Early induction of antibacterial activities distinguishes response of mice to infection with non-permissive from response to permissive *Salmonella*

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Running Title: Antibacterial activities in *S.* Typhi-infected mice

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Abstract

Salmonella enterica serovar Typhi (*S.* Typhi), the causative agent of typhoid in humans, shares very high homology with closely related serovar, *S.* Typhimurium. Yet, unlike *S.* Typhimurium, *S*. Typhi does not establish infection in mice, the reasons for which are not well understood. Here, we present evidence that unlike *S*. Typhimurium, *S*. Typhi brings about induction of extracellular and intracellular antibacterial activities in mice. Cell-free peritoneal fluids from *S*. Typhi but not *S*. Typhimurium-infected mice inhibited replication of *Salmonella ex vivo*. The production of this activity was reduced in presence of serine protease inhibitor, phenylmethylsulfonlyl fluoride (PMSF). PMSF also inhibited generation of antibacterial activity released from *in vitro S*. Typhi - infected peritoneal macrophages in a cell death – dependent manner. Intracellularly, infection with *S*. Typhi *in vitro* as well as *in vivo* resulted in increased mRNA levels of iron-regulating molecules, ferroportin and lipocalin. These results suggest that induction of antibacterial molecules early on in mice in response to *S*. Typhi may prevent establishment of infection with this *Salmonella* serovar.

34 **INTRODUCTION**

35 *S.* Typhi produces systemic infection, typhoid, almost exclusively in humans. Only in
36 chimpanzees, infection with a very high inoculum (1 x 10^{11} CFU) of this pathogen
37 produces a disease that resembles human typhoid [1]. Due to this extreme host specificity,
38 there is no animal model for *S*. Typhi infection because of which pathogenesis of human
39 typhoid, host-pathogen interactions that ensue during infection with *S*. Typhi and the
40 immune responses that might be relevant to protection against this infection are poorly
41 understood. Unlike *S*. Typhi, non-typhoidal *Salmonella* serovar, *S*. Typhimurium, which
42 causes only localized gastroenteritis in humans, produces a systemic disease in mice that
43 is analogous to human typhoid. Comparative analysis of the genomes of these two closely
44 related *Salmonella* serovars has shown that these two share close to 90% homology at the
45 genome level. This analysis also revealed that *S.* Typhi has lost several genes due to
46 genome degradation or formation of pseudogenes, which might have led to its host
47 restriction [2]. How these differences contribute to host specificity and different clinical
48 manifestations is not clear. Recent studies have shown that *S.* Typhimurium expresses a
49 serine protease, GtgE, that degrades Rab29 and Rab32 which are known to regulate
50 intracellular trafficking. The degradation of these Rabs promotes survival of *S.*
51 Typhimurium inside macrophages, which led to the suggestion that these Rabs might also
52 be involved in delivering antimicrobial molecules to the *Salmonella* - containing vacuole
53 [3]. In fact, a recent study has identified itaconate as one of the antimicrobial molecules
54 that is delivered by Rab32 to this vacuole [4]. GtgE is not present in *S*. Typhi and its
55 ectopic expression increases intracellular survival of this serovar inside mouse
56 macrophages [3, 5, 6]. Therefore, absence of this enzyme might be one of the reasons
57 responsible for the inability of *S*. Typhi to establish infection in mice.

58 Several studies have reported use of immune-deficient mice reconstituted with human
59 immune cells (humanized mice) for investigating infection with *S*. Typhi [7,8]. Unlike
60 WT mice, infection of these mice with *S*. Typhi resulted in replication of this pathogen in
61 various organs including spleen, liver and gall bladder reaffirming that the presence of
62 human immune cells supports infection with this *Salmonella* serovar. However, due to
63 heterogeneities in donor individuals and variable extents of cell engraftment, this model
64 has found limited use in carrying out long term investigations with *S*. Typhi [7, 9].
65 Nevertheless, what these studies have reiterated is that the inability of *S.* Typhi to
66 establish infection in mice is primarily due to the ability of mouse cells to prevent
67 replication and residence of this pathogen. The mechanism of this selective prevention
68 remains unclear.
69 In the present study, we analyzed early inflammatory and antibacterial responses in mice
70 infected with *S*. Typhi and *S*. Typhimurium to understand the reasons for different
71 outcomes of infection with these two closely related serovars. Our findings suggest that
72 generation of antibacterial activities, both intracellular and extracellular, early on with *S*.
73 Typhi might contribute to non-establishment of infection with this serovar in mice.
74

