

1 **GH18 family glycoside hydrolase Chitinase A of *Salmonella* facilitates bacterial invasion**
2 **and survival by modulating host immune responses**

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23 **Abstract**

24 ***Salmonella* is a facultative intracellular pathogen that has co-evolved with its host and**
25 **has also developed various strategies to evade the host immune responses. *Salmonella***
26 **recruits an array of virulence factors to escape from host defense mechanisms.**
27 **Previously *chitinase A (chiA)* was found to be upregulated in intracellular *Salmonella*.**
28 **Although studies show that chitinases and chitin binding proteins (CBP) of many**
29 **human pathogens have a profound role in various aspects of pathogenesis, like**
30 **adhesion, virulence and immune evasion, the role of chitinase in strict intravacuolar**
31 **pathogen *Salmonella* has not yet been elucidated. In this study, we deciphered the role**
32 **of chitinase of *Salmonella* in the pathogenesis of the serovars, Typhimurium and Typhi.**
33 **Our data propose that ChiA mediated modification of the glycosylation on the epithelial**
34 **cell surface facilitates the invasion of the pathogen into the epithelial cells. Further we**
35 **found that ChiA aids in reactive nitrogen species (RNS) and reactive oxygen species**
36 **(ROS) production in phagocytes, leading to MHCII downregulation followed by**
37 **suppression of antigen presentation and antibacterial responses. In continuation of the**
38 **study in animal model *C. elegans*, *Salmonella* Typhi ChiA was found to facilitate**
39 **attachment to the intestinal epithelium, gut colonization and persistence by**
40 **downregulating antimicrobial peptides.**

41

42 **Introduction**

43 ***Salmonella* is one of the major foodborne pathogens that causes enteric disturbances in**
44 **humans and other mammals. Although *Salmonella*-mediated enteric illnesses can be treated,**

45 the high occurrences of drug-resistant strains challenge the pathogen eradication. Human
46 gastrointestinal tract is covered with two distinct types of glycan layers- mucin and complex
47 oligosaccharides (glycocalyx) that protects the enterocytes from the environment [1]. To gain
48 access to the enterocytes, an enteric pathogen like *Salmonella* should be able to cleave the
49 mucinous layer. In various human pathogens, glycoside hydrolases such as sialidases,
50 muraminidases, glucosaminidases, pullulanases, GalNAcases etc. are known to facilitate the
51 bacterial attachment to the host cells [2]. GH18 family protein chitinases and chitin binding
52 proteins were also found to be involved in pathogenesis of several human enteric (*Vibrio*
53 *cholerae*, *Listeria monocytogenes*, *Serratia marcescens*) [3-7] and non-enteric pathogens
54 (*Pseudomonas aeruginosa*, *Legionella pneumophila*) [8-10]. In all these pathogens,
55 commonality of the presence of mucin-rich environment hinted towards a potentially
56 significant role of chitinases and chitin binding proteins in breaching mucosal barrier.
57 *Salmonella* causes infection in the gut mucosal region which also has a protective mucinous
58 layer. A BLAST search revealed that *Salmonella* Typhimurium exochitinase ChiA (encoded
59 by STM14_0022) showed 20-40% identity with the abovementioned pathogenic proteins.
60 Further *Salmonella* Typhi chitinase (ChiA; STY0018) is 98% similar to the *S. Typhimurium*
61 SL1344 *chiA* (STM0022) that was reported to be upregulated ~12-20 fold in the infected
62 macrophages and ~4-5 fold in the epithelial cells [11, 12].
63 We infected epithelial cells and phagocytes with the mutant strain and interestingly we found
64 that the mutant was invasion defective in epithelial cells. *Salmonella* is known to remodel the
65 host cell surface glycans to facilitate invasion in the epithelial cells [13-15], we checked the
66 host cell surface glycan modification by lectin-binding assay. Our data suggest that chitinase
67 aids in glycan remodeling by cleaving the terminal sialic acid (Neu5Ac), and Gal- β 1,4-
68 GalNAc, thus making the mannose residues accessible to the bacteria for binding. Further we
69 found that the phagocytes infected with the mutant bacteria produced less antibacterial

70 molecules. Interestingly, the mutants were significantly less virulent, less persistent, and were
71 unable to dampen host antibacterial and immune responses in the *in vivo* infection models.
72 Moreover, in this study we demonstrated a novel role of ChiA in facilitating extra-intestinal
73 colonization of *Salmonella* Typhi in *C. elegans*. Together our data suggest that chitinase A
74 plays a multifaceted role in *Salmonella* pathogenesis ranging from aiding bacterial invasion
75 in epithelial cells, enhancing antibacterial NO production *ex vivo*, to increasing bacterial
76 persistence in the nematodes and regulating cellular and humoral immune responses *in vivo*.

77

78 **Results**

79 **Chitinase deletion impairs bacterial invasion in human epithelial cells**

80 Since previous reports suggest that chitinases and chitin-binding proteins (CBPs) were
81 involved in adhesion, invasion and *in vivo* pathogenesis of several human pathogens [3-10]
82 and STM ChiA and STY ChiA are 19-24% identical to these pathogenic proteins (**Fig. S1A**),
83 we made isogenic mutants of *chiA* using lambda red recombinase method [16]. The mutants
84 as well as the trans-complemented strain (STY Δ *chiA*:*chiA*) did not show any growth
85 difference *in vitro* (**Fig. S1B, S1C**), suggesting that Chitinase A is non-essential for
86 extracellular life of *Salmonella* sp. Upon entering the host and surviving through the acidic
87 stomach environment, *Salmonella* reaches gut epithelium, where SPI1-T3SS effectors induce
88 membrane ruffling in the enterocytes. This facilitates bacterial entry in the epithelial cells and
89 marks the beginning of *Salmonella* infection [17]. Since earlier report suggested that *chiA* of
90 *S. Typhimurium* SL1344 strain (STM0022) was highly upregulated in intracellular bacteria
91 from epithelial cells, we checked bacterial invasion and intracellular proliferation in Caco2
92 cells. We found that the *chiA* deletion rendered the bacteria less invasive and
93 hyperproliferative in epithelial cells (**Fig. 1A-D, S1D, S1E**). We next checked the expression
94 of SPI1 and SPI2 effector genes in intracellular bacteria as SPI1 effectors facilitate bacterial

95 invasion in epithelial cells and SPI2 effectors are required for intracellular survival and
96 proliferation. Surprisingly we found that SPI1 effectors *invF* and *hilA* were significantly
97 upregulated during the early phase of infection in the $\Delta chiA$ mutant bacteria, whereas no
98 significant difference was observed in the expression of SPI2 effector *ssaV* after 16 hours of
99 infection (**Fig. 1E-F**), suggesting that the reduced bacterial invasion in the epithelial cells by
100 $\Delta chiA$ mutant is independent of SPI1 gene expression.

101 **Chitinase A facilitates bacterial entry in epithelial cells by cell surface glycan**

102 **modification** The intestinal epithelium is covered with mucus and the cells are layered with
103 oligosachharide molecules that forms the glycocalyx. α 2-6, α 2-3, α 1-3, β 1-3 or β 1-6 linked
104 Glycosylation on the host epithelial cells follow a particular array, such as the outermost
105 glycosylation is N- acetylneuraminic acid (Neu5Ac or sialic acid), followed by galactose, N-
106 acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc) and the innermost moieties
107 mannose and fucose (**Fig. 2A**) [14]. To initiate infection, an enteric pathogen must breach the
108 protective glycocalyx layer. Particularly in the pathogenesis of *S. Typhi*, host cell surface
109 glycoproteins are known to play an important role in typhoid toxin mediated inflammation
110 [18]. Therefore, we checked the abundance of various glycosyl molecules present on Caco2
111 cells after *Salmonella* infection. Interestingly we observed that after 120 min of infection with
112 the WT bacteria, the host cell surface showed a significant decrease in the abundance of
113 sialylation on the surface glycome, which was not observed for the cells infected with $\Delta chiA$
114 mutants, suggesting chitinase is involved in removal of the terminal sialic acids (**Fig. 2B; top**
115 **panel**). This was further validated by the shift of the Neu5Ac-bound SNA-FITC lectins
116 towards lower abundance in flow cytometry analysis (**Fig. 2C; first column**). Consequently,
117 we observed a significant increase in the abundance of Gal- β 1,3-GalNAc on the cells infected
118 with WT bacteria as compared to the cells infected with $\Delta chiA$ strains (**Fig. 2B; middle**
119 **panel**), which was further corroborated by a significant shift of Gal-bound PNA-FITC lectins

120 towards higher abundance in flow cytometry analysis (**Fig. 2C; second column**). Finally, we
121 observed a substantial increase in the abundance of mannose-bound concanavalin A-FITC
122 fluorescence on the cell surface of WT bacteria infected cells as compared to the $\Delta chiA$
123 mutant infected cells (**Fig. 2B; bottom panel**) that was supported by a significant shift of
124 mannose-bound ConA-FITC lectins towards higher abundance in flow cytometry analysis
125 (**Fig. 2C; third column**). The cell surface glycan bound lectin fluorescence was further
126 quantified which further validated these observations (**Fig. S1F**) Together these data
127 suggested that *Salmonella* chitinase helps in the host cell surface remodeling.

128 ***Salmonella* ChiA is required for stabilization of the SCVs in epithelial cells**

129 In the epithelial cells, *Salmonella* resides in a double membrane compartment known as
130 *Salmonella*-containing vacuoles (SCVs). *Salmonella* replicates in the epithelial cells by
131 inhibiting the fusion of SCVs with lysosomes. SCVs are specialized endosomes that are
132 marked with the LAMPs, Rab7, Rab11 and vATPases. Since several reports suggested that
133 disruption of SCV leads to bacterial hyperproliferation in the cytoplasm [19], we checked
134 whether the enhanced proliferation of the mutant bacteria after the loss of chitinase is caused
135 by defect in SCV maintenance. We found that after 16 hours of infection, $\Delta chiA$ mutant
136 bacteria did not co-localize with the late-endosomal marker LAMP1 (**Fig. 3A, S1G, S1H**),
137 suggesting disruption of SCVs in the mutant bacteria infected cells. Upon counting the
138 number of SCV-bound and cytoplasmic bacterial population, we found that 81.6% of STM
139 $\Delta chiA$ and 87.2% of STY $\Delta chiA$ quit the vacuole, while very less WT bacteria (STM WT
140 12.2%, STY WT 8.2%) did not co-localize with LAMP1 (**Fig. 3B-C**). We further quantified
141 the cytosolic bacterial population by chloroquine resistance assay and found significantly
142 higher number of cytosolic mutant bacteria after 16 hours of infection (**Fig. 3D-E**) suggesting
143 that chitinase deletion leads to SCV destabilization in epithelial cells and hyper-proliferation
144 of the cytoplasmic bacteria.

145 **Chitinase aids in bacterial survival in phagocytes by suppressing antimicrobial**
146 **responses**

147 After successfully invading the epithelial cells, *Salmonella* reaches lamina propria, where it is
148 taken up by macrophages, DCs and neutrophils which marks the beginning of systemic
149 spread of the pathogen. To understand the role of chitinase in phagocytic cell infection, we
150 infected U937 monocytes and BMDCs. We found that $\Delta chiA$ mutants were invasion
151 defective in U937 monocytes, while the mutants showed enhanced survival in the monocytes
152 as compared to the WT strains (**Fig. 4A-D, S2A, S2B**). While STM WT and STM $\Delta chiA$
153 showed similar invasiveness, surprisingly STY $\Delta chiA$ showed increased invasion and better
154 survival in BMDCs (**Fig. 4E-H, S2C, S2D**). Higher invasiveness of STY $\Delta chiA$ in naturally
155 phagocytic cells hints towards that this strain might be highly immunogenic. Phagocytic cells
156 are known to inhibit intracellular bacterial growth by production of reactive nitrogen species
157 and reactive oxygen species [20]. Estimation of nitric oxide produced by the infected BMDCs
158 suggested that $\Delta chiA$ mutant infected cells produced significantly less nitric oxide (**Fig. 4I**).
159 We further checked the survival of the WT and $\Delta chiA$ in the absence of NO using NOS2^{-/-}
160 BMDCs and observed that WT bacteria survived similar to the $\Delta chiA$ mutants (**Fig. 4J-K**),
161 suggesting that chitinase might be involved in the induction of NO in DCs. Furthermore,
162 $\Delta chiA$ infected peritoneal macrophages (PM) showed significantly less ROS level as
163 compared to WT infected cells (**Fig. 4L**), indicating that chitinase might be regulating RNI
164 and ROS level in the infected cells. NO is an important cell signaling molecule that is
165 produced to kill many human pathogens, such as *Salmonella*, *Mycobacterium* and *Listeria* etc
166 [20]. Previous studies suggested that low level of NO enhances T cell survival [21], while
167 very high NO is capable of inhibiting T cell proliferation [22]. To check whether lesser
168 production of NO in response to $\Delta chiA$ mutant infection has any effect on antigen
169 presentation and T cell expansion, we looked into the proliferation of CD8⁺ T cell population

170 using OT1 transgenic mouse (C57BL/6-Tg(TcraTcrb) 1100Mjb/J). The TCR of this
171 transgenic mouse recognizes OVA₂₅₇₋₂₆₄ when presented by MHC-I molecules. This TCR
172 recognition of MHC-I bound cognate peptide results in CD8⁺ T cell proliferation, which can
173 be measured by incorporation of ³H thymidine in the DNA of the proliferating population.
174 We found that with $\Delta chiA$ mutant infection, the expansion of CD8⁺ T cell population was
175 significantly higher in response to the antigen stimulation (**Fig. 4N**). Since APCs such as
176 macrophages and DCs possess MHC-I and MHC-II molecules on the cell surface in order to
177 induce both CD8⁺ T cells and CD4⁺ T cells population, respectively, we detected the surface
178 MHC-II molecules on activated PMs. We found that upon $\Delta chiA$ infection, the surface MHC-
179 II level was similar to uninfected cells, while WT infection significantly reduced the surface
180 MHC-II level (**Fig. 4O, S2E**). Further immunofluorescence analysis verified that $\Delta chiA$
181 infection did not change the surface MHC-II molecules on macrophages (**Fig. 4M, S2F**).
182 Together these data suggests that *Salmonella* ChiA dampens host antimicrobial responses
183 leading to enhanced pathogen survival.

