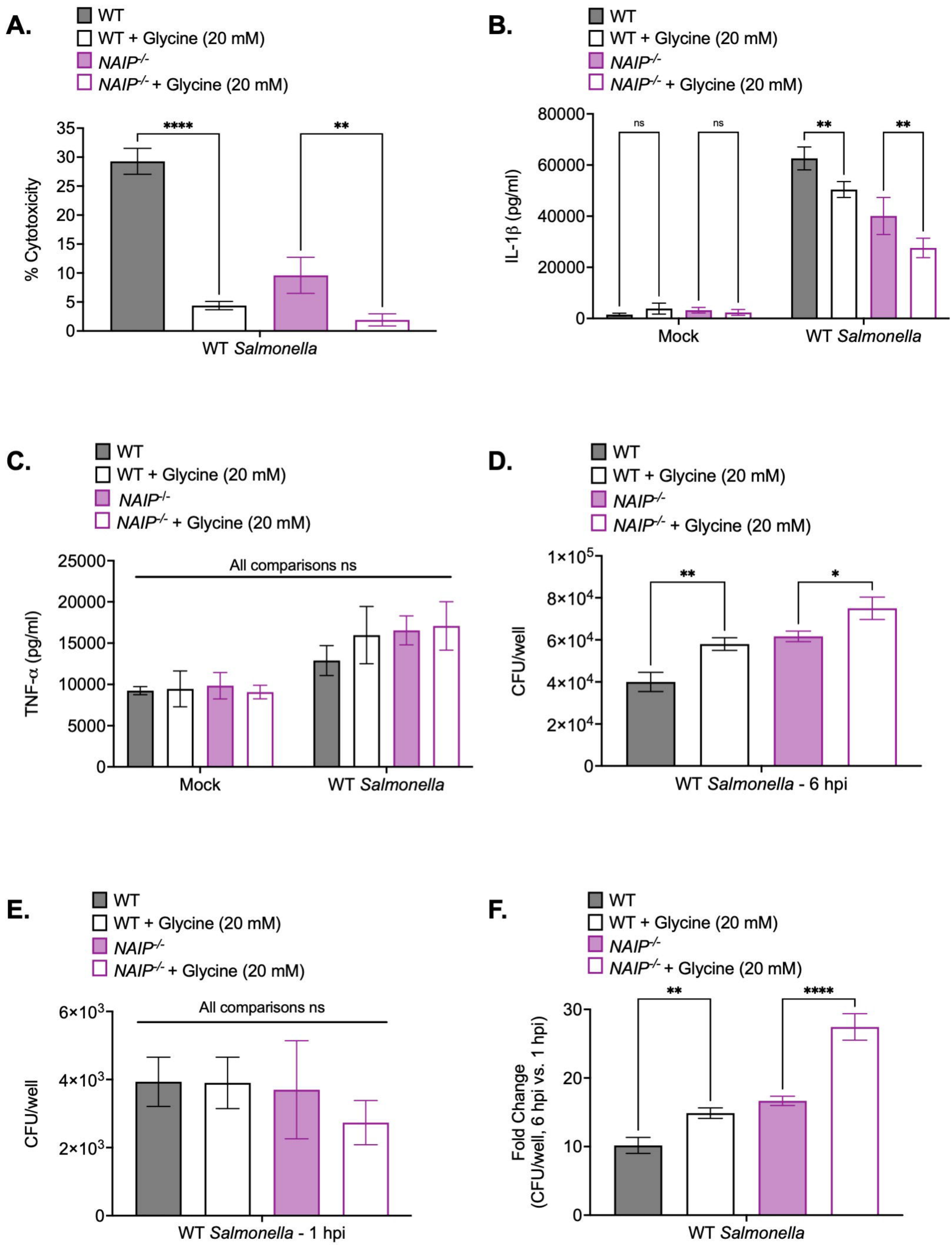

Fig. 4—Fig. Sup. 1

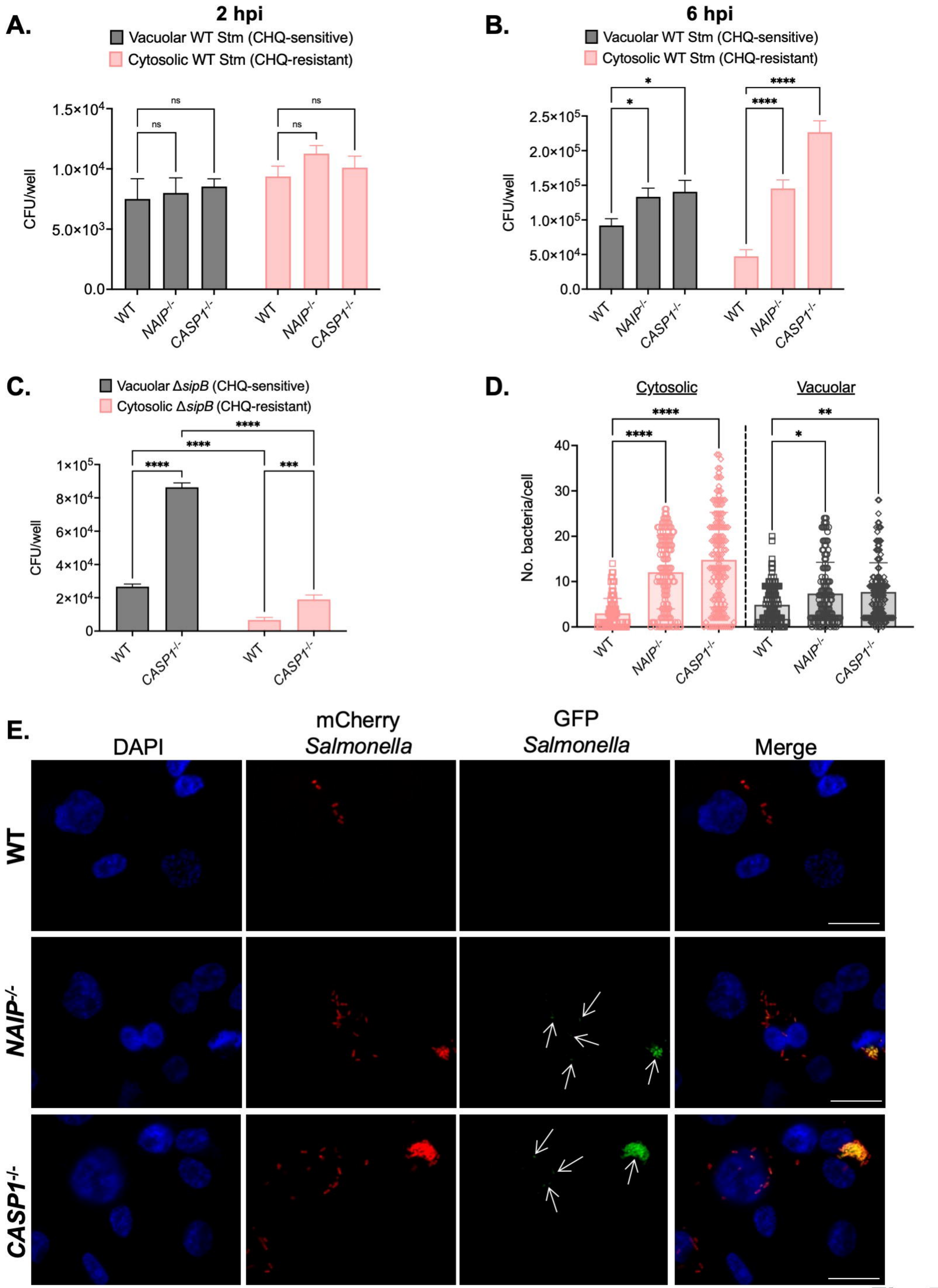
A.
2 hpi
Vacuolar WT Stm (CHQ-sensitive)
Cytosolic WT Stm (CHQ-resistant)
CFU/well
1.5×10^4
1.0×10^4
5.0×10^3
0.0
ns
WT
NAIP-/-
CASP1-/-
B.
6 hpi
Vacuolar WT Stm (CHQ-sensitive)
Cytosolic WT Stm (CHQ-resistant)
2.5×10^5
2.0×10^5
1.5×10^5
1.0×10^5
5.0×10^4
0.0
*
****
C.
Vacuolar ΔsipB (CHQ-sensitive)
Cytosolic ΔsipB (CHQ-resistant)
1×10^5
8×10^4
6×10^4
4×10^4
2×10^4
0
***
D.
Cytosolic
Vacuolar
No. bacteria/cell
40
30
20
10
0
**
E.
DAPI
mCherry Salmonella
GFP Salmonella
Merge
WT
NAIP-/-
CASP1-/-