75 **Materials and Methods:**

76 **Mice and bacterial strains**

77 Wild type, T and B cell deficient, MyD88$^{-/-}$ (on C57BL/6 background), CBA/J, C3H/OuJ
78 and C3H/HeJ mice were procured from the Jackson laboratory, USA and maintained at
79 the Small Animal Facility of the National Institute of Immunology. The experiments with
80 mice were carried out according to the guidelines provided by the Institutional Animal
81 Ethics Committee (IAEC). Vi negative *Salmonella* Typhi was provided by Prof. Geeta
82 Mehta, Department of Microbiology, Lady Hardinge Medical College, New Delhi.
83 *Salmonella* Typhimurium SL1344 strain was obtained from Prof. Emmanuelle
84 Charpentier, Department of Microbiology and Genetics, University of Vienna, Austria
85 (now at the Max Planck Institute for Infection Biology in Berlin). Bacteria were cultured
86 in LB medium at 37ºC with shaking at 220 rpm for 10-12h. Bacterial suspension was
87 centrifuged at 8000xg for 5 min to pellet down bacteria. Bacterial pellet was washed two
88 times with serum-free RPMI-1640, resuspended in RPMI-1640 and then used for mouse
89 infection. Bacterial loads were determined by plating tissue lysates on *Salmonella–*
90 *Shigella* (SS) agar plates. Other bacteria used in this study were obtained from the
91 laboratories of Dr. Kanwaljit Kaur and Dr. Vinay Nandicoori, National Institute of
92 Immunology, New Delhi. These bacteria were grown using standard culture protocols.

93

94 **Serum ELISA**

95 Mice were infected with 5x10^{6} CFU of *S.* Typhi or *S.* Typhimurium. At 1 h, 4 h and 8 h
96 post infection, serum samples were collected and analyzed for IL-6 (BD OptEIA™), IL-
97 12p40 (BD OptEIA™), KC (R&D Systems) and TNF-α (BD OptEIA™) by ELISA as
98 per manufacturer's instructions.

99

100 **Flow cytometric analysis of cell populations in the peritoneum**

101 Macrophages, B cells, and T cell populations in the peritoneum of uninfected, *Salmonella*
102 Typhi and *Salmonella* Typhimurium-infected mice were analyzed by flow cytometry
103 using cell type specific antibodies. Briefly, Peritoneal cells were adjusted to $1x10^6$ cells
104 and stained with specific antibodies against cell surface markers CD11b (M1/70 clone),
105 Gr1 (RB6-8C5 clone), F4/80 (BM8 clone), CD4 (GK1.5 clone), CD8 (53-6.7 clone) and
106 B220 (RA3-6B2 clone), or isotype matched antibodies (BD, San Jose, CA) for 60 min at
107 4ºC. After washing, 20,000 cells were analyzed in a flow cytometer (FACS Verse or
108 Accuri C6; BD Pharminogen, USA). Single colour control stained cells were used for
109 compensation.

110

111 **Analysis of antibacterial activity in the cell - free peritoneal lavages of infected mice**

112 Mice were infected intraperitoneally with $5x10^6$ CFU of *S.* Typhi or *S.* Typhimurium.
113 Uninfected mice received equivalent volume of RPMI-1640. At 4 h post injection,
114 peritoneal lavages were collected, centrifuged at 800xg for 5 min and the supernatants
115 were collected and passed through 0.22 µ membrane filter. 10,000 CFU of *S.* Typhi or *S.*
116 Typhimurium (in 100 µl) were incubated with 900 µl of the cell -free peritoneal lavage in
117 triplicate at 37ºC and bacterial growth was monitored by measuring absorbance at 630
118 nm. In some experiments mice were treated with PMSF (1 mg/mouse) half an hour before
119 infecting with *Salmonella*. DMSO was used as vehicle control.

120

121 **Analysis of antibacterial activity from *in vitro*-infected macrophages**

122 6-8-week-old C57BL/6 mice were euthanised by cervical dislocation and peritoneal cells
123 were recovered with the help of ice cold RPMI. Cells were centrifuged at 800xg for 5 min
124 and resuspended in RPMI containing 10% FCS. Cells were plated in a 6-well plate at a

125 density of $4x10^6$ cells per well and incubated at 37°C in 5% CO_2. After 48 h, cells were
126 washed 3 times with RPMI and infected with *S.* Typhi or *S.* Typhimurium at MOI 5 for
127 150 min. For experiments with antibiotic-treated bacteria, *S.* Typhi and *S.* Typhimurium
128 were incubated with gentamicin (1 mg/10^8 bacteria/ml) for 1 hour, washed to get rid of
129 the antibiotic and then used for stimulating peritoneal cells. In some other experiments,
130 cells were treated with PMSF (100 µM) or glycine (10 mM) for half an hour before
131 infecting with *Salmonella*. The supernatants from uninfected cells and infected cultures
132 were collected and passed through 0.22 µ membrane to get rid of bacteria or cells. 10,000
133 CFU of *S.* Typhimurium were incubated with 900 µl of these supernatants in triplicate at
134 37ºC for 9 h and bacterial growth was determined by measuring absorbance at 630 nm.
135