184 **Chitinase facilitates *in vivo* invasion, survival and pathogenesis of *Salmonella*** 185 **Typhimurium**

186 To further delineate the role of chitinase in *Salmonella* infection *in vivo*, we orally infected 6
187 weeks old C57BL/6J mice with lethal dose of the bacterial strains (10^8 CFU/animal) and
188 observed for survival. *Salmonella enterica* serovar Typhimurium mimics the systemic
189 typhoidal disease in murine model. STM $\Delta chiA$ infected cohort showed enhanced animal
190 survival as compared to the STM WT infected cohort (**Fig. 5A**), suggesting a role of chitinase
191 A during infection *in vivo*. We also found that STM $\Delta chiA$ mutant bacteria were shed prior to
192 the STM WT and the $\Delta chiA$ mutant was defective in PP colonization after 2 hours of oral
193 gavage (**Fig. 5B-C**). Further, we orally infected C57BL/6J mice with sublethal dose of
194 *Salmonella* strains (10^7 CFU/animal) and bacterial CFU from liver, spleen, mesenteric lymph

195 node (MLN) and PP was enumerated after sacrificing the animals after indicated time
196 intervals. We found that STM $\Delta chiA$ mutant infected animals showed less bacterial burden in
197 each of the organs and higher body weight as compared to the STM WT infected animals
198 (**Fig. 5D-H**). Since our *ex vivo* data suggested that the $\Delta chiA$ mutant was unable to induce
199 NO production which is known to affect T cell survival, we checked whether chitinase
200 mediated NO upregulation has any effect *in vivo*. We also found a significant increase in the
201 spleen length after 20 days of infection with STM $\Delta chiA$ bacteria, as compared to the STM
202 WT infected mice (**Fig. S3A, S3B**). We isolated total splenocytes from these infected spleens
203 and checked the activated CD4⁺ T cell population and T cell proliferation by flow cytometry.
204 We found that STM $\Delta chiA$ infection leads to a significant increase in CD4⁺ T cell population,
205 as well as an increase in the activated CD25⁺ T cells (**Fig. 5K**). Analysis of T cell mediated
206 cytokine response revealed that there was a significant increase in the pro-inflammatory
207 cytokines IL2 and IFN γ , in the serum isolated from $\Delta chiA$ infected animals (**Fig. 5I-J**),
208 whereas there was no difference in the anti-inflammatory cytokine levels (**Fig. S3C, S3D**).
209 Lesser IL2 in STM WT infected mice serum as compared to that in $\Delta chiA$ mutant infected
210 serum further strengthens the previous finding that ChiA aids in dampening of T cell
211 activation, as IL2 is a key marker of CD8⁺ T cell proliferation [23]. Previous reports
212 suggested that high level of IFN γ can induce B cell proliferation and enhance IgG2a and
213 IgG3 production [24]. Therefore, we looked into the role of chitinase in anti-*Salmonella*
214 immune response by detecting the anti-*Salmonella* IgG titer from infected mice serum.
215 Interestingly, we found a significant increase in the anti-*Salmonella* antibody titer in the
216 serum obtained from STM $\Delta chiA$ mutant infected cohort (**Fig. 5L**). We further used the
217 polyclonal convalescent sera isolated from STM $\Delta chiA$ infected mice to probe against STM
218 WT-mCherry whole cell lysate to test the polyclonality of the sera. Multiple dense bands
219 against various *Salmonella* proteins were obtained after incubating the membrane with sera

220 collected from STM $\Delta chiA$ mutant infected cohort (**Fig. S3E**). Together these data suggest
221 that *Salmonella* chitinase A is essential for restricting innate and humoral immune responses
222 against *Salmonella*.

223 **Chitinase helps in *Salmonella* Typhi pathogenesis in *C. elegans***

224 *Salmonella* Typhi is a human obligatory pathogen that does not cause a significant infection
225 in mice because of the presence of TLR11 [25]. Long before TLR11^{-/-} mice model came into
226 existence, Labrousse *et al.* suggested *Caenorhabditis elegans* can be used as an alternative
227 host to study *S. Typhi* pathogenesis [26]. Given that *C. elegans* pharyngeal lumen is rich in
228 chitin, it served as a suitable host to study the role of chitinase in bacterial pathogenesis [27].
229 We began with checking the bacterial CFU in the infected worms after 24 hours and 48 hours
230 of continuous feeding. We found that the STY $\Delta chiA$ and STY $\Delta chiA:pQE60$ strains showed
231 a higher bacterial burden after 24 hours of continuous feeding (**Fig. S3F**), but the fold change
232 of bacteria were lesser than that of the STY WT and STY $\Delta chiA:chiA$ strains (**Fig. 6A**). We
233 further checked animal survival after infecting the worms with different bacterial strains and
234 found that all the *Salmonella* Typhi strains are pathogenic to the animals as compared to *E.*
235 *coli* OP50, while STY $\Delta chiA$ showed slower death (TD₅₀ 330±8hrs) in the worms as
236 compared to the STY WT (TD₅₀ 190±10hrs) and STY $\Delta chiA:chiA$ (TD₅₀ 270±12hrs) strains
237 (**Fig. 6B**). Together these data suggest that chitinase deletion renders *Salmonella* Typhi less
238 virulent in *C. elegans* infection. We further checked bacterial colonization in the worm's gut
239 using the transgenic worm FT63 strain that expresses GFP in the epithelial cells. We
240 visualized the bacterial colonization in the worms gut after 24 hours and 48 hours of
241 continuous feeding. We used STM $\Delta invC$ mutant as a control which is known to be invasion
242 defective in nonphagocytic cells [28]. We found that *S. Typhi* $\Delta chiA$ showed less
243 colonization than STY WT after 24 hours continuous feeding, while the colonization was
244 significantly reduced after 48 hours feeding (**Fig. 6C**), suggesting chitinase is required for

245 successful gut colonization in *C. elegans*. Percent colonization was measured as the ratio of
246 the diameter of the lumen occupied by the bacteria to the total diameter of the gut (**Fig. S3G**).
247 Interestingly STM $\Delta invC$ did not show any defect in colonization in the *C. elegans* gut,
248 suggesting SPI1 effector InvC is not essential for colonization in the worms gut. We next
249 checked if *S. Typhi* utilizes chitinase to colonize the chitin-rich pharyngeal lumen by
250 infecting N2 worms with different strains of *Salmonella* and stained the chitin-rich parts of
251 the worms using eosin Y. We found that after 24 hours of continuous feeding, luminal STY
252 WT and STM $\Delta invC$ bacteria colocalized with the chitin-rich regions of the pharyngeal wall
253 and terminal bulb (grinder), whereas STY $\Delta chiA$ bacteria did not show any colocalization
254 with the pharyngeal wall (**Fig. 6D**), suggesting *Salmonella Typhi* utilizes chitinase to
255 colonize the chitin-rich pharynx and terminal bulb. Additionally, we looked into the role of
256 chitinase in bacterial persistence in the worms gut by infecting the worms with different STY
257 strains, followed by feeding onto *E. coli* OP50. We hypothesized that if a bacterial strain can
258 adhere to the gut lumen effectively, it will remain adhered to the gut lumen and proliferate
259 even when the worms are fed with *E. coli* OP50. We observed that after 24 hours of feeding
260 on STY $\Delta chiA$ followed by 24 hours feeding on *E. coli* OP50, the STY $\Delta chiA$ was unable to
261 persist in the gut, whereas STY WT showed significantly higher colonization in the
262 pharyngeal lumen (**Fig. S3H**). When we further extended the infection for 48 hours, followed
263 by 24 hours of *E. coli* OP50 feeding, we observed that STY WT showed profound
264 colonization of the gut lumen, while STY $\Delta chiA$ colonization was diminished (**Fig. 6E**),
265 suggesting *Salmonella* utilizes chitinase to attach to the lumen wall for enhanced persistence
266 in the worms. Interestingly, after 24 hours of continuous feeding STY WT attached to the
267 luminal wall, but not STY $\Delta chiA$ strain. (**Fig. S4A**). After 48 hours of continuous feeding, we
268 detected STY WT and STM $\Delta invC$ bacteria in the extra-intestinal tissues of the worms, while
269 STY $\Delta chiA$ did not show extra-intestinal colonization (**Fig. 7A, S4A, S4B**), suggesting that

270 chitinase might be required to invade extra-intestinal tissues of the worms. To the best of our
271 knowledge, this study is the first report suggesting an extra-intestinal invasion/colonization
272 by *Salmonella* Typhi in *C. elegans*.

273 ***Salmonella* chitinase is important for alteration of metabolism and antibacterial defense**
274 **in *C. elegans***

275 Grinder, a part of the terminal bulb, is the complex structure that helps in uptake and grinding
276 of bacteria before it passes to the intestine where the nutrients get absorbed. In a healthy and
277 well-fed state, worms feed at the average rate of 200 pumps/min. Since we observed that
278 *Salmonella* uses chitinase to colonize chitin-rich organs (**Fig. 6D**), we next looked into the
279 nutritional state of the worms by counting the number of pharyngeal pumps per min. We
280 found a significant reduction in the number of pharyngeal pumps/min after 72 hours of STY
281 WT and STY $\Delta chiA:chiA$ infection (**Fig. 7B**). Further, *in vivo* oxidative stress was quantified
282 using CL2166 worms, that possess oxidative stress inducible GFP. STY WT and STY
283 $\Delta chiA:chiA$ infected worms showed significantly higher oxidative stress and ‘bag of worms’
284 phenotype (**Fig. S4C, S4D**). We next checked the nutritional fitness of the worms by
285 measuring the lipid content using oil red O staining. Oil red O (ORO) is a fat-soluble dye that
286 stains neutral lipids [29]. Interestingly we observed significant fat-loss in the worms fed with
287 STY WT and STY $\Delta chiA:chiA$ for 24 hours and 48 hours as compared to STY $\Delta chiA$
288 infected wormed (**Fig. 7C, S4E**). We further checked whether chitinase deletion has any
289 effect on the immune response of the worms. Although our data suggest both STY WT and
290 STY $\Delta chiA$ downregulated p38 MAP kinase pathway genes *pmk1* and *mek1* equally (**Fig.**
291 **S4F**), p38 MAP kinase pathway regulated antimicrobial peptides were differentially
292 expressed. While *clec85*, *lys7*, *ilys2* expressions were severely downregulated in both STY
293 WT and STY $\Delta chiA$ infection, all of them showed significant rescue in the worms infected
294 with STY $\Delta chiA$ bacteria (**Fig. 7D**). Interestingly, we observed that STY WT infection

295 induced downregulation of antimicrobial peptide *spp1* was completely rescued upon STY
296 $\Delta chiA$ infection, while *abf2* was significantly upregulated upon infection with STY $\Delta chiA$
297 (**Fig. 7E**), indicating an important contribution of chitinase in dampening the antimicrobial
298 responses of the host.

299

300 **Discussion**

301 *Salmonella* is a facultative intracellular human pathogen that has co-evolved with its host and
302 has also developed various strategies to evade the host's immune responses. The detailed
303 understanding of the metabolism and the ease of genetic manipulation has made *Salmonella*
304 an excellent the model to study the role of metabolism related proteins in the light of host-
305 pathogen interaction. Although *Salmonella* pathogenesis is governed by classical virulence
306 factors such as adhesins, invasins and toxins, emerging reports suggest that various unique
307 metabolic proteins are important in various aspects of *Salmonella* pathogenesis. Several
308 reports suggest that *Salmonella* can utilize a large pool of chemically diverse host nutrients,
309 such as carbohydrates, lipids, amino acids etc [30]. One such carbon metabolism related
310 protein encoding gene *chiA* (STM0022) was found to be highly upregulated in intracellular
311 *Salmonella* Typhimurium str. SL1344 isolated from infected macrophages and epithelial cells
312 [11, 12]. Bacterial chitinases belong to GH18 and GH19, which are getting recognized as
313 bacterial virulence factors along with several other structurally similar glycosidases such as
314 sialidases, muraminidases, N-acetylgalactosidases etc [2]. Although *Salmonella chiA* was
315 upregulated during infection, the role of this chitinase in *Salmonella* pathogenesis remains
316 elusive. To answer this question, we generated isogenic $\Delta chiA$ mutant by one-step gene
317 inactivation method. We did not find any significant difference in the *in vitro* growth among
318 the two strains. To test whether *chiA* upregulation in infected cells has any significance in the

319 pathogenesis, we infected epithelial cells and phagocytes with the mutant strain. Interestingly,
320 we found that the mutant was invasion defective in epithelial cells. Previous reports
321 suggested that *Salmonella* remodels the host cell surface glycans to facilitate invasion in the
322 epithelial cells [13-15]. Our observations from the lectin-binding assay suggests that chitinase
323 aids in glycan remodeling by cleaving the terminal glycosyl molecules and making the
324 mannose residues accessible to the bacteria for binding. We further found that absence of
325 ChiA leads to destabilization of the SCV and hyper-proliferation of the mutant bacteria in the
326 cytoplasm of the epithelial cells. The $\Delta chiA$ mutants survived better than WT strains in the
327 phagocytes, suggesting the $\Delta chiA$ mutants were protected from phagocytes mediated bacterial
328 killing. Additionally, we found that the phagocytes infected with the mutant bacteria
329 produced less antimicrobial molecules such as NO and ROS. We also found that ChiA was
330 important for downregulating the MHC-I molecules on the dendritic cells, leading to the
331 inhibition of CD8⁺ T cell proliferation and subsequent antigen presentation. In coherence
332 with the available literatures [22], the enhanced T cell proliferation could be attributed to the
333 absence of NO induction by the $\Delta chiA$ mutant strains. We further showed that absence of
334 *chiA* failed to downregulate the surface MHC-II molecules on the activated macrophages,
335 which is a well-known phenomenon during *Salmonella* infection [31]. *In vivo* infection in
336 C57BL/6 mice suggested that STM $\Delta chiA$ mutant was unable to invade the Peyer's patches,
337 leading to an early fecal shedding and enhanced pathogen clearance. STM $\Delta chiA$ mutant
338 infected cohort presented significantly less bacterial burden in the liver, spleen, MLN and PP,
339 as well as they showed increased survival, suggesting that the STM $\Delta chiA$ mutant is less
340 virulent *in vivo*. Analysis of total splenic lymphocytes by flow cytometry suggested that the
341 $\Delta chiA$ mutant infected cohort had an increased activated T cell population (CD4⁺CD25⁺) in
342 the spleens, suggesting an intensified immune response in these mice. This was corroborated
343 by significant increment in the pro-inflammatory cytokines and anti-STM IgG antibody titer

344 in the STM $\Delta chiA$ infected mice sera. In the invertebrate *C. elegans* model, chitinase helps in
345 bacterial attachment to the pharyngeal lumen. Additionally, we found that the chitinase helps
346 in *Salmonella* Typhi colonization and persistence in the worms, since deletion of *chiA* leads
347 to bacterial clearance from the worm's gut. In addition, our data suggest that *Salmonella*
348 Typhi chitinase might help in extra-intestinal tissue invasion in the worms. Chitinase was
349 found to be regulating the fat-responsive immune response such as antibacterial peptide
350 synthesis in the worms. We found significantly higher expression of antimicrobial peptides
351 genes *spp1* and *abf2* when the worms were infected with STY $\Delta chiA$ strain, hinting towards a
352 potential role of chitinase in modulating innate immune response in the worms. Together our
353 data suggest that *Salmonella* Chitinase regulates different aspects of pathogenesis, ranging
354 from aiding in invasion in the epithelial cells, impairing the activity of professional antigen
355 presenting cells to as diverse as immune response regulation in various hosts (**Fig. 7F**) and
356 emerges as a novel virulence factor.

357

358 **Materials and Methods**

359 **Bacterial strains**

360 All *Salmonella* Typhimurium strains used in this study are listed below with their genetic
361 description. *Salmonella enterica* serovar Typhimurium strain 14028S was used as the wild
362 type strain, and was also the parental background for all the mutant strains used in this study,
363 i.e. $\Delta chiA$ and $\Delta invC$. All strains were grown and maintained in Lennox broth (LB; 0.5%
364 NaCl, 1% casein enzyme hydrolysate and 0.5% yeast extract) at 37°C under shaking
365 conditions. *Salmonella enterica* serovar Typhi strain CT18 was used as the wild type strain,
366 and was also the parental background for the mutant strain used in this study, i.e. $\Delta chiA$. *S.*
367 Typhi *chiA* was trans-complemented in pQE60 plasmid in 5' NotI-*chiA*-BamHI 3' direction.

368 This plasmid was transferred to STY $\Delta chiA$ strain to make complement strain.
369 Complemented strain STY $\Delta chiA:chiA$ and empty vector strain STY $\Delta chiA;pQE60$ strains
370 were maintained on LBA supplemented with ampicillin (50 $\mu\text{g/ml}$). The mCherry expressing
371 strains were cultured in Lennox broth with 50 $\mu\text{g/ml}$ Ampicillin at 37°C in shaking condition.
372 List of strains used in this study.