Fig. 5

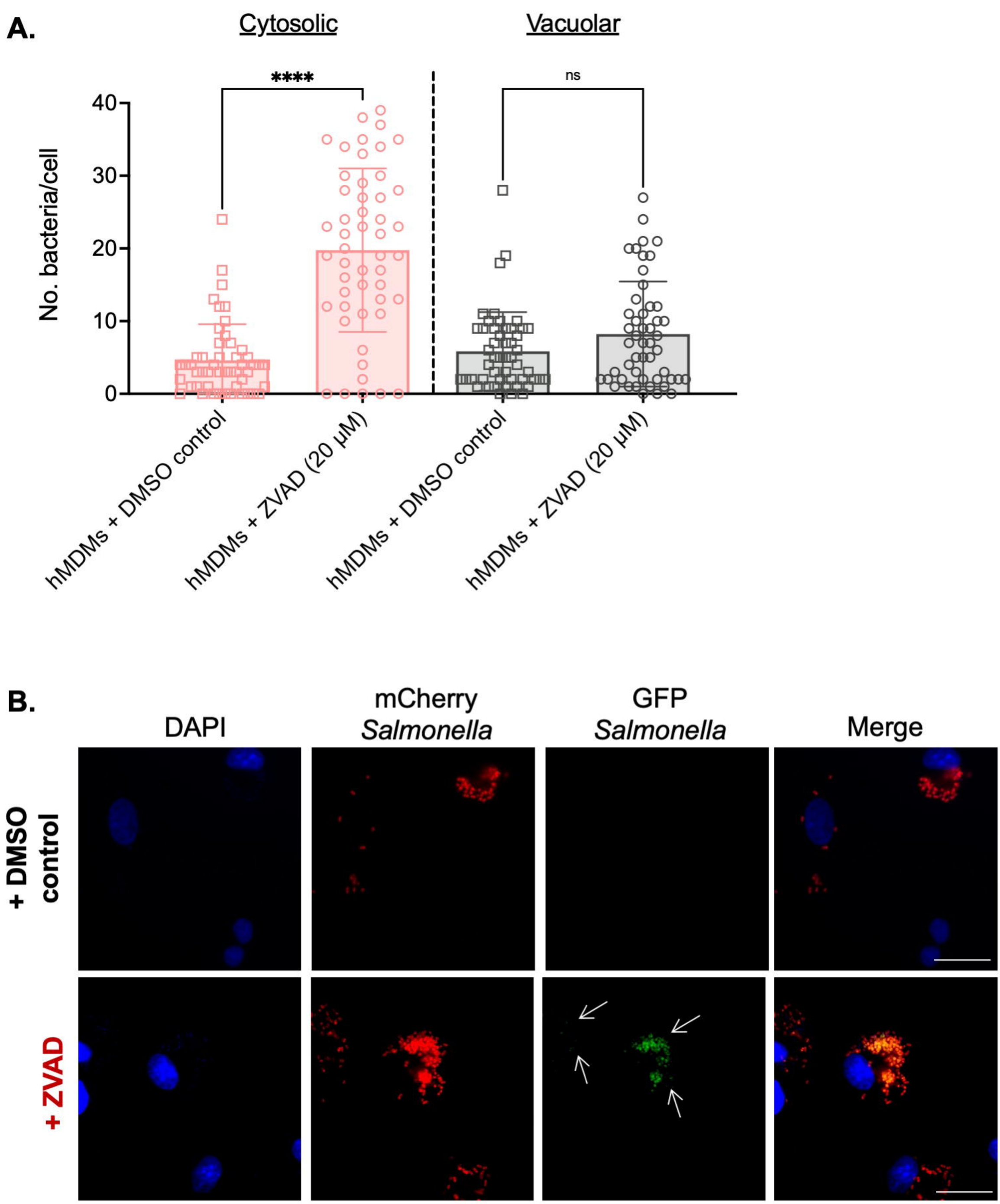

Fig. 5—Fig. Sup. 1

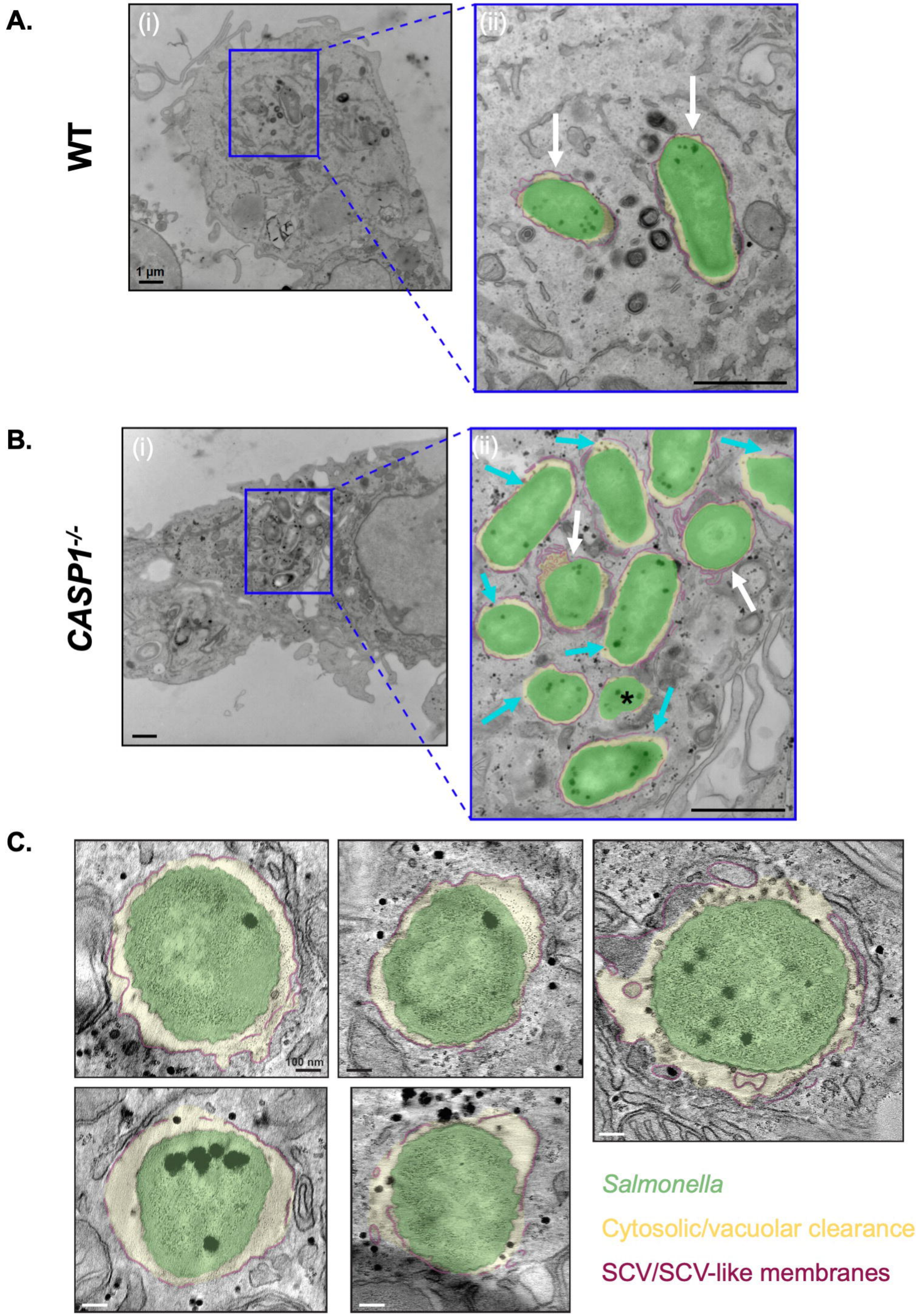
A.
WT
(i)
(ii)
1 µm
B.
CASP1-/-
(i)
(ii)
*
C.
100 nm
Salmonella
Cytosolic/vacuolar clearance
SCV/SCV-like membranes