136 **Analysis of LDH-release from infected macrophages**

137 Peritoneal macrophages were infected with *Salmonella* in triplicate (5 MOI for 2.5 h).
138 The culture supernatants were collected and analysed for lactate dehydrogenase (LDH) by
139 a commercially available CytoTox 96 Non-radioactive Cytotoxicity Assay kit according
140 to the manufacturer's (Promega) instructions. Percent cell death was calculated as =
141 (LDH released upon infection/LDH released by TritonX-100 treated cells) x 100. LDH
142 obtained from cells upon lysis with 1% TritonX-100 was taken as 100% cell death.
143

144 **mRNA quantification**

145 *In vitro* or *in vivo*-infected peritoneal cells were lysed using RLT-buffer (Qiagen, Hilden,
146 Germany). Total RNA was extracted following the manufacturer's instructions. RNA was
147 eluted in RNase-free water (Qiagen). The quantity and quality of mRNA were checked by
148 nanodrop. cDNA was synthesized using Thermo Script RT-PCR kit according to the
149 instructions provided by the manufacturer (Invitrogen). Real-time PCR reactions were set

150 up with a SYBR green power up kit (Applied Biosystems) on an Applied Biosystems
151 7500 Real Time Quantitative PCR System workstation. Target genes were normalised to
152 stably expressed reference gene 18S rRNA. The mean threshold cycles were used for
153 further analysis. For the *in vitro* study, RNA from uninfected cells was used as calibrator
154 sample. For *in vivo* investigation, since there was a significant change in cellularity
155 following infection with both serovars, comparative analysis was carried out between
156 samples harvested at two different time points from *S*. Typhi - infected mice and those
157 from *S*. Typhimurium - infected mice. PCR was performed with following sets of primers
158 designed using NCBI/Primer-BLAST.
159
160 CRAMP: Forward 5'–CCCAAGTCTGTGAGGTTCCG
161 Reverse 5'–CTTGAACCGAAAGGGCTGTG
162 Lipocalin: Forward 5'–AATGCGGTCCAGAAAAAAACA
163 Reverse 5'–TGACCAGGATGGAGGTGACA
164 Feroportin: Forward 5'–TCCGTGAACTTGAATGTGAACAA
165 Reverse 5'–GGAAGGGCTCTGCCATCTG
166 Hepcidin: Forward 5'–CCACCTATCTCCATCAACAGATGA
167 Reverse 5'–TTCTTCCCCGTGCAAAGG
168 18S rRNA Forward 5'-CGAAAGCATTTGCCAAGAAT-3′
169 Reverse 5'-AGTCGGCATCGTTTATGGTC-3′
170
171 **Proteinase K digestion**
172 Cell-free peritoneal lavage from *S.* Typhi-infected mice was treated with Proteinase K
173 (100 µg/ml) for 1h and passed through 0.22 µ membrane. Antibacterial activity was

174 determined by incubating 10,000 *S.* Typhimurium at 37ºC with undigested and Proteinase
175 K-digested cell-free peritoneal lavage.
176
177 **2D gel electrophoresis of the cell-free peritoneal lavages**
178 Mice were infected intraperitoneally with $5x10^6$ CFU of *S.* Typhi or *S.* Typhimurium. At
179 8h post infection, peritoneal lavages were collected and passed through 0.22µ membrane.
180 Cell-free lavages were precipitated with trichloroacetic acid. 2D sample buffer was added
181 to the precipitate and iso-electric focusing was carried out according to the protocol
182 described by Adams and Gallagher [10], using GE IPGphor device. After iso-electric
183 focusing, the strip was placed on top of SDS-polyacrylamide gel and subjected to
184 electrophoresis in the second dimension at 40mA for 4h. The spots in the 2D gel were
185 visualized by silver staining as described by Sasse and Gallagher [11].
186
187 **Mass Spectrometric analysis**
188 Silver-stained 2D spot was processed for mass spectrometric analysis as described by
189 Shevchenko et al., 2006 [12]. The protein sample was analysed using EASY-nLC system
190 (Thermo Fisher Scientific, USA) coupled to LTQ Orbitrap-Velos mass spectrometer
191 (Thermo Fisher Scientific). The resolution of peptide mixture was carried out using a 10
192 cm PicoFrit Self-Pack microcapillary column (inner diameter = 75 µm, outer dimeter =
193 360 µm, 15 µm tip) filled with 5 µm C18-resin (New Objective, USA). Analysis of
194 protein sample was performed at the Mass Spectrometry Facility of the National Institute
195 of Immunology, New Delhi.
196
197 **Statistical analysis**

Statistical analyses were performed using the Graph Pad Prism 8 software package. Statistical differences were evaluated using Mann-Whitney test (for comparing two groups) or Kruskal-Wallis test (for three or more groups) followed by Dunn's multiple comparison test for *in vivo* experiments. For *in vitro* experiments, Student's two tailed t-test was used for comparing two groups and one-way ANOVA was used when comparing three or more than three groups, using appropriate post hoc analyses. A p value of less than 0.05 (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) was considered statistically significant. p values for the data shown in each panel have been included in figure legends. Error bars represent standard error mean.