Strain name	Description	Reference
<i>S. Typhimurium</i> ATCC 14028S (STM WT)	Wild type (WT)	Kind gift from Prof. M. Hensel (Division of Microbiology, University of Osnabrück, Germany)
STM $\Delta chiA$	Isogenic knockout strain for the gene <i>chiA</i> ; Kan ^r	This study
STM $\Delta invC$	Isogenic knockout strain for the gene <i>invC</i> ;	Kind gift from Prof. M. Hensel (Division of Microbiology, University of Osnabrück, Germany)
<i>S. Typhi</i> CT18 (STY WT)	Wild type (WT)	PGIMER, Chandigarh
STY $\Delta chiA$	Isogenic knockout strain for the gene <i>chiA</i> ; Kan ^r	This study
STY $\Delta chiA:chiA$	Isogenic complement strain for $\Delta chiA$ expressing <i>chiA</i> under the T5	This study

	promoter present in the pQE60 plasmid; Kan ^r Amp ^r	
STY Δ <i>chiA</i> :pQE60	Isogenic complement strain with empty pQE60 plasmid; Kan ^r Amp ^r	This study
mCherry tagged strains	Respective strains carrying pFPV-mCherry plasmid, Amp ^r	This study

373

374 **Isolation and maintenance of primary cells and cell lines**

375 Human colorectal adenocarcinoma cell line Caco2 (ATCC HTB-37) was cultured in
376 complete DMEM media (Lonza), whereas human monocyte cell line U937 (ATCC CRL-
377 1593.2) was maintained in complete RPMI 1640 media (Lonza) with 100 μ M β -
378 mercaptoethanol and differentiated to macrophages using 20 ng/ml PMA for 24 hours prior to
379 infection. Bone-marrow was isolated from either wildtype (NOS2^{+/+}) C57BL/6J mice or
380 NOS2^{-/-} C57BL/6J mice as described previously [31]. Briefly, tibia and femur bones were
381 carefully taken out, caps were removed and the marrow was flushed with RPMI 1640 media
382 using a 26G needle. After making single cell suspension, RBCs were lysed using RBC lysis
383 buffer. Cells were pelleted and grown in complete RPMI 1640 media supplemented with 20
384 ng/ml mGM-CSF (Peprotech), antibiotics and 100 μ M β -mercaptoethanol. After every 2
385 days, the media was replenished. Once approximately 65-70% of the cells were differentiated
386 to dendritic cells (loosely adherent spheres), the cells were collected and used for further
387 experiments. To obtain peritoneal macrophages (PMs), thioglycolate was injected in the
388 peritoneal cavity of C57BL/6J mice. After 5 days these mice were sacrificed and ice cold
389 PBS was injected in the peritoneum to collect the peritoneal exudate. Any residual

390 erythrocytes were lysed using RBC lysis buffer and the cells were maintained in complete
391 RPMI 1640 media for further experiments.

392 **Generation of deletion mutant**

393 $\Delta chiA$ mutant strains were made using one-step deletion strategy as mentioned by Datsenko
394 and Wanner [16]. Briefly, wild-type *Salmonella* (*S. Typhimurium* 14028S or *S. Typhi* CT18)
395 bacteria transformed with a ‘lambda red recombinase’ expressing plasmid under arabinose
396 inducible promoter (pKD46), was grown in LB with 50 $\mu\text{g/ml}$ ampicillin and was induced
397 with 10 mM L-arabinose at 30 °C to an OD_{600} of 0.35-0.4. Electrocompetent cells were
398 prepared by pelleting the bacterial cells and washing the pellet three times with ice cold,
399 sterile MiliQ water and 10% glycerol, followed by resuspension in 50 μl of 10% glycerol.
400 Kanamycin resistance cassette was amplified from pKD4 plasmid using primers containing
401 upstream and downstream sequences of *S. Typhimurium chiA* gene (STM14_0022) and *S.*
402 *Typhi chiA* gene (STY0018) fragment. 500 ng of this PCR product was purified and used for
403 electroporation. Transformants were selected on LB agar containing kanamycin plates and
404 were further confirmed with confirmatory primers, *chiA* specific RT primers and kanamycin
405 resistance cassette internal primers.

406 **Infection and gentamicin protection assay**

407 Epithelial Caco-2 cell line was infected with mid-log phase culture of bacteria grown in LB
408 (OD_{600} 0.3), whereas phagocytic U937 derived monocytes and BMDCs were infected with
409 overnight culture (OD_{600} 0.3). The multiplicity of infection (MOI) of 10 was used in each
410 case. Bacterial attachment to host cells was enhanced by centrifuging at 600 rpm for 10 min.
411 After 25 min of infection, cells were treated with gentamicin (100 $\mu\text{g/ml}$ in complete media)
412 for 1 hour to remove extracellular bacteria and then maintained with 25 $\mu\text{g/ml}$ gentamicin for
413 rest of the experiment. 0.1% Triton-X 100 (v/v in 1x PBS) was used to lyse the cells and the

414 lysate was plated on *Salmonella-Shigella* (SS) agar for *S. Typhimurium* strains and Wilson
415 Blair (WB) agar for *S. Typhi* strains. For invasion assay, cells were lysed after incubation in
416 100 µg/ml gentamicin treatment (i.e. 1 hour post infection) and percent invasion was
417 calculated with respect to the pre-inoculum used for infection. For intracellular survival assay
418 (ICSA), infected cells were lysed at 2 hours and 18 hours post infection. CFU at 18 hours was
419 divided by CFU at 2 hours to obtain fold replication of the intracellular bacteria. For
420 estimating the cytoplasmic bacterial population, chloroquine resistance assay was performed
421 [32]. Briefly, Caco2 cells were infected by different bacterial strains as mentioned previously.
422 The infected cells were treated with 800 µM chloroquine 1 hour prior to cell lysis and
423 absolute CFU were calculated by plating the cell lysate on selective media.

424 **Quantitative RT-PCR**

425 Bacterial RNA was isolated from infected cells as described previously by Eriksson *et*
426 *al.*[11]. Briefly, *Salmonella* infected cells were lysed at different time intervals on ice by
427 incubating for 30 minutes with 0.1% SDS, 1% acidic phenol and 19% ethanol in sterile water.
428 Eukaryotic cell debris was removed by centrifuging the cell lysate at 300g for 10 minutes,
429 followed by pelleting bacterial cells at 5000 rpm for 5 minutes. At each time point, bacteria
430 were recovered from a 6-well plate of infected U937-derived monocytes and pooled to isolate
431 RNA. *In vitro* grown bacterial RNA was obtained by growing bacteria statically at 37 °C in
432 RPMI 1640 medium, under 5 % CO₂. The bacterial pellet was resuspended in TRIzol reagent
433 (Takara) and stored at -80 °C. Young adult hermaphrodites were infected with respective
434 bacterial strains for 48 hr. Infected worms were harvested by washing the plates with M9
435 buffer and pelleting at 1000g for 1 min. The extracellular bacteria were removed by
436 repeatedly washing the pellet for 5-6 times. The worms pellet was resuspended in TRIzol
437 reagent (Takara) and stored at -80 °C. RNA was isolated by phase separation method using
438 chloroform. cDNA was synthesized with reverse transcriptase (GCC Biotech). Quantitative

439 PCR was carried out using SYBR Green Q-PCR kit (Takara). Relative expression with
 440 respect to control (*act2* gene) was plotted as fold change. Relative expression with respect to
 441 control (16s rRNA gene for bacterial genes and *act2* for *C. elegans* genes) was plotted as fold
 442 change.

Gene	Sequence (5'-3')
STM <i>chiA</i> KO FP	TTATGGACCCCGCAGAACGAGCTGCGACAATTTTG AAACGTAAAAGGAAATTTGAAAGTGTAGGCTGGAG CTGCTTC
STM <i>chiA</i> KO RP	GGTAAACCAGGGCTTGAATCATGAAGCCCAATACA TCGGCTTAATACCGTGTACATATGAATATCCTCCTT AG
STM <i>chiA</i> conf FP	GCTGCGACAATTTTGAAAC
STM <i>chiA</i> conf RP	GAAGCCCAATACATCGG
STY <i>chiA</i> KO FP	GGACCCCGCAGAACGAGCTGCGACAATTTTGAAAC GTAAAAGGAAATTTGAAAGTGTAGGCTGGAGCTGC TTC
STY <i>chiA</i> KO RP	CCCCGGTAAACCGGGGCTTGAATCATGAAGCCCAA TACATCGGCTTAATACCGTGTACATATGAATATCCT CCTTAG
STY <i>chiA</i> conf FP	CTGCGACAATTTTGAAACG
STY <i>chiA</i> conf RP	CCAATACATCGGCTTAATACC
STY <i>chiA</i> :pQE60- <i>chiA</i> FP	TACGCCATGGATGGCTACAAGCAAAGTCAAG
STY <i>chiA</i> :pQE60- <i>chiA</i> RP	AGTCGGATCCTTAGTAAGCGCCAAGATCGG
STM/STY <i>chiA</i> RT FP	CGGAAGAGGAAGAAGAGATT

STM/STY <i>chiA</i> RT RP	CATAGACCACCATTTCACCT
<i>invF</i> FP	AGATCGTAAACGCTGCGAGT
<i>invF</i> RP	CTGCTGCACAAACGACGAAA
<i>hilA</i> FP	GCCGGTGACCATTACGAAGA
<i>hilA</i> RP	AAGAGAGAAGCGGGTTGGTG
<i>ssaV</i> FP	TATTGATAGGCGCGGACGCTA
<i>ssaV</i> RP	CGCCTTATGGGCCATGTCTTT
<i>phoP</i> FP	GATCTCTCACGCCGGAATT
<i>phoP</i> RP	TGACATCGTGCGGATACTGG
<i>sodA</i> FP	CCTGCCGGTTGAAGAACTGA
<i>sodA</i> RP	GGTTGCTGCTGCTTTTTTCGA
STM 16s rRNA FP	GTGAGGTAACGGCTCACCAA
STM 16s rRNA RP	TAACCGCAACACCTTCCTCC
<i>C. elegans act2</i> FP	ATCGTCCTCGACTCTGGAGAT
<i>C. elegans act2</i> RP	TCACGTCCAGCCAAGTCAAG
<i>C. elegans pmk1</i> FP	CCAAAAATGACTCGCCGTGA
<i>C. elegans pmk1</i> RP	CTTTTGCAGTTGGACGACGA
<i>C. elegans mek1</i> FP	AGCAGCCAATTCCAGAGAGA
<i>C. elegans mek1</i> RP	CGATCAGTCTGCCAGCAATA
<i>C. elegans clec85</i> FP	CCAATGGGATGACGGAACCA
<i>C. elegans clec85</i> RP	CTTCTGTCCAGCCAACGTCT
<i>C. elegans lys7</i> FP	GTACAGCGGTGGAGTCACTG
<i>C. elegans lys7</i> RP	GCCTTGAGCACATTTCCAGC
<i>C. elegans ilys2</i> FP	TGTTGGATCGCTTTCTTGTG

<i>C. elegans ilys2</i> RP	CATTATGGTTCGGGCCATC
<i>C. elegans spp1</i> FP	TGGACTATGCTGTTGCCGTT
<i>C. elegans spp1</i> RP	ACGCCTTGTCTGGAGAATCC
<i>C. elegans abf2</i> FP	CCGTTCCCTTTTCCTTGCAC
<i>C. elegans abf2</i> RP	GACGACCGCTTCGTTTCTTG

443

444 **Lectin binding assay for cell surface glycan modification**

445 Human colorectal carcinoma cells Caco2 were infected with different bacterial strains as
446 mentioned before. For confocal imaging, cells were seeded on coverslips prior to infection.
447 After infection for the specified time, the cells were fixed with 3.5% PFA for 20 min on ice.
448 For flow cytometry, cells were washed with PBS and treated with 1x Trypsin-EDTA (TE) for
449 15 min, under 5% CO₂ at 37 °C. After the cells were dislodged from the wells, the TE was
450 removed and cells were incubated with 1 ml complete media for 20 min under 5% CO₂ at 37
451 °C for recovery. To avoid non-specific lectin binding the cells were treated with blocking
452 buffer (PBS+2% FBS) at RT for 15 min. Specific lectins (50µg/ml lectin solution in blocking
453 buffer for every 10⁶ cells) (Vector Laboratories; #FL-1301, #FL-1071, #FL-1001) were
454 added to each samples and incubated for 30 min at RT, followed by washing with blocking
455 buffer. Cells treated with only FITC dye (Merck; #46950) were used as controls.

456 **Flow cytometry and immunofluorescence**

457 Cells were fixed with 3.5% PFA for 20 min on ice. All staining except for the surface
458 markers (MHC II, CD4 and CD25), were performed in the presence of permeabilizing agent,
459 0.01% saponin (Sigma) dissolved in 2.5% BSA containing PBS. Flow cytometry analysis
460 was carried out using BD FACSVerse and BD FACSAria and data were analyzed using BD
461 FACSDiva software. Immunofluorescence images were obtained using Zeiss LSM 710
462 and/or Zeiss LSM 880. The images were analyzed using ZEN Black 2012 platform. For

463 analysis of activated T cell population (CD4⁺ CD25⁺) from infected mice spleen, splenocytes
464 were isolated from mice that survived through 20 days of infection. Total splenocytes were
465 fixed using 3.5% PFA on ice for 20 min, followed by incubation for 1 hour at RT with
466 fluorophore conjugated antibody cocktail in dark. The cells were washed with 1x PBS and
467 analyzed by flow cytometry. Anti-mouse LAMP1 (DSHB; #1D4B) antibody was used for
468 immunofluorescence microscopy. Anti-mouse I-A/I-E (or MHC II) (clone 2G9) FITC (BD
469 Pharmingen; #553623) antibody was used for immunofluorescence microscopy and flow
470 cytometry. Anti-mouse CD4 FITC (Invitrogen; #11-0041-85), Anti mouse CD25 PE
471 (Invitrogen; #12-0251-82) antibodies were used for flow cytometry.

472 **Nitric oxide estimation**

473 Sodium nitrite (Sigma) standards of 100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM and 3.13 µM
474 were prepared by diluting 0.1 M stock in deionized distilled water. Conditioned media from
475 infected cells were collected after indicated time intervals for estimation of nitrite by Greiss
476 assay [33]. 1% sulphanilamide solution was made in 5% phosphoric acid. To 50 µl of the
477 standards and the samples (in triplicates), 50 µl acidic sulphanilamide was added and
478 incubated at RT, in dark for 10 min. After incubation, 50 µl of 0.1% NED (N-1-
479 naphthylethylene diamine dihydrochloride) solution was added to it and incubated for 10 min
480 in dark at RT. OD₅₂₀ was measured within 30 min of appearance of purple/magenta colored
481 product using TECAN Infinite Pro 200 microplate reader.

482 **ROS measurement**

483 Intracellular ROS was detected by 2', 7'-dichlorofluorescein diacetate (H₂DCFDA; Sigma)
484 staining. Cells were stained with 10µM DCFDA at 37 °C in dark. After 30 min, cells were
485 washed with ice cold PBS and harvested followed by flow cytometry analysis at 495/530 nm
486 in BD FACSVerse.