Fig. 6

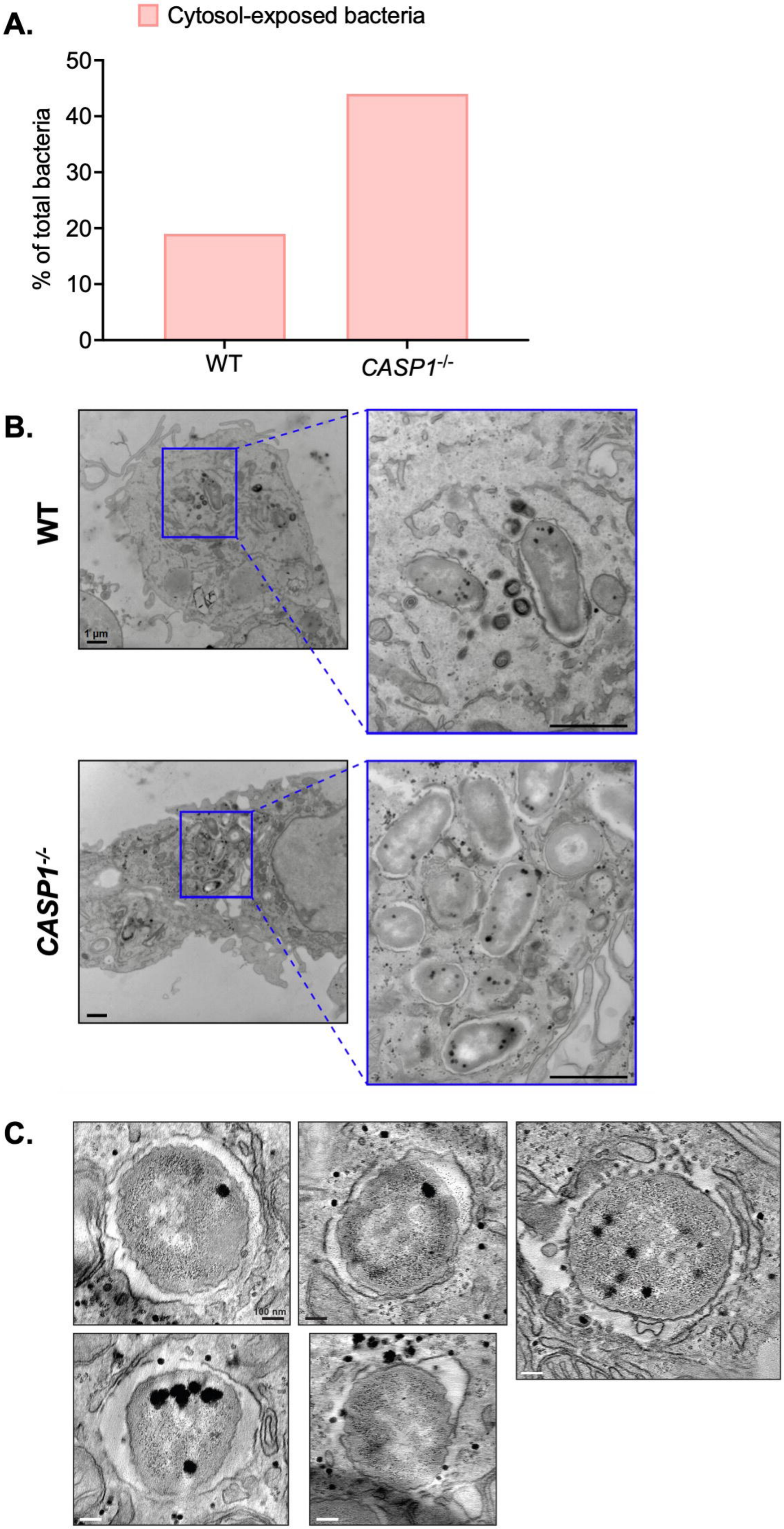

Fig. 6—Fig. Sup. 1