208 **RESULTS**

209 **Cell-free fluids from peritoneal cells infected with *S.* Typhi *in vivo* or *in vitro* inhibit**
210 **bacterial replication**

211 To investigate early host responses upon infection with *S.* Typhi and *S.* Typhimurium, we
212 infected mice intraperitoneally with these two *Salmonella* serovars and analyzed
213 peritoneal bacterial load, peritoneal cellularity and circulating cytokines. As expected,
214 bacterial loads, both intracellular (peritoneal cells) and extracellular (cell-free peritoneal
215 lavages), were higher in mice infected with *S.* Typhimurium (Fig. 1 A). The difference
216 was close to 100 folds in extracellular bacteria and more than 10 folds in intracellular
217 bacteria. Infection with *Salmonella* resulted in changes in the proportions of various cell
218 types in the peritoneum but these differences were comparable in the two serovars. The
219 numbers of F4/80^{+} macrophages decreased significantly most likely due to pyroptotic cell
220 death (Fig. 1 B; 13). The numbers of CD11b^{lo}Gr1^{hi} cells were also reduced with time
221 (Fig. 1 B). On the other hand, CD11b^{hi}Gr1^{int} cell proportions increased with time (Fig. 1
222 B). There was also reduction in the proportions B220^{+} B cells, CD4^{+} T cells and CD8^{+} T
223 in the peritoneal cavities of mice infected with either serovar (Supplementary Fig. 1).
224 Similar changes in cellularity have been previously reported in the peritoneal cavities and
225 spleens of mice infected with pathogenic *Salmonella* and these have been associated with
226 immunomodulatory roles in the establishment of infection with this pathogen [14-16].

227 In contrast to cellularity, there were significant differences in the levels of cytokines upon
228 infection of mice with these two *Salmonella* serovars. *S.* Typhimurium-infected mice
229 showed significantly higher levels of IL-6, IL-12p40 and KC, as compared to *S.* Typhi-
230 infected mice (Fig. 1 C). IL-6 was comparable at 1h post infection in mice infected with
231 either serovar (Fig. 1 C); the levels increased further at 4h and 8h in *S.* Typhimurium-
232 infected mice but dropped to baseline in *S.* Typhi-infected mice (Fig. 1 C). In a similar

233 fashion, IL-12p40 levels were comparable at 1h and 4h of infection, increased further at
234 8h in *S.* Typhimurium–infected mice while reducing close to baseline in *S.* Typhi-infected
235 mice (Fig. 1 C). KC levels were 5 and 30 fold higher at 4h and 8h respectively in *S.*
236 Typhimurium–infected mice (Fig. 1 C). Serum TNF-α at 1h post infection was 2-fold
237 higher in *S.* Typhimurium–infected mice as compared to *S.* Typhi-infected mice (Fig. 1
238 C). While some of these cytokines are involved in clearance of bacteria early on, their
239 increased levels with the progression of infection serve as an indicator of increased
240 bacterial load and inflammation [17].
241 The reduction in extracellular as well as intracellular bacterial load in mice infected with
242 *S*. Typhi suggested that infection with this serovar might induce antibacterial activities in
243 the peritoneum. To explore this possibility, we first determined antibacterial activity in
244 cell-free peritoneal lavages prepared from infected mice. This analysis showed striking
245 differences between peritoneal fluids from *S.* Typhi-infected mice and *S.* Typhimurium-
246 infected mice (Fig. 2 A). Peritoneal fluids from *S.* Typhi-infected mice prevented growth
247 of *Salmonella* (*S*. Typhi as well as *S*. Typhimurium) significantly better than those
248 obtained from *S.* Typhimurium-infected mice (Fig. 2 A). The production of this activity
249 was not dependent on TLR activation as peritoneal fluids obtained from MyD88-deficient
250 mice infected with *S.* Typhi also showed antibacterial activity (Supplementary Fig. 2). T
251 and B lymphocytes also did not contribute to generation of this antibacterial factor(s) as
252 inhibition in bacterial growth was also observed with cell-free peritoneal lavages obtained
253 from *S.* Typhi-infected mice deficient in either of these two cell types (Supplementary
254 Fig. 3). Significantly, the ability of this antibacterial activity to prevent bacterial growth
255 was not restricted to *Salmonella*; this activity suppressed replication of other Gram-
256 negative bacteria including *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas*
257 *aeruginosa*, Gram-positive bacteria such as *Staphylococcus aureus* and *Listeria*