487 **T cell proliferation assay**

488 WT BMDCs were infected by incubating the bacteria with DCs for 90min, followed by
489 removal of the bacteria and incubating the infected cells with 25µg/ml gentamicin. Total
490 splenocytes were isolated from the spleen of C57BL/6-Tg (TcraTcrb) 1100Mjb/J mice by
491 mechanical disruption. Erythrocytes were lysed by RBC lysis buffer (Sigma) and cells were
492 maintained in complete RPMI-1640. Finally, non-adherent cells were collected and were
493 used for mixed lymphocyte proliferation assay. The proliferation of the lymphocytes in
494 response to antigen stimuli, was detected by incorporation of the $^3\text{H}_1$ as measured by the
495 scintillation counter.

496 ***In vivo* experiment**

497 6 weeks old male C57BL/6J mice were used for all the *in vivo* mice experiments. All animal
498 experiments were approved by the Institutional Animal Ethics Committee
499 (CAF/Ethics/670/2019) and the National Animal Care Guidelines were strictly followed. 10^8
500 CFUs of overnight grown STM WT and STM $\Delta chiA$ mutant bacteria were used for oral
501 infection for animal survival assay. The control group was orally administered with sterile 1x
502 PBS. Animals were observed for 20 days for survival and body weight was documented. For
503 *in vivo* invasion, the animals were euthanized after 2 hours of gavage, and the bacterial CFUs
504 in Peyer's patches (PP) were estimated. To check the bacterial shedding, fecal pellets were
505 collected aseptically from infected cohorts after indicated time. Homogenates were plated on
506 SS agar plates and CFUs were counted. For estimating *in vivo* bacterial burden in different
507 organs, a sublethal dose of 10^7 CFUs of each bacterial strain were used and bacterial CFUs
508 from liver, spleen, MLN and PP were enumerated after indicated time intervals. Spleens were
509 isolated from the animals after 20 days and the length was measured.

510 **ELISA for serum cytokines and anti-*Salmonella* IgG**

511 Blood collected from infected animals by cardiac puncture under aseptic conditions, was
512 incubated at RT to facilitate coagulation. Serum was then isolated by centrifugation at
513 5000 rpm for 10 min at RT and stored at -20 °C for further use. Estimation of serum level of
514 different pro-inflammatory cytokines (IL2 and IFN γ) and anti-inflammatory cytokines (IL10
515 and IL4) was performed according to the manufacturer's instructions. Anti-*Salmonella* IgG
516 titer was measured by sandwich ELISA as mentioned previously [34]. Briefly, wells were
517 coated with *Salmonella* LPS (200 ng/well; Sigma) at 4 °C overnight. Next day, LPS was
518 removed and the wells were washed with PBST (PBS+0.05% Tween 20), followed by
519 blocking for 1 hour at RT with 5% FBS in PBS to avoid non-specific binding. After blocking,
520 wells were washed with PBST. The serum samples, diluted in blocking buffer, were added to
521 the wells in triplicates and incubated for 2 hours. Subsequently, wells were washed with
522 PBST and anti-mouse IgG (HRP conjugate) was then added to the wells and incubated for 1
523 hour at RT. Tetramethylbenzidine (TMB; Sigma) was added and the plate was incubated in
524 dark for 20-30 min. The reactions were stopped with 2 N H₂SO₄ and the absorbance was
525 measured at 450 nm.

526 ***In vivo* colonization in *Caenorhabditis elegans***

527 *C. elegans* var. Bristol worms wildtype strain N2, FT63 [xnIs17; dlg-1::GFP + rol-
528 6(su1006)], and CL2166 [dvls19 III; dvls (pAF15)gst-4p::GFP::NLS III] strains were
529 maintained on NGM media at 20 °C. L4 or Young adult N2 hermaphrodite worms were used
530 for *in vivo* experiments. 10⁷ CFU of different bacterial strains were seeded on NGM plates
531 and grown for 16 hours. Young adult N2 worms were fed at 20 °C with the different bacterial
532 strains for 24 hours or 48 hours to check bacterial colonization in the worms [35]. Bacterial
533 CFU was enumerated by plating worms' lysate from equal number of infected worms on WB
534 agar plates. Fold change was calculated as the ratio of CFU after 48 hours to CFU after 24

535 hours. For confocal analysis of the worm gut colonization, FT63 worms were used. mCherry
536 expressing bacterial strains were used to visualize the gut colonization.

537 Further to check worms survivability, 10^7 CFUs of overnight grown bacterial strains were
538 seeded on 30 mm dishes containing Brain Heart Infusion (BHI) agar media. ~30-40 young
539 adult worms were added at the center of each plate and survival was monitored [36]. Animals
540 were transferred to fresh bacterial plates every day for first 5 days and then after every 5
541 days. The worms were scored as live or dead at regular intervals throughout the course of the
542 assay. Worms were considered dead when they failed to respond to touch stimulus.

543 Chitin-rich organs were visualized using Eosin Y stain. After 24 hours of infection, worms
544 were harvested and washed 5 times with M9 buffer, followed by washing the worms pellet
545 with citrate phosphate buffer (0.2 M Na_2HPO_4 , 0.1 M potassium citrate, pH 6.0). The worms
546 were resuspended in 500 μL citrate-phosphate buffer and 15 μL of 5 mg/ml eosin Y (in 70%
547 ethanol) was added to it. Tubes were incubated at RT, in dark for 10 minutes, followed by
548 centrifugation at 1000g for 1 min for washing. The supernatant was discarded and the pellet
549 was washed with citrate phosphate buffer 5 times to remove excess eosin Y.

550 Effect of bacterial colonization was determined by infecting CL2166 worms for 48 hours
551 with different strains. CL2166 worms possess oxidative stress inducible GFP. Fluorescence
552 of the infected worms was visualized using Zeiss LSM 880 with Multiphoton mode.

553 **Bacterial persistence assay in *C. elegans***

554 Young adult N2 worms were infected as mentioned previously. After 24 hours or 48 hours of
555 infection, the worms were harvested and washed 5 times with M9 buffer. After indicated
556 time, ~30 worms were mounted for confocal imaging. Rest of the worms were transferred to
557 *E. coli* OP50 plate for further 24 hours. These worms were harvested, washed and imaged as
558 mentioned previously.

559 **Quantification of pharyngeal pumps**

560 The effect of bacterial colonization on the chitin-rich grinder integrity was determined by
561 counting number of pharyngeal pumps per min. Young adult worms were infected as
562 mentioned previously. After indicated infection time, no. of pharyngeal pumps/min was
563 counted for ~25 worms from each infected plate.

564 **Fat estimation by Oil red O**

565 Neutral lipids present in the worms was estimated by Oil Red O (ORO; Sigma) staining [29].
566 Briefly, solution of Oil Red O was prepared in isopropanol (5 mg/ml) and diluted to 60% in
567 water before use. Synchronized L4 animals were allowed to feed on *E. coli* and STY strains
568 for 48 hr. Worms were harvested in M9 buffer, followed by fixing and permeabilizing using
569 MRWB buffer (160 mM KCl, 40 mM NaCl, 14 mM Na₂-EGTA, 1 mM spermidine-HCl, 0.4
570 mM spermine, 30 mM Na-PIPES [Na-piperazine N, N'-bis(2-ethanesulfonic acid); pH 7.4],
571 0.2% β -mercaptoethanol, 0.2% paraformaldehyde) for 1 hour at RT. The animals were
572 stained with 60% ORO at RT. Excess stain was removed by washing twice with 1x PBST
573 (PBS+0.01% Tween 20). Stained animals were mounted on agar pads.

574 **Statistical analysis**

575 Data were plotted using GraphPad Prism 6 software. Statistical analysis was performed using
576 Student's t-test or ANOVA as indicated. The results are expressed as mean \pm SEM. p values
577 <0.05 was considered to be significant (p values: ****<0.0001, ***<0.001, **<0.01,
578 *<0.05).

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590 **Author Contribution**

591 KC and DC conceived the study and designed experiments. KC performed experiments,
592 analyzed the data, prepared the figures and wrote the manuscript. DC supervised the work.
593 All the authors read and approved the manuscript.

594

595 **Competing Interests**

596 The authors declare no competing interests.

597

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692

693 **Figure Legends**

694 **Fig 1. Chitinase deletion impairs bacterial invasion in human epithelial cells.** (A) %
695 invasion and (B) Intracellular proliferation of STM WT and STM $\Delta chiA$ strains in Caco2
696 cells by gentamicin protection assay. Data are represented as mean \pm SEM of 3 independent
697 experiments (N=3, n=3). Unpaired Student's t test was used to analyze the data. (C) %
698 invasion and (D) Intracellular proliferation of STY WT, STY $\Delta chiA$, STY $\Delta chiA:chiA$ and
699 STY $\Delta chiA:pQE60$ strains in Caco2 cells by gentamicin protection assay. Data are
700 represented as mean \pm SEM of 3 independent experiments (N=3, n=3). One-way ANOVA

701 was used to analyze the data. **(D-E)** RNA expression level of SPI1 and SPI2 genes in from
702 STM WT and STM $\Delta chiA$ during **(D)** early phase and **(E)** late phase of infection in Caco2
703 cells. Data are represented as mean \pm SEM of 3 independent experiments (N=3, n=3). Two-
704 way ANOVA was used to analyze the data.

705 **Fig 2. Chitinase helps in glycan remodeling in host epithelial cells. (A)** Cell surface
706 glycan assembly. **(B)** Representative confocal images of Caco2 cells stained with SNA-FITC
707 (top panel), PNA-FITC (middle panel) and ConA-FITC (bottom panel) lectin after indicated
708 time intervals of STM WT, STM $\Delta chiA$, STY WT and STY $\Delta chiA$ infection (UI-
709 Uninfected). **(C)** Representative flow cytometry histogram showing the cell surface Neu5Ac-
710 bound SNA-FITC (first column), Gal bound PNA-FITC (second column) and mannose
711 bound ConA-FITC (third column) lectin (UI- Uninfected). Solid lines represent MFI after 30
712 min and dashed lines represent MFI after 120 min. Data are represented as mean \pm SEM of 2
713 independent experiments (N=2).

714 **Fig 3. Chitinase deletion destabilizes the *Salmonella*-containing vacuoles in the epithelial**
715 **cells leading to hyperproliferation of the cytosolic bacteria. (A)** Representative confocal
716 image of Caco2 cells infected with STM WT, STM $\Delta chiA$, STY WT and STY $\Delta chiA$ strains
717 to visualize the intracellular niche of the bacteria. The SCVs were stained for LAMP1 in
718 presence of 0.01% saponin and 2.5% BSA (UI- Uninfected). **(B)** % of STM WT and STM
719 $\Delta chiA$, **(C)** % of STY WT and STY $\Delta chiA$ bacteria inside and outside the LAMP1-stained
720 SCVs after 16 hours of infection was calculated. Data are represented as mean \pm SEM of 3
721 independent experiments (N=3). **(D-E)** Absolute CFU/ml values of **(D)** STM WT and STM
722 $\Delta chiA$, **(E)** STY WT, STY $\Delta chiA$ and STY $\Delta chiA:chiA$ in Caco2 cells in chloroquine
723 resistance assay after 16 hours of infection. Data are represented as mean \pm SEM of 3
724 independent experiments (N=3, n=3). One-way ANOVA was used to analyze the data.

725 **Fig 4. Chitinase induces NOS and ROS generation in phagocytic cells and inhibits of**
726 **antigen presentation. (A)** % invasion and **(B)** fold proliferation of STM WT and STM
727 $\Delta chiA$ strains in U937 derived monocytes by gentamicin protection assay. Data are
728 represented as mean \pm SEM of 3 independent experiments (N=3, n=3). Unpaired Student's t
729 test was used to analyze the data. **(C)** % invasion and **(D)** fold proliferation of STY WT, STY
730 $\Delta chiA$, STY $\Delta chiA:chiA$ and STY $\Delta chiA:pQE60$ strains in U937 derived monocytes by
731 gentamicin protection assay. Data are represented as mean \pm SEM of 3 independent
732 experiments (N=3, n=3). One-way ANOVA was used to analyze the data. **(E)** % invasion and
733 **(F)** fold proliferation of STM WT and STM $\Delta chiA$ strains in U937 derived monocytes by
734 gentamicin protection assay. Data are represented as mean \pm SEM of 3 independent
735 experiments (N=3, n=3). Unpaired Student's t test was used to analyze the data. **(G)** %
736 invasion and **(H)** fold proliferation of STY WT, STY $\Delta chiA$, STY $\Delta chiA:chiA$ and STY
737 $\Delta chiA:pQE60$ strains in U937 derived monocytes by gentamicin protection assay. Data are
738 represented as mean \pm SEM of 3 independent experiments (N=3, n=3). One-way ANOVA
739 was used to analyze the data. **(I)** Extracellular NO was estimated by Greiss assay from
740 conditioned media obtained from STM WT, STM $\Delta chiA$, STY WT, STY $\Delta chiA$ and STY
741 $\Delta chiA:chiA$ infected DCs. (UI- Uninfected). Data are represented as mean \pm SEM of 3
742 independent experiments (N=3, n=3). Two-way ANOVA was used to analyze the data. **(J-K)**
743 Intracellular survival of **(J)** STM WT and STM $\Delta chiA$ and **(K)** STY WT and STY $\Delta chiA$
744 strains were calculated in WT and NOS2^{-/-} BMDCs by gentamicin protection assay. Data are
745 represented as mean \pm SEM of 3 independent experiments (N=3, n=3). Unpaired Student's t
746 test was used to analyze the data. **(L)** Representative flow cytometry plot for ROS estimation
747 by DCFDA assay from STM WT, STM $\Delta chiA$, STY WT, STY $\Delta chiA$ and STY $\Delta chiA:chiA$
748 infected and LPS treated peritoneal macrophages. (UI US- Unstained uninfected, UI-
749 Uninfected. LPS- Lipopolysaccharide). **(M)** Quantification of the MHC-II density per unit

750 length of the cell membrane of STM WT, STM $\Delta chiA$, STY WT, STY $\Delta chiA$ and STY
751 $\Delta chiA:chiA$ infected PMs after indicated time (UI- Uninfected). Data are represented as mean
752 \pm SEM of 2 independent experiments. One-way ANOVA was used to analyze the data. (UI
753 US- Unstained uninfected, UI- Uninfected). (N) 3H thymidine incorporation assay to assess
754 CD8⁺ T cell proliferation after 20 hours of infection with STM WT, STM $\Delta chiA$, STY WT,
755 STY $\Delta chiA$ and STY $\Delta chiA:chiA$ (UI- Uninfected, OVA- Ovalbumin, ConA- Concanavalin
756 A). Data are represented as mean \pm SEM of 3 independent experiments. One-way ANOVA
757 was used to analyze the data. (O) Representative flow cytometry plot showing the level of
758 surface MHC-II molecules on PMs infected with STM WT and STM $\Delta chiA$ for 20 hours (UI
759 US- Unstained uninfected, UI- Uninfected).