258 *monocytogenes* as well as acid fast bacillus, *Mycobacterium smegmatis* (Supplementary
259 Fig. 4). The ability to inhibit *Salmonella* growth was also seen with peritoneal fluids from
260 mice infected with Vi positive *S*. Typhi (data not shown).
261 Antibacterial activity obtained from *S*. Typhi-infected mice did not readily inhibit
262 bacterial replication in the first one hour of incubation but by 4 h, the difference in
263 antibacterial activity between peritoneal fluids obtained from *S*. Typhi and *S*.
264 Typhimurium-infected mice was very evident and it increased further by 8 h (Fig. 2 B).
265 Bacterial numbers in *S*. Typhi – derived lavage at 8 h post incubation were close to 50
266 times lower as compared to those in *S*. Typhimurium – derived lavage. However,
267 bacterial numbers neither reduced nor increased in presence of the cell-free lavage from
268 *S*. Typhi – infected mice between 4 h and 8 h suggesting that this antibacterial activity
269 may be largely bacteriostatic although simultaneous bactericidal effects can't be ruled
270 out. Importantly, the antibacterial activity obtained from *S*. Typhi – infected mice was
271 also effective against *S*. Typhimurium isolated from infected mice suggesting that unlike
272 resistance to other antimicrobial peptides such as polymyxin B, susceptibility to this
273 activity is not altered in the course of infection (Supplementary Fig. 5) [18]. These results
274 established that infection of mice with *S*. Typhi is accompanied by generation of
275 extracellular antibacterial factor(s), and suggested that the innate immune cells and not
276 the lymphocytes were the likely source of these factors. Microscopic examination
277 revealed filamentation of *S*. Typhimurium in presence of antibacterial factor(s) present in
278 the peritoneal fluids of *S*. Typhi-infected mice (Fig. 2 C). Interestingly, *S*. Typhi did not
279 undergo filamentation upon treatment with this antibacterial factor(s) even though its
280 growth was inhibited as much as that of *S*. Typhimurium (data not shown; Fig. 2 A), the
281 reasons for which are not clear.

282 The induction of antibacterial activity with *S*. Typhi was also observed during its *in vitro*
283 infection of peritoneal macrophages. Cell-free supernatants prepared from *S.* Typhi-
284 infected cells showed higher antibacterial activity as compared to those obtained from
285 cells infected with *S.* Typhimurium (Fig. 2 D). This induction required metabolically
286 active bacteria as cells incubated with gentamycin-treated *S.* Typhi did not release any
287 detectable antibacterial activity (Supplementary Fig. 6). Similar to what was observed
288 during *in vivo* infection, the production of this antibacterial activity was not dependent on
289 the activation of MyD88-dependent TLR signaling (Fig. 2 D). Moreover, the induction of
290 antibacterial activity was not restricted to C57BL/6 mice; it was also observed during *S.*
291 Typhi - infection of macrophages from other strains of mice including CBA/J, C3H/OuJ
292 and LPS-hyporesponsive strain, C3H/HeJ (Supplementary Fig. 7).
293
294 **Induction of extracellular antibacterial activity with *S.* Typhi is dependent on serine**
295 **protease**
296 Serine proteases produced by macrophages and neutrophils have been previously shown
297 to be involved in generating active antimicrobial peptides [19-25]. Therefore, we
298 examined the role of these proteases in regulating production of antibacterial activity with
299 *S*. Typhi. Treatment of mice with serine protease inhibitor, PMSF, prior to infection
300 significantly reduced the ability of peritoneal fluid from *S.* Typhi-infected mice to inhibit
301 replication of *Salmonella ex vivo* (Fig. 3 A). Similar reduction in this activity was seen
302 during infection of peritoneal cells with *S*. Typhi *in vitro* (Fig. 3 B). These results
303 indicated that one or more serine proteases might be contributing to the production of
304 antibacterial activity by cells infected with *S*. Typhi. At concentrations used in this study,
305 PMSF did not show any direct effect on bacterial growth (data not shown).

306 As infection with *Salmonella* is known to bring about pyroptotic cell death that is mainly
307 driven by caspase-1 [13], we also looked at possible role of this cell death in the release
308 of this antibacterial activity. *S.* Typhi did not differ from *S.* Typhimurium in bringing
309 about cell death of macrophages (Supplementary Fig. 8). Interestingly, treatment of cells
310 with glycine, which prevents cell lysis by blocking plasma membrane permeabilization
311 [26], significantly reduced antibacterial activity in cell-free supernatants from *S.* Typhi-
312 infected cells showing that cell death was required for the release of this activity (Fig. 3
313 C, D). PMSF, which inhibited generation of antibacterial activity during *in vivo* as well as
314 *in vitro* infection (Fig. 3 A, B), did not affect cell death produced by *S.* Typhi
315 (Supplementary Fig. 9). These results suggested that the production of antibacterial
316 factor(s) by peritoneal cells upon infection with *S.* Typhi is regulated by one or more
317 serine proteases, and its release from cells requires cell death. Alternatively, these
318 proteases might be generating this activity extracellularly after getting released as a result
319 of cell death.
320 Preliminary characterization of the antibacterial activity in the peritoneal fluids of *S.*
321 Typhi-infected mice showed that it was more than 10 KDa and proteinaceous in nature as
322 it lost its activity upon digestion with Proteinase K (Supplementary Fig. 10 a, b). Analysis
323 by 2D gel electrophoresis showed that a large majority of proteins were conserved in the
324 peritoneal fluids obtained from mice infected with *S.* Typhi and *S.* Typhimurium
325 (Supplementary Fig. 10 c). A very prominent 50 KDa spot present only in the peritoneal
326 fluid of *S.* Typhi-infected mice was identified as cleaved serotransferrin by mass
327 spectrometry (Supplementary Fig. 10 c).
328
329 **Infection with *S.* Typhi results in increased expression of lipocalin and ferroportin**
330 **mRNAs**

331 To analyze possible intracellular antibacterial activities, we focused on molecules
332 involved in regulating iron, which is known to play a crucial role in controlling
333 *Salmonella* replication in macrophages [27-29]. We examined mRNA expression of
334 lipocalin, ferroportin and hepcidin, and also analyzed expression of murine cathelin-
335 related antimicrobial peptide (CRAMP). Lipocalin, ferroportin and hepcidin regulate the
336 levels and metabolism of iron, and CRAMP possesses direct antibacterial activity [27-
337 29]. Lipocalin checks bacterial growth by binding iron-loaded siderophores while
338 ferroportin transports iron from inside of the cell to outside of the cell thus making it
339 unavailable for intracellular bacteria during infection [27,28]. Hepcidin regulates iron
340 levels by binding to ferroportin [30]. Considering significant changes in the proportions
341 of various cell types including F4/80^{+} macrophages (Fig. 1 B), which might be the first
342 cell type that gets infected with *Salmonella* during intraperitoneal infection, we didn't
343 determine expression of these molecules in the peritoneal cells from uninfected mice.
344 Comparison was carried out between mice infected with *S*. Typhi and those infected with
345 *S*. Typhimurium. The expression of lipocalin and ferroportin was similar at 4 h in
346 peritoneal cells isolated from *S.* Typhi and *S.* Typhimurium-infected mice. However, at 8
347 h of infection, these levels reduced significantly in *S.* Typhimurium-infected mice but not
348 in *S*. Typhi – infected mice (Fig. 4 A). The levels of hepcidin mRNA were not different in
349 the two sets of infected mice (Fig. 4 A). The levels of CRAMP mRNA were similar in the
350 two sets of mice at 4 h of infection. However, by 8 h, these levels reduced in *S*. Typhi-
351 infected mice but increased significantly in *S*. Typhimurium-infected mice (Fig 4 A).
352 The expression of CRAMP, lipocalin, ferroportin and hepcidin was also analysed during
353 *in vitro* infection of peritoneal macrophages with these two *Salmonella* serovars (Fig. 4
354 B). The mRNA levels of CRAMP, ferroportin, and hepcidin increased significantly in
355 macrophages infected with either serovar (Fig. 4 B). Unlike what was seen with

356 peritoneal cells from infected mice, the mRNA expression of CRAMP was similar in
357 peritoneal cells infected with *S*. Typhi or *S*. Typhimurium *in vitro* (Fig. 4 B). Lipocalin-2
358 increased 2-3 times upon infection with *S*. Typhi but not with *S*. Typhimurium (Fig. 4 B).
359 The levels of ferroportin and hepcidin mRNAs increased upon infection with *S*. Typhi as
360 well *S*. Typhimurium but the increase was significantly more with *S*. Typhi (Fig. 4 B).
361 The reasons for differences between *in vivo* and *in vitro* analyses in the pattern of
362 CRAMP and hepcidin expression are not clear at the moment. These might be due to
363 differences in the responses of different cell types recruited to the peritoneum during
364 infection with the two *Salmonella* serovars. It is also possible that their expression is
365 modulated by inflammatory cytokines, which show significant differences in mice
366 infected with the two serovars (Fig. 1 C). Irrespective of these differences, the results
367 showed that infection with *S*. Typhi induces higher and sustained expression of lipocalin
368 and ferroportin, which might contribute to clearance of this serovar in mice.
369