760 **Fig 5. Chitinase facilitates *in vivo* invasion, survival and pathogenesis of *Salmonella***
761 **Typhimurium.** (A) Survival of the mice infected with lethal dose of STM WT and STM
762 $\Delta chiA$ (PBS= Phosphate Buffered Saline). Data are presented from one independent
763 experiment, representative of 3 independent experiments (N=3). (B) Bacterial shedding in the
764 feces of the animals infected with STM WT and STM $\Delta chiA$. Data are presented as mean \pm
765 SD of one independent experiment, representative of 3 independent experiments (N=3). (C)
766 *In vivo* invasion in Peyer's patches 2 hour post oral gavage by either STM WT or STM
767 $\Delta chiA$. Data are presented as mean \pm SEM of 3 independent experiments. Unpaired Student's
768 t test was used to analyse the data. (D-G) Bacterial burden in (D) liver, (E) spleen, (F) MLN
769 and (G) PP of the infected mice on different days post infection with sublethal dose of STM
770 WT and STM $\Delta chiA$. Data are presented as mean \pm SEM of 3 independent experiments.
771 Unpaired Student's t test was used to analyze the data. (H) Body weight of the infected mice
772 was measured 5 days post infection with sublethal dose of STM WT and STM $\Delta chiA$. Data
773 are presented from 3 independent experiments. (PBS- Phosphate Buffered Saline). (I-J) Pro-
774 inflammatory cytokines (I) IL2 and (J) IFN γ level in serum from STM WT and STM $\Delta chiA$

775 infected mice after indicated time. Data are presented as mean \pm SEM of 3 independent
776 experiments (N=3). One-way ANOVA was used to analyze the data. **(K)** Flow cytometry
777 analysis of total splenocytes isolated from the spleens isolated from STM WT and STM
778 $\Delta chiA$ infected mice after 20 days. Splenocytes were stained for CD4 and CD25 markers
779 (US-PBS- Unstained splenocytes from PBS infected mouse). Data are presented from one
780 independent experiment, representative of 3 independent experiments (N=3). **(L)** Serum anti-
781 *Salmonella* antibody level was checked by sandwich ELISA after indicated time. Data are
782 presented as mean \pm SEM of 3 independent experiments (N=3). Two-way ANOVA was used
783 to analyze the data.

784 **Fig 6. Chitinase enhances *Salmonella* Typhi pathogenesis in *C. elegans*.** **(A)** Bacterial
785 proliferation measured by the ratio of bacterial CFU obtained from infected *C. elegans* after
786 24 hours and 48 hours continuous feeding on STY WT, STY $\Delta chiA$, STY $\Delta chiA:chiA$ and
787 STY $\Delta chiA:pQE60$ strains. Data are represented as mean \pm SEM of 4 independent
788 experiments. One way ANOVA was used to analyze the data. **(B)** Survival of the worms fed
789 on *E. coli* OP50, STY WT, STY $\Delta chiA$ and STY $\Delta chiA:chiA$. Data are presented from one
790 independent experiment, representative of 4 independent experiments. **(C)** Representative
791 confocal images of bacterial colonization in worms gut as observed by infecting transgenic
792 FT63 worms by bacteria expressing mCherry for indicated time. Yellow arrows show
793 presence of intact bacteria in the terminal bulb of the worm. **(D)** Representative confocal
794 images of bacterial colonization on the chitin-rich organs of the worms as detected by eosin
795 Y staining. Yellow arrows colocalization of the bacteria (red) and the eosin-stained chitin
796 containing regions (green). White arrow shows absence of colocalization of the bacteria and
797 chitin-rich organs. **(E)** Representative confocal images showing bacterial colonization in the
798 worms gut after 48 hours of STY WT and STY $\Delta chiA$ feeding followed by feeding on *E. coli*
799 OP50 for subsequent 24 hours.

800 **Fig 7. *Salmonella* chitinase is important for alteration of metabolism and antibacterial**
801 **defense in *C. elegans*.** (A) Representative images of bacterial colonization of the worms gut
802 at higher magnification. Yellow arrows show presence of the STY WT and STM $\Delta invC$
803 bacteria outside the gut lumen. (B) Quantification of no. of pharyngeal pumps/min in *E. coli*
804 OP50, STY WT, STY $\Delta chiA$ and STY $\Delta chiA:chiA$ fed worms. Data are represented as mean
805 \pm SD of 3 independent experiments. Two-way ANOVA was used to analyze the data. (C)
806 Representative images of Oil Red O stained worms fed with *E. coli* OP50, STY WT, STY
807 $\Delta chiA$ and STY $\Delta chiA:chiA$ for 48 hours. (D-E) Quantitative RTPCR analysis of the p38
808 MAPK dependent antimicrobial peptide genes (D) *clec85*, *lys7* and *lys2* (E) *spp1* and *abf2* in
809 worms fed with *E. coli* OP50, STY WT, STY $\Delta chiA$ and STY $\Delta chiA:chiA$ for 48 hours. Fold
810 change was normalized over *act2*. Data represents mean \pm SEM of 4 independent
811 experiments. One-way ANOVA was used to analyze the data. (F) Model depicting the role of
812 Chitinase A in bacterial invasion and regulation of host immune response during *Salmonella*
813 pathogenesis in mouse and *C. elegans* host (Created with Biorender.com).

Fig 1

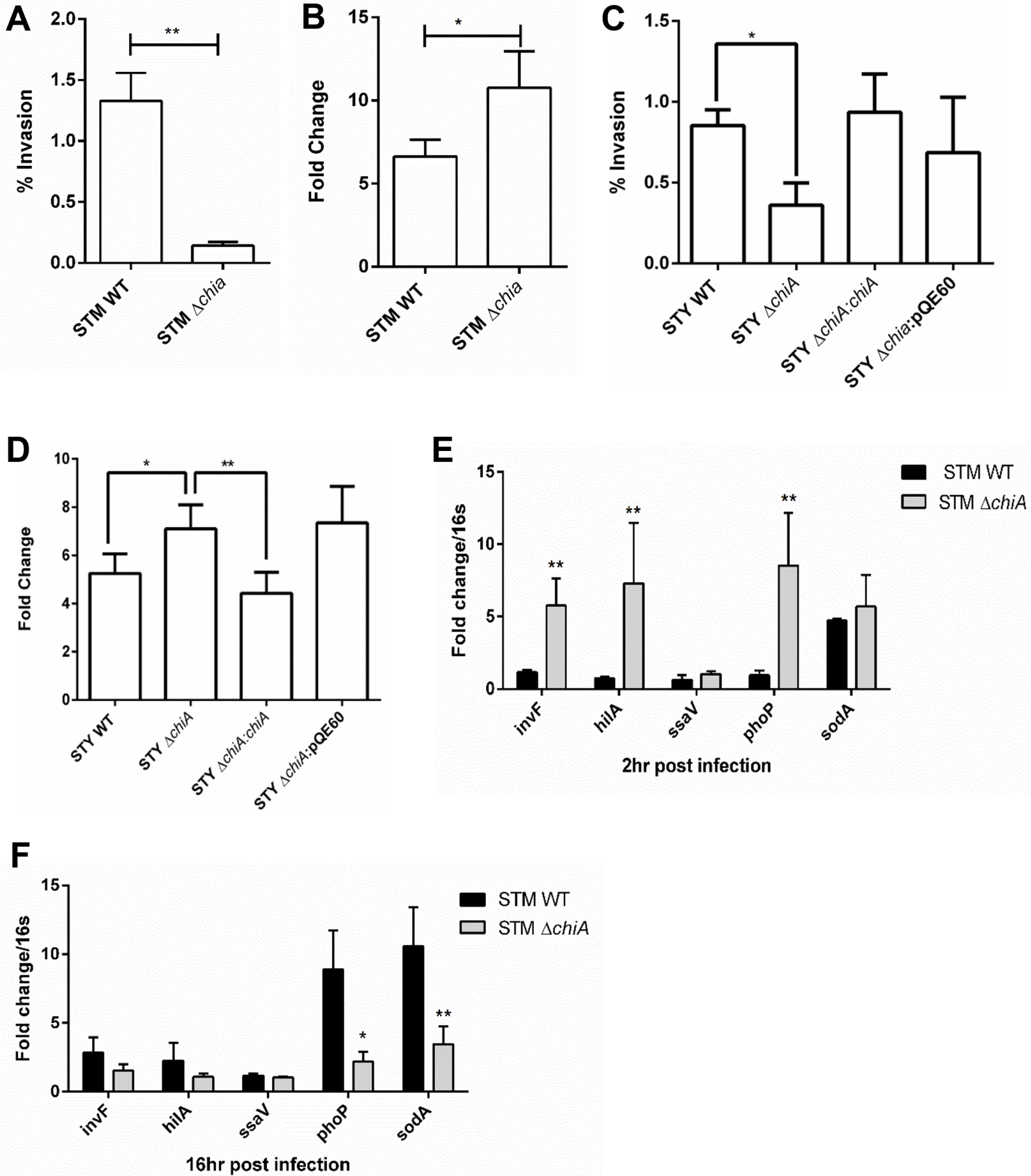
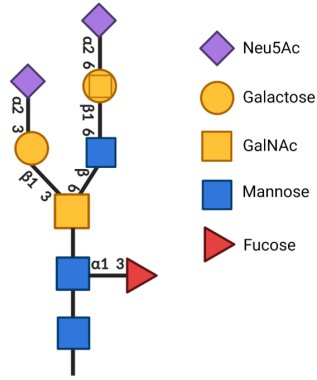
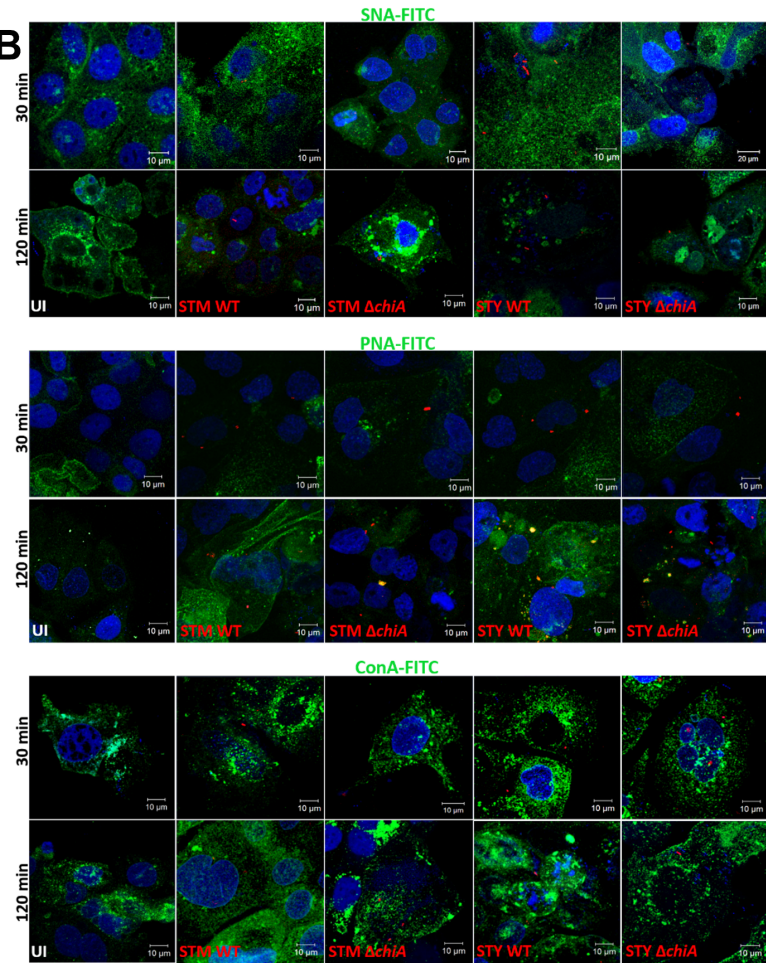