370 **DISCUSSION**

371 Our results reveal induction of extracellular and intracellular antibacterial activities
372 during clearance of *S.* Typhi from the peritoneal cavity of mice. The production of
373 extracellular pan anti-bacterial activity seems to be in part driven by one or more serine
374 proteases. The exact identity of the factor(s) present in this activity is not established at
375 the moment. Preliminary analysis showed presence of cleaved serotransferrin in this
376 activity. This molecule could contribute to inhibiting bacterial growth through depletion
377 of iron as its proteolytically cleaved high and low molecular weight fragments have been
378 previously shown to have antibacterial and proinflammatory activities respectively
379 [31,32]. Significantly, a serine protease-generated antibacterial activity similar to the one
380 found during *in vivo* infection was also observed during *in vitro* infection of macrophages
381 with *S.* Typhi. Serine proteases have been shown to participate either directly or through
382 generation of antimicrobial molecules in host defense against bacterial pathogens [25].
383 Interestingly, the release of the antibacterial activity from *S.* Typhi-infected macrophages
384 was found to be dependent on pathogen-induced cell death. Considering that the
385 magnitudes of macrophage cell death produced by *S.* Typhi and *S.* Typhimurium were not
386 different, this observation raises an interesting possibility that cell death produced by the
387 two *Salmonella* serovars might be qualitatively different.

388 Intracellularly, both *in vivo* and *in vitro*, infection with *S.* Typhi resulted in increased
389 induction of lipocalin and ferroportin mRNAs. Lipocalin is expressed by macrophages,
390 neutrophils, and epithelial cells and is up-regulated in response to infectious and
391 inflammatory stimuli [33]. It inhibits bacterial growth by impeding siderophore-mediated
392 iron uptake [28]. Ferroportin is the only known iron transporter which can transport iron
393 from intracellular milieu to the extracellular environment thereby limiting intracellular
394 iron levels [34]. The acquisition of iron is known to be crucial for survival of *Salmonella*

395 in macrophages [35], and *S.* Typhi has been shown to be more sensitive to iron limitation
396 than *S.* Typhimurium [36]. *In vivo* administration of iron to mice has been previously
397 reported to promote systemic replication of *S.* Typhi [37]. In J774 murine macrophage
398 cell line, ferroportin overexpression lead to iron deprivation and subsequent reduction in
399 intracellular growth of *Salmonella* [27]. Therefore, it is likely that iron deprivation due to
400 enhanced lipocalin and ferroportin expression during infection with *S.* Typhi infection
401 selectively restricts growth of this *Salmonella* serovar in mouse cells. The reasons for
402 lack of upregulation of these two antibacterial proteins during infection with *S.*
403 Typhimurium need to be investigated.
404 Spanò and Galán have suggested that *S.* Typhi might be cleared in mouse macrophages
405 by one or more antibacterial factors that are delivered to *Salmonella*-containing vacuole
406 by Rab32 [6]. In a recent study from the Galan laboratory, Chen et al. have shown that
407 one of the antibacterial molecules delivered by Rab32 to the *Salmonella*-containing
408 molecule in macrophages is itaconate [4]. Rab29 and Rab32 are specifically cleaved by a
409 protease, GtgE, present in *S.* Typhimurium but not in *S.* Typhi [3,6]. Ectopic expression
410 of this enzyme in *S.* Typhi resulted in its increased replication inside mouse macrophages
411 [6]. The study by Chen et al. suggested that Rab32 might deliver additional antibacterial
412 factors to bring about clearance of pathogenic *Salmonella* [4]. Our results indicate that
413 antibacterial molecules involved in the regulation of iron might also critically contribute
414 to the inability of *S.* Typhi to establish infection in mice [6].
415 Taken together, our findings suggest that early anti-bacterial activities generated
416 selectively with *S.* Typhi might play a major role in preventing establishment of infection
417 with this *Salmonella* serovar in mice. Further investigations on the identity of
418 extracellular antibacterial activity and the mechanism involved in the induction of

419 lipocalin and ferroportin should facilitate development of a suitable animal model for *S.*