Fig 2

A



B



C

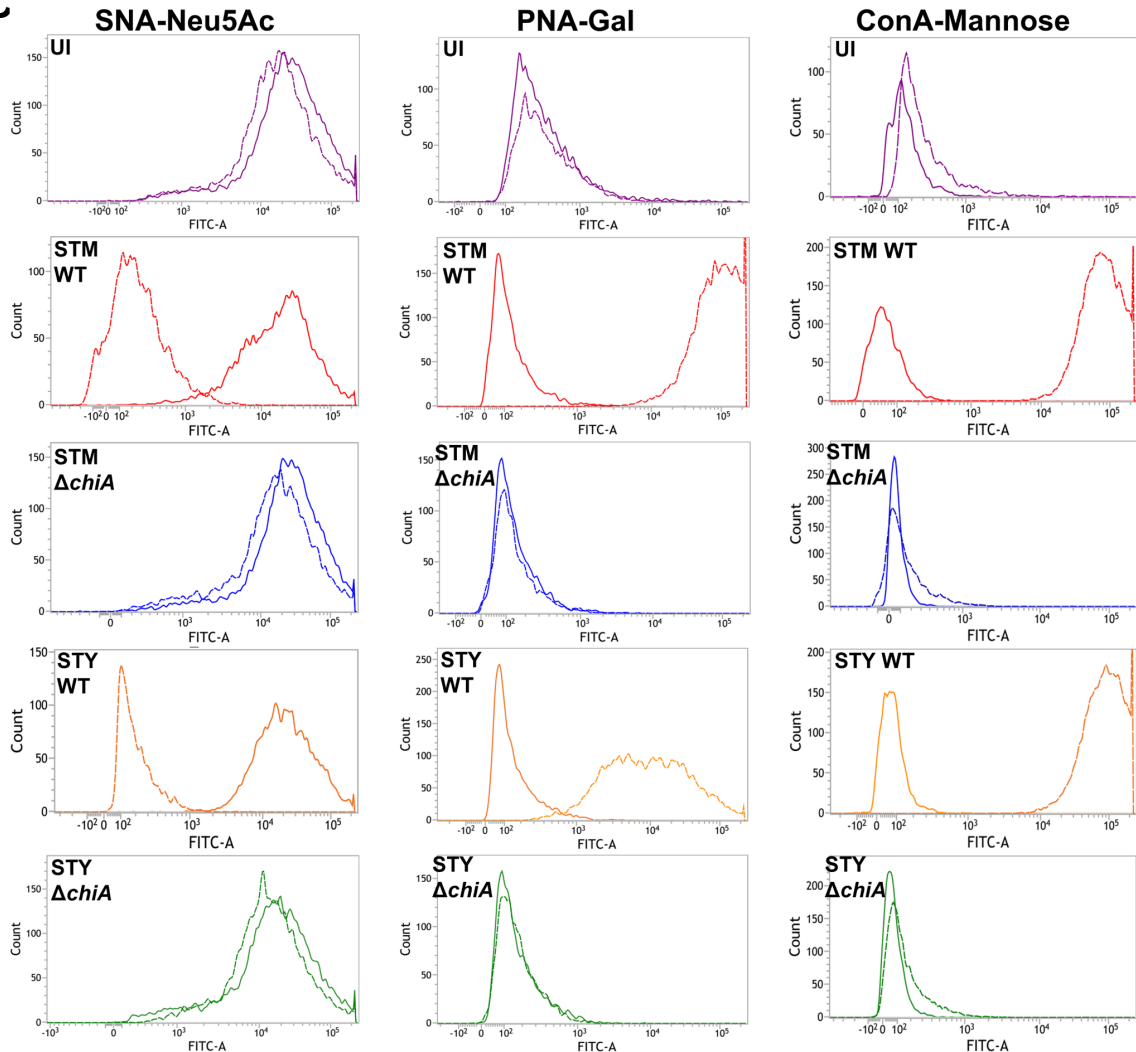


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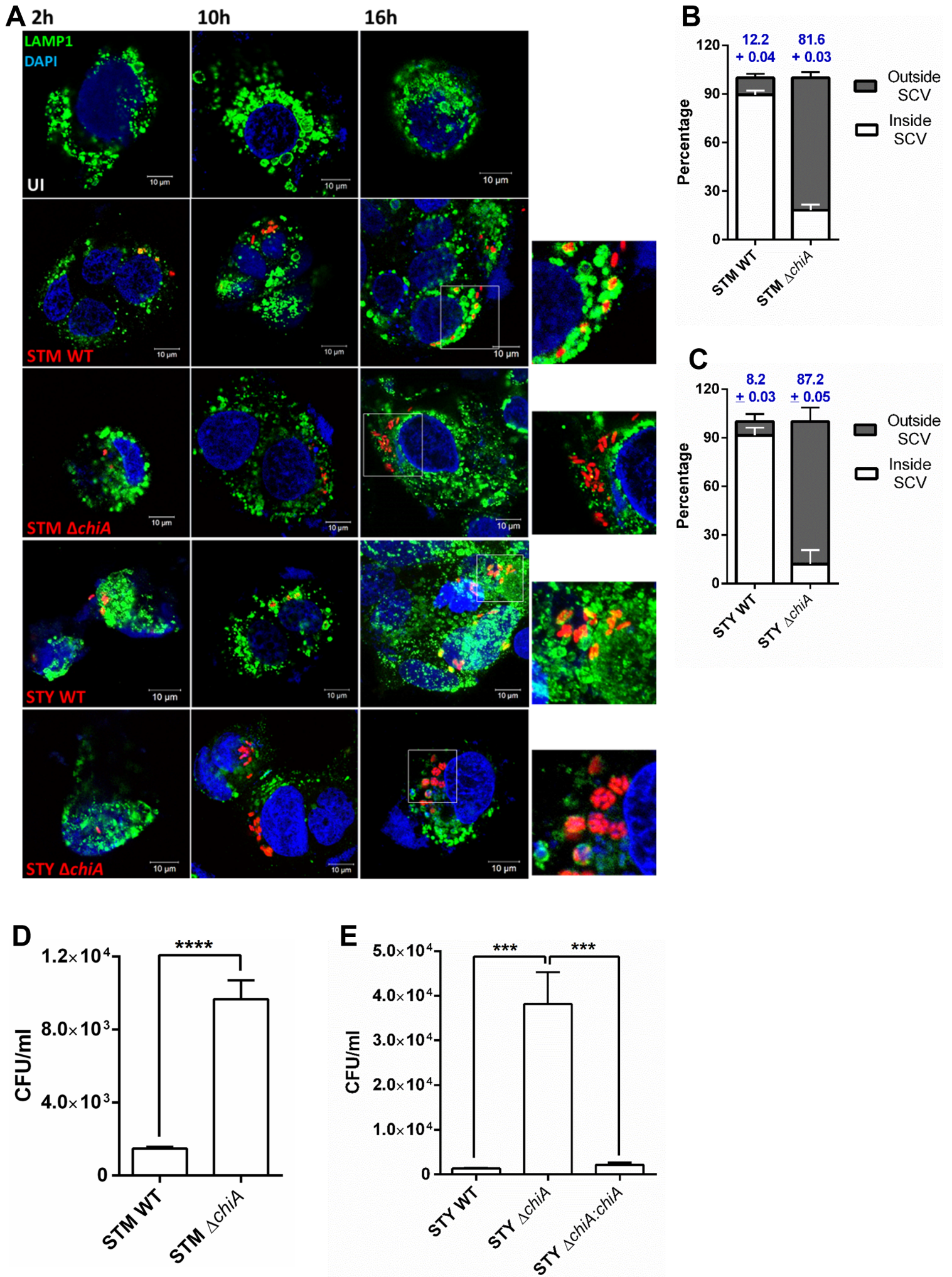


Fig 4

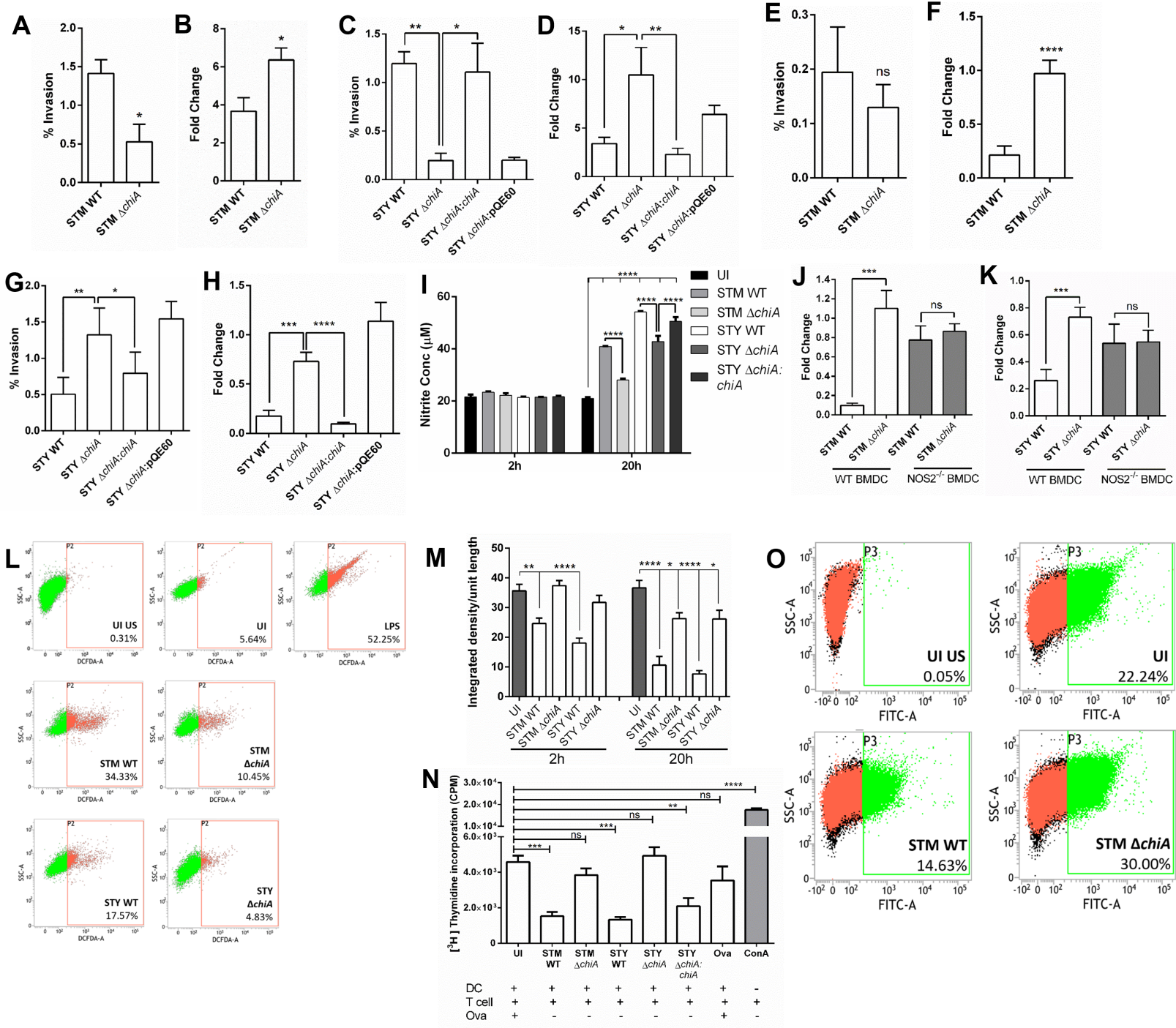


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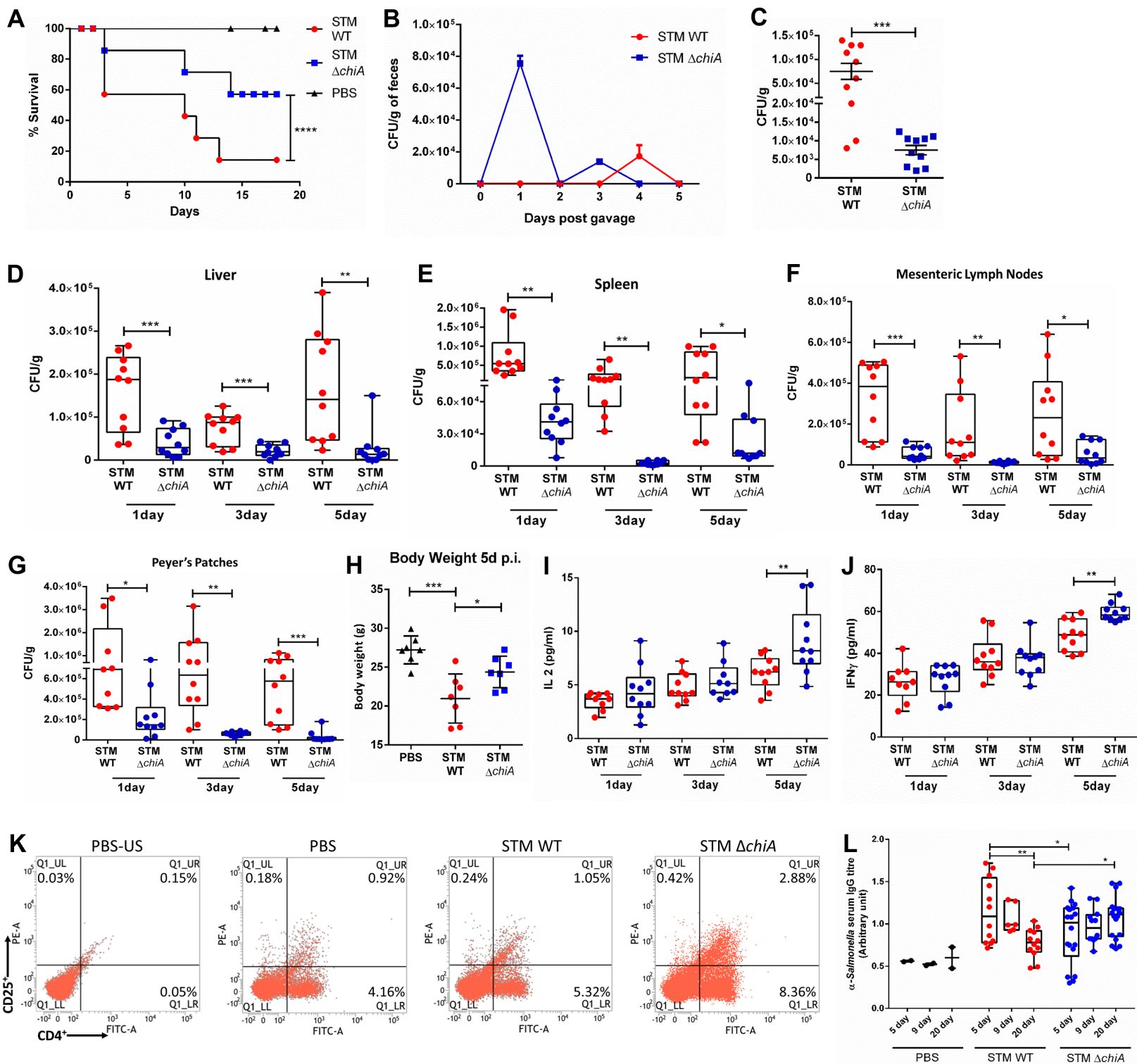


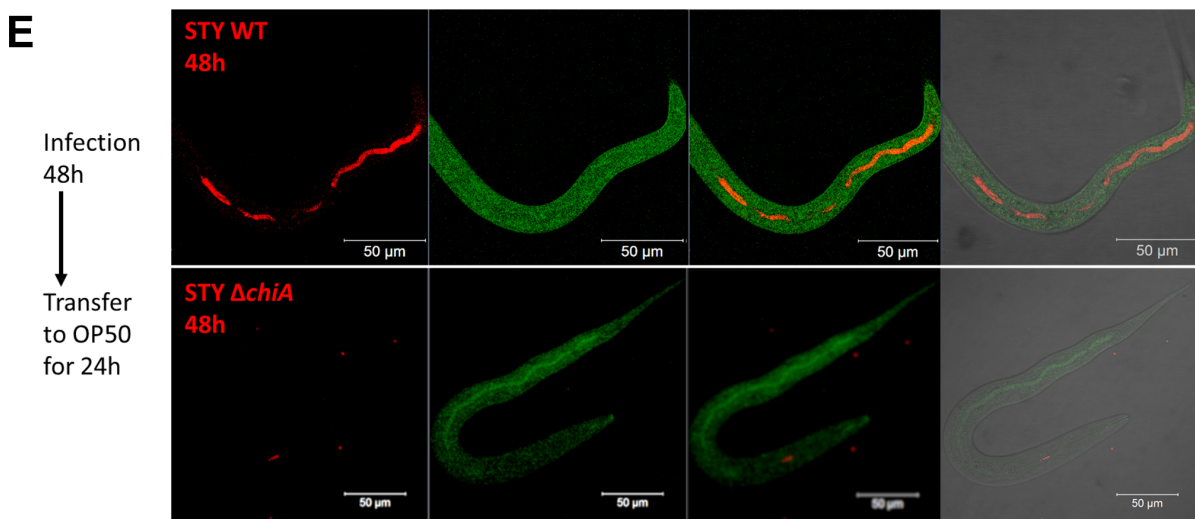
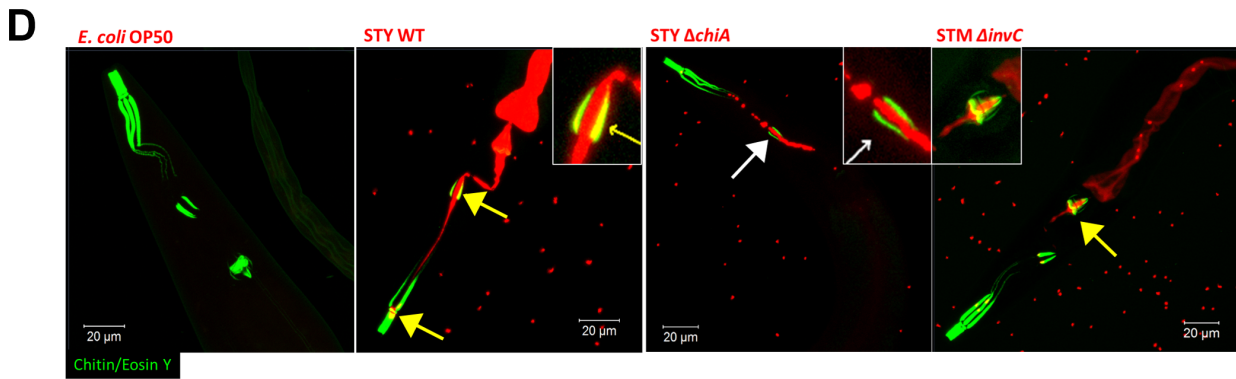
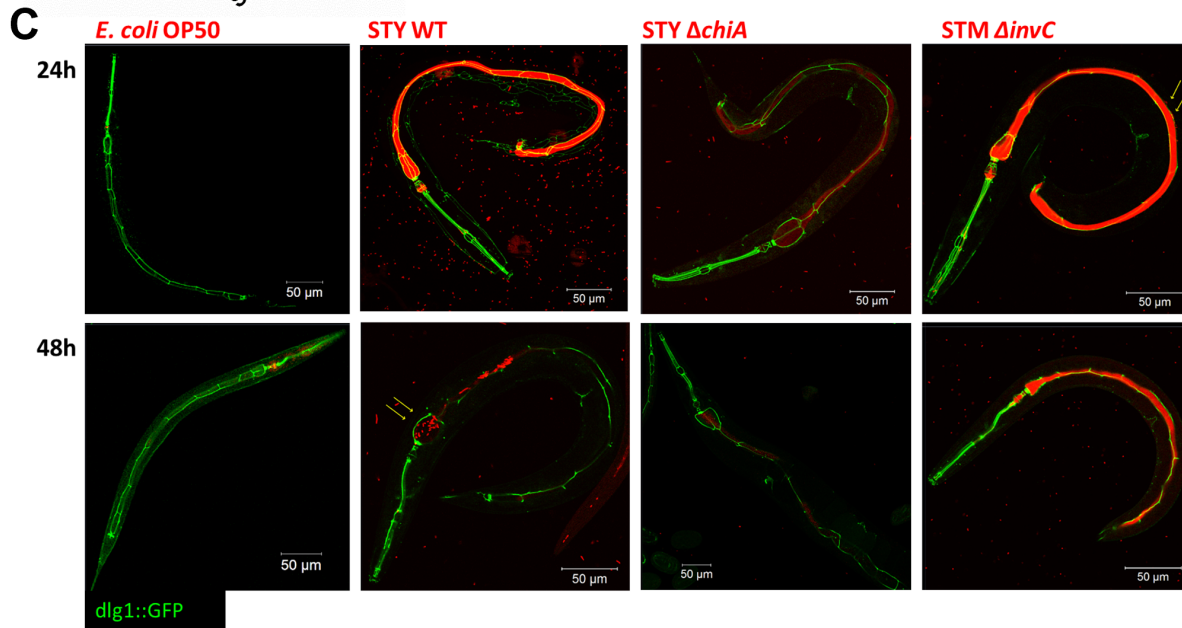
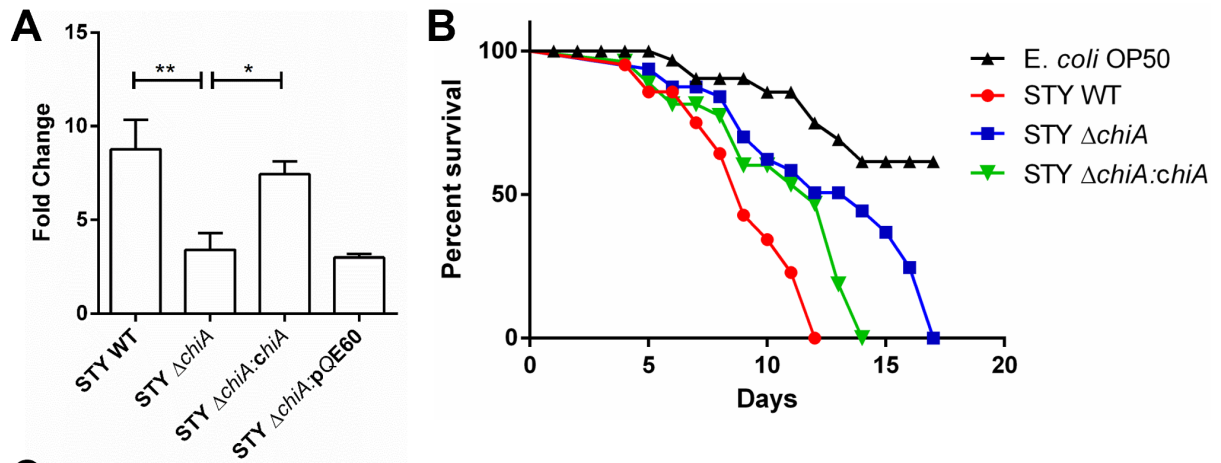
Fig 6

Fig 7

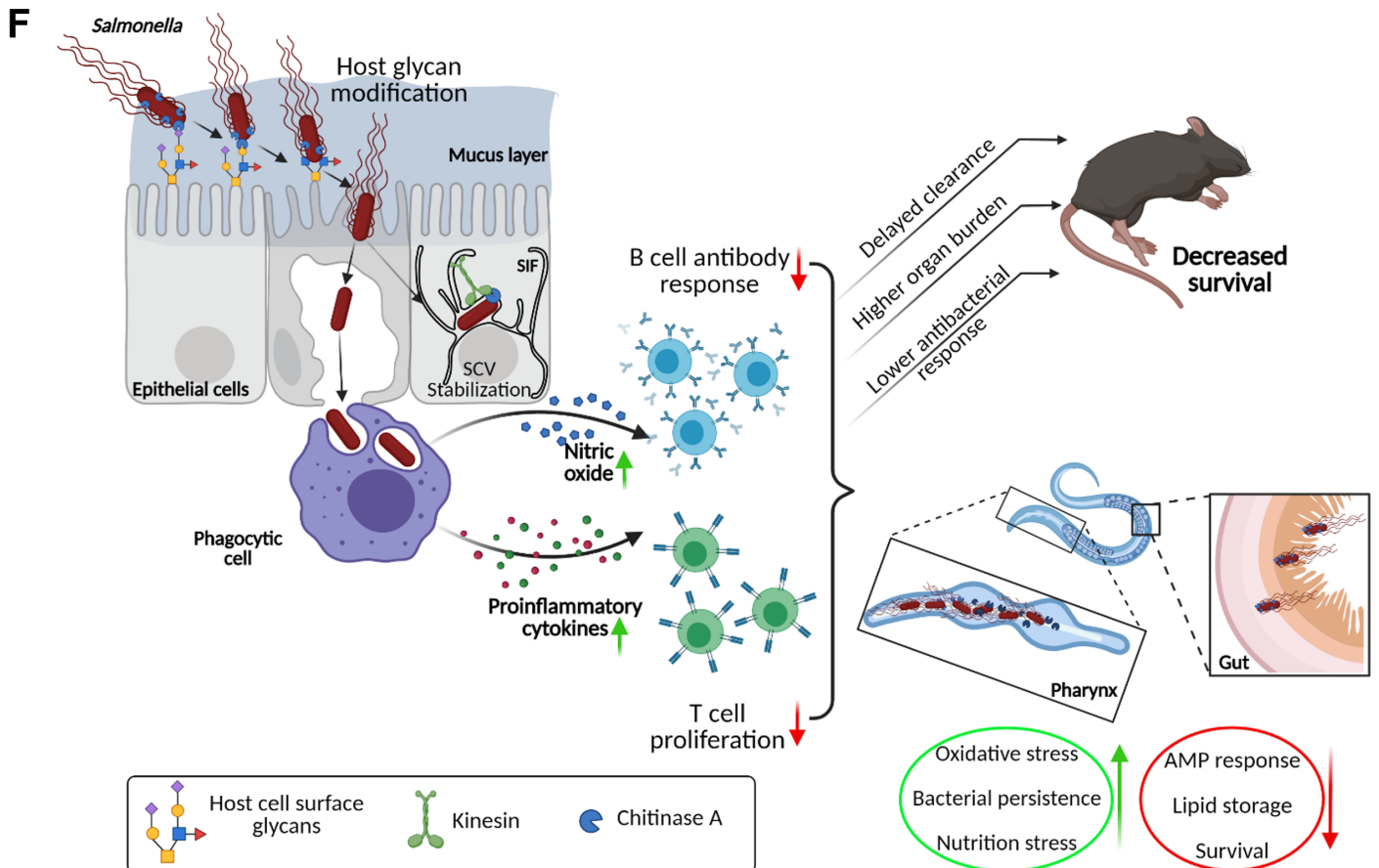
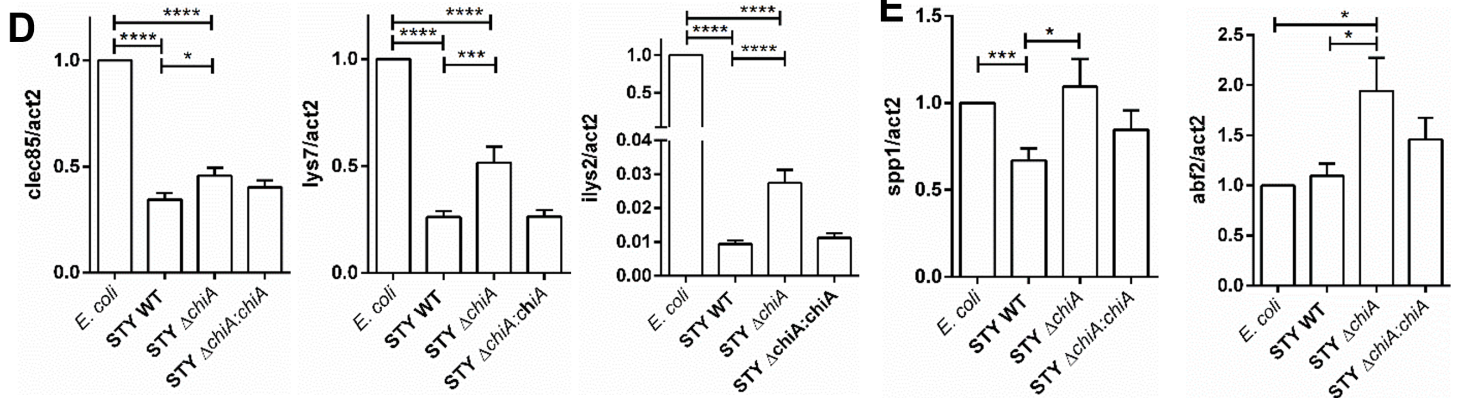
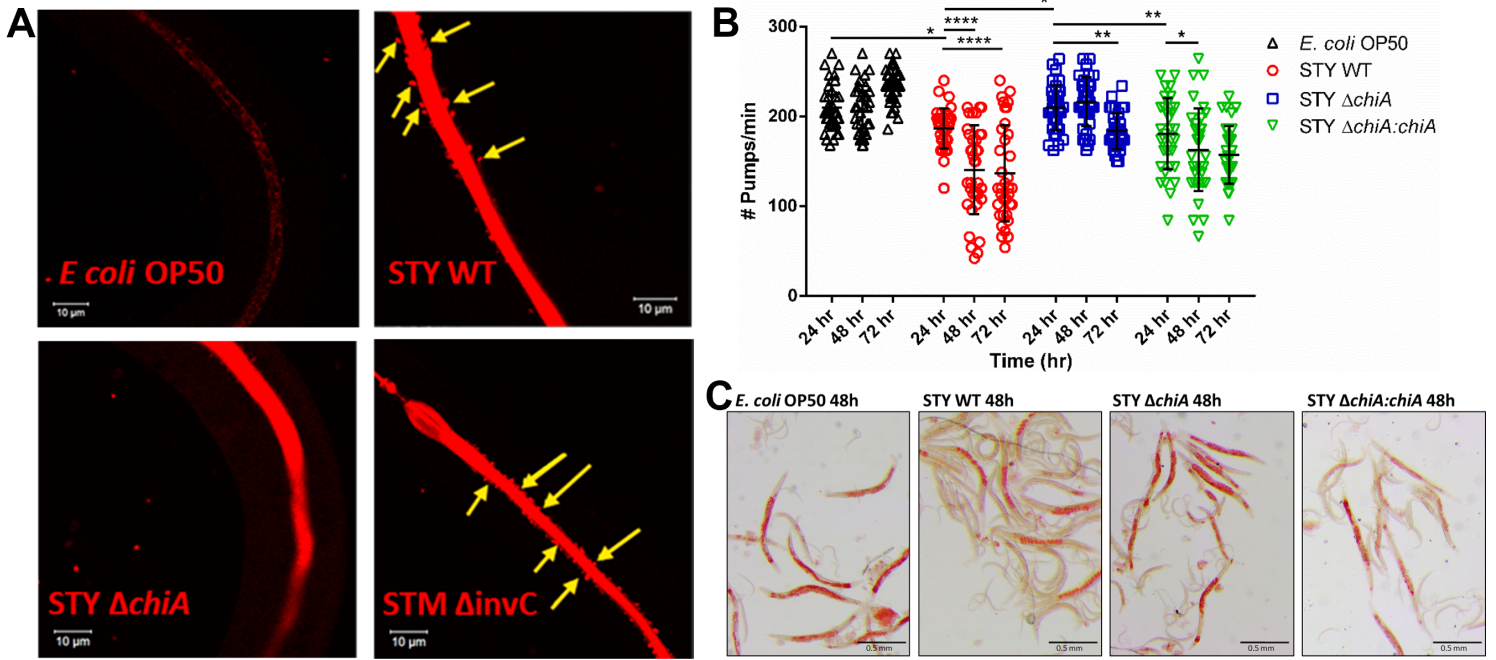


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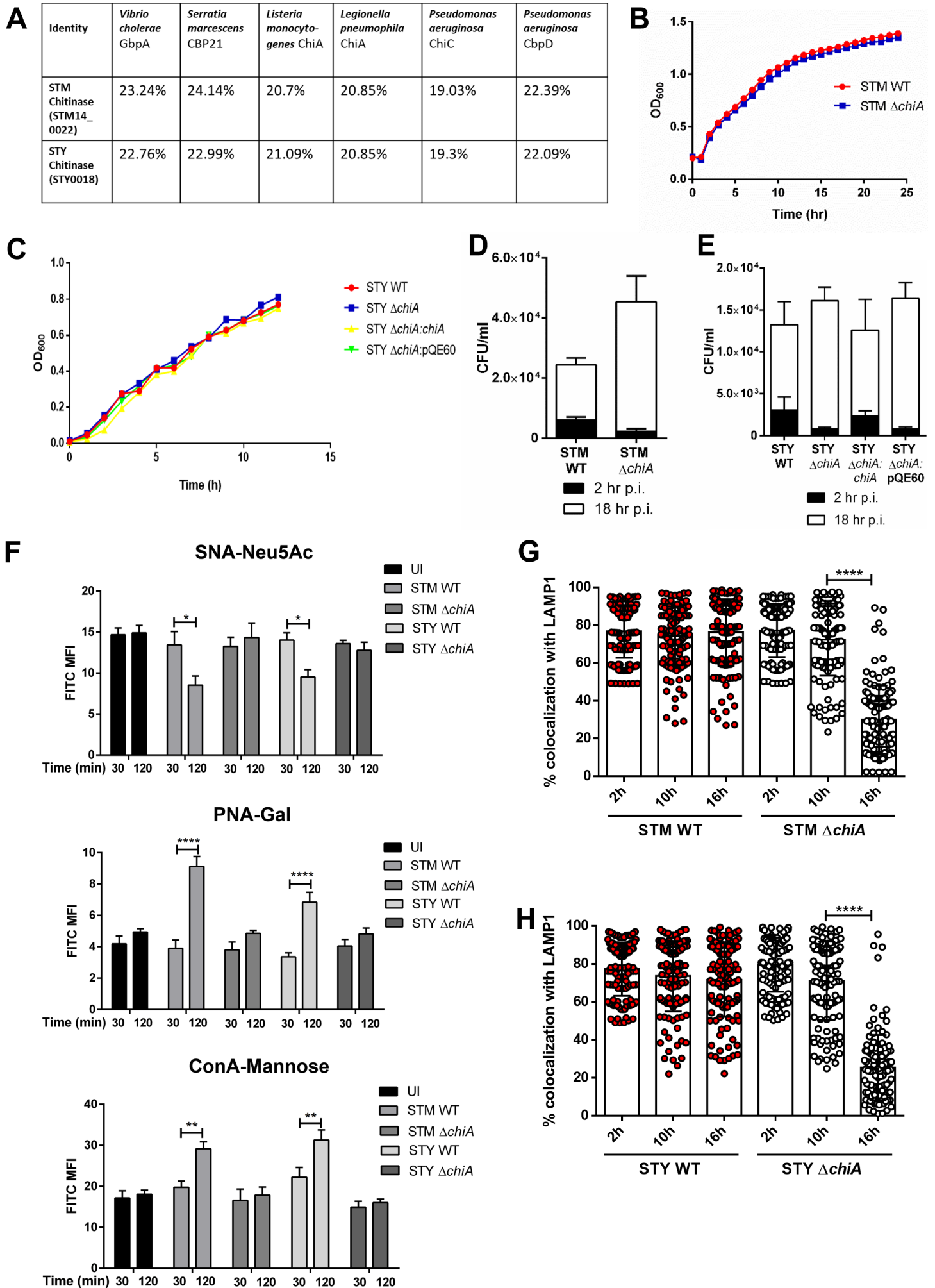


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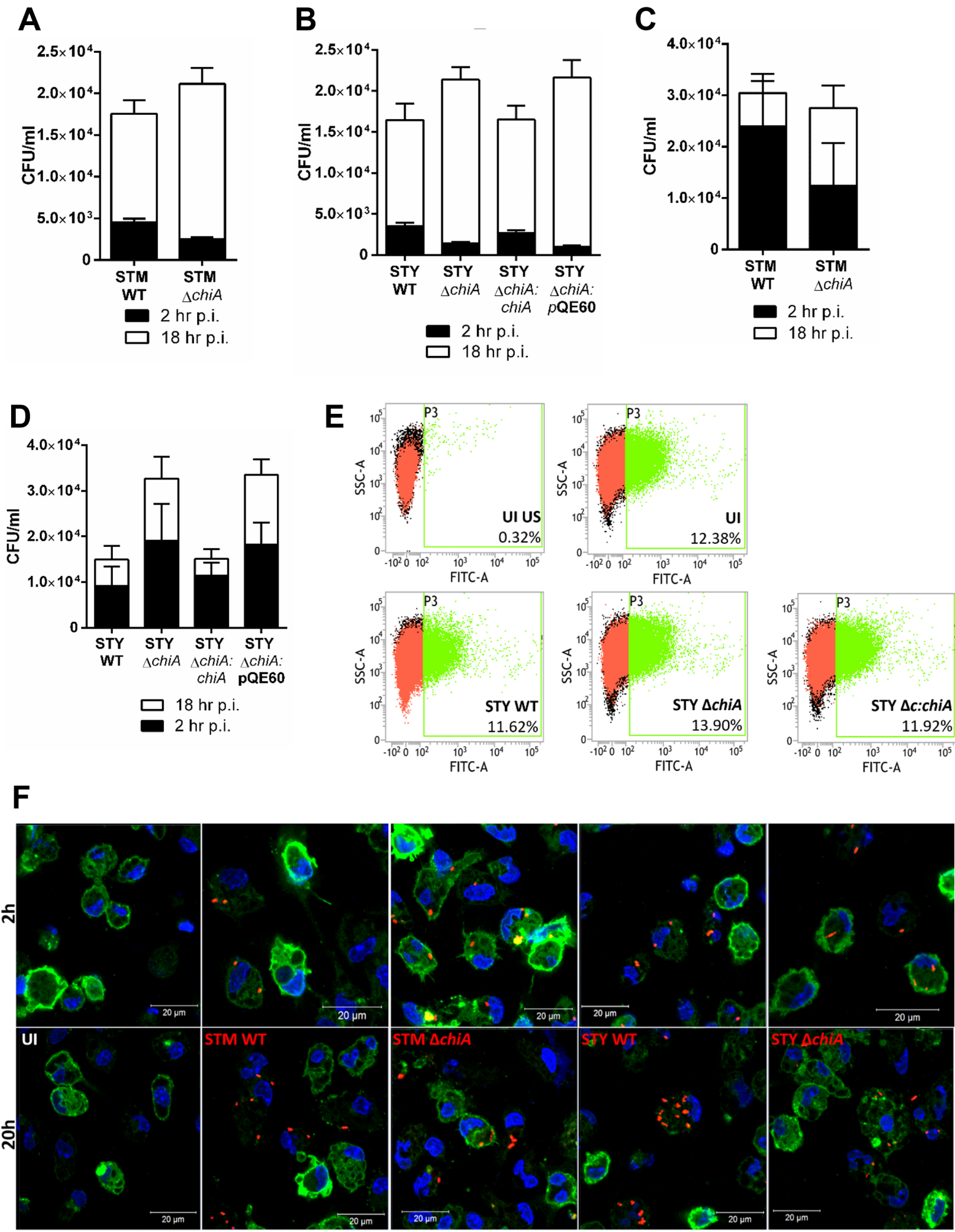


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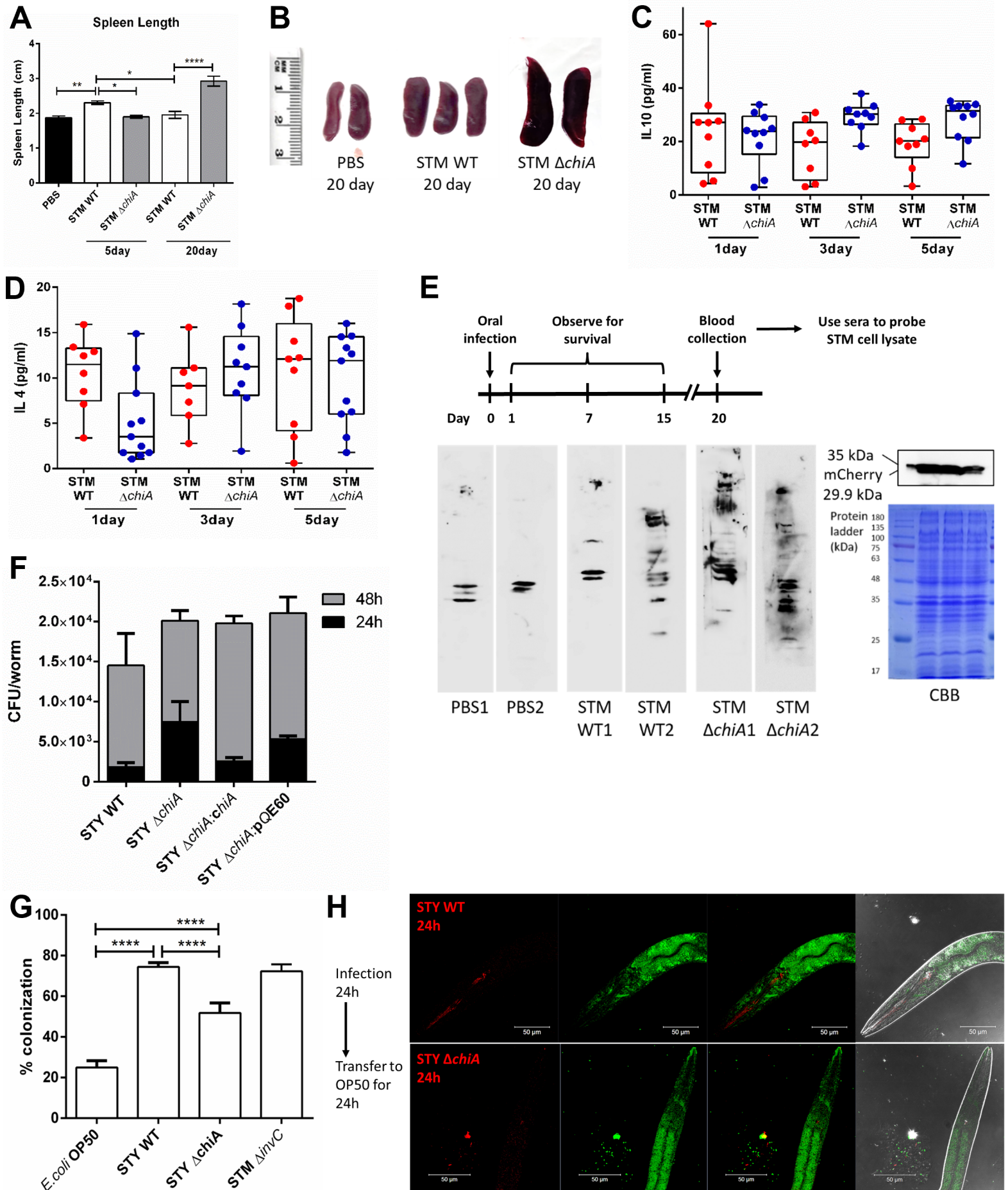
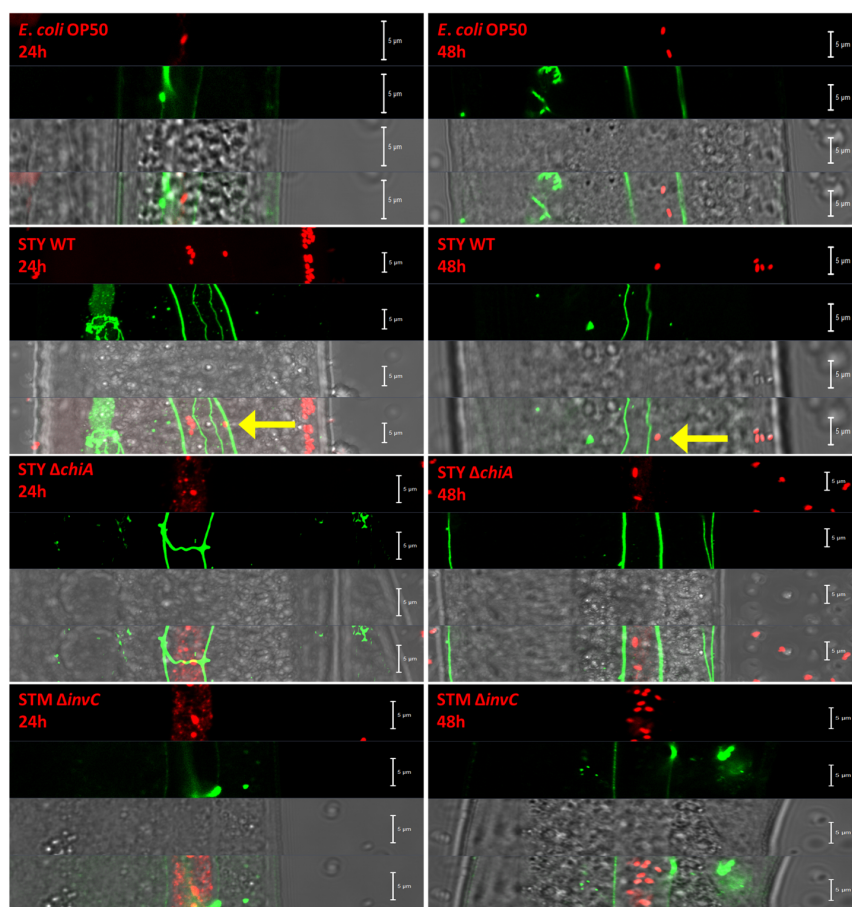
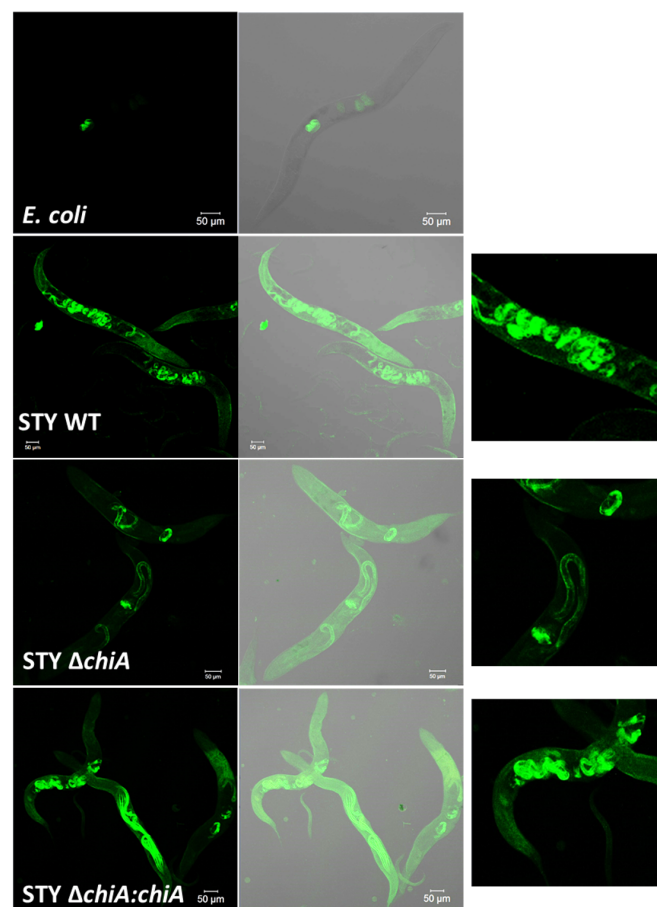


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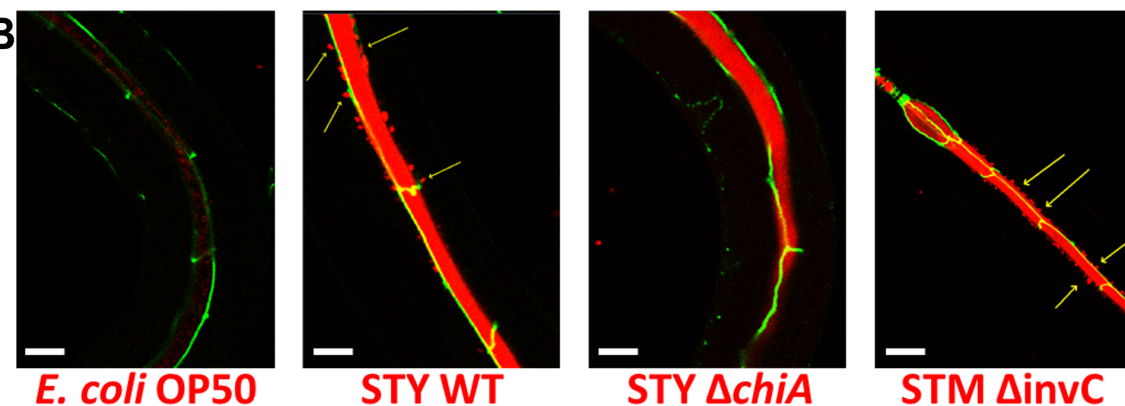
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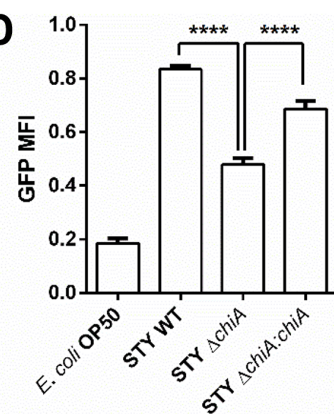
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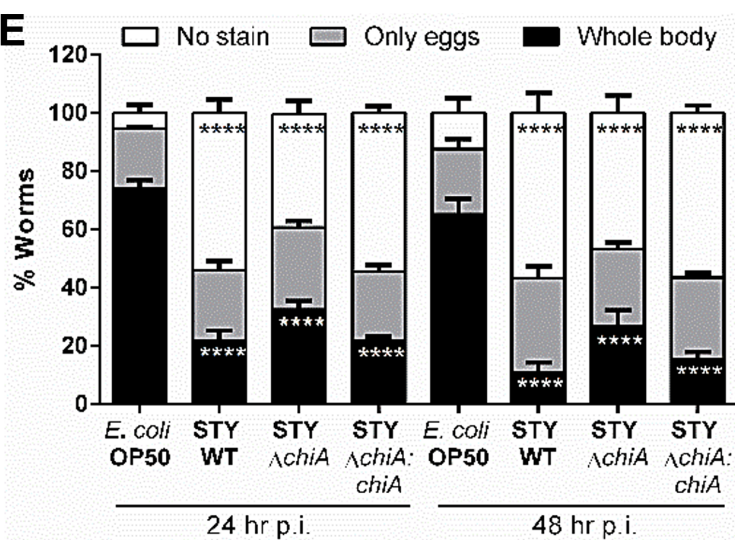
B



D



E



F