420 Typhi infection.

421

422 **Figure Legends**

423 **Figure 1: *S*. Typhi is cleared from the peritoneal cavity of mice.**

424 (**A**) C57BL/6 mice (6 per group) were infected intraperitoneally with $5x10^6$ *S.*
425 Typhimurium or *S.* Typhi. The number of extracellular and intracellular bacteria
426 recovered from the peritoneum 4 h after infection was determined by plating on *SS* agar
427 plates. (**B**) C57BL/6 mice were infected intraperitoneally with $5x10^6$ *S.* Typhimurium or
428 *S.* Typhi. Cells were isolated at 1 h, 4 h and 8 h after infection and stained with
429 fluorophore-labelled antibodies against CD11b (FITC-labelled), Gr1 (PE-labelled) and
430 F4/80 (V450-labelled). 20,000 cells were acquired in a flow cytometer (BD Verse). The
431 results obtained with three mice in each group are representative of two independent
432 experiments (UI - Uninfected, STP - *S.* Typhi, STM - *S.* Typhimurium). (**C**) Sera were
433 collected at 1 h, 4 h and 8 h after intraperitoneal infection with $5x10^6$ of *S.* Typhimurium
434 or *S.* Typhi (4 mice per group), and the levels of different cytokines were determined by
435 ELISA. Data representative of 2-3 independent experiments is shown as mean ± sem. *
436 $p<0.05$, ** $p< 0.01$, *** $p<0.005$, ns – not significant.

437

438 **Figure 2: Cell-free peritoneal fluids from mice infected with *S*. Typhi and cell-free**
439 **supernatants from macrophages infected *in vitro* with *S.* Typhi inhibit bacterial**
440 **growth *ex vivo*.**

441 (**A**) WT C57BL/6 mice were infected intraperitoneally with $5x10^6$ CFU of *S.* Typhi or *S.*
442 Typhimurium. Peritoneal lavages were collected from uninfected and infected mice (4 h
443 after infection) and filtered through 0.22 μ membrane. 10,000 *S.* Typhi or *S.*
444 Typhimurium were grown in triplicate for 23 h at 37ºC in RPMI or in cell-free peritoneal
445 lavages derived from uninfected and infected mice. Bacterial growth was determined by
446 measuring absorbance at 630 nm. Each bar in this figure represents one mouse (UI –

447 uninfected). (**B**) *S.* Typhimurium were incubated with cell-free peritoneal lavage from *S.*
448 Typhi or *S.* Typhimurium-infected mice (3 mice per group) for different time points (1 h,
449 4 h and 8 h) and bacterial numbers were determined by plating on SS agar plates. (**C**)
450 10,000 *S.* Typhimurium were incubated in triplicate with peritoneal lavage from *S.* Typhi
451 or *S.* Typhimurium-infected mice at 37ºC. After 21 h, bacteria were observed under a
452 microscope (Nikon TE2000) and images were acquired using ACT software. The results
453 are from two independent experiments. (**D**) Peritoneal cells pooled from 3 WT or
454 MyD88$^{-/-}$ C57BL/6 mice were seeded in a 6 well plate (4x10^{6} per well). 48 h later, cells
455 were infected in triplicate with *S.* Typhi or *S.* Typhimurium. Cell supernatants were
456 collected at 4 h after infection and filtered through a 0.22 μ membrane. 10,000 *S.*
457 Typhimurium were incubated with these supernatants at 37ºC. Bacterial growth was
458 determined after 9 h by measuring absorbance at 630 nm. Data representative of 2
459 independent experiments is shown as mean ± sem. * p value <0.05, ** p value <0.01, ***
460 p<0.005, ns – not significant.
461
462 **Figure 3: Induction of antibacterial factor(s) with *S.* Typhi is dependent on serine**
463 **protease activity, and its release is dependent on cell death.**
464 (**A**) C57BL/6 mice were pretreated with serine protease inhibitor, PMSF, (1 mg/mouse)
465 or with the vehicle (DMSO) and 1 h later, mice were infected with *S.* Typhi. *S.*
466 Typhimurium was incubated for 21 h with cell-free peritoneal lavages (obtained after 4
467 hours of infection) from untreated and PMSF-pretreated, *S.* Typhi-infected mice.
468 Bacterial growth was determined by measuring absorbance at 630 nm. (**B**) Peritoneal
469 macrophages were pretreated with PMSF (100 μM) or with the vehicle (DMSO) in
470 triplicate, and 30 min later, cells were infected with *S.* Typhi for 2.5 h. Cell-free
471 supernatants were collected and passed through 0.22 μ membrane. 10,000 *S.*

472 Typhimurium were incubated in triplicate with these cell-free supernatants and bacterial
473 growth was determined by measuring absorbance at 630 nm. The results are
474 representative of two independent experiments. (UI – uninfected, UT – untreated). (**C, D**)
475 Resident peritoneal cells from C57BL/6 were seeded in a 6 well plate ($4x10^6$ per well). 48
476 h later, cells were pretreated with glycine (10 mM) in triplicate and 30 min later, these
477 were infected with *S.* Typhi for 2.5h. Cell death was determined by release of LDH in the
478 supernatant. Culture supernatants collected at 2.5h were passed through 0.22 μ membrane
479 and evaluated for antibacterial activity by incubating with *S.* Typhimurium. Bacterial
480 growth was determined after 9 h by measuring absorbance at 630 nm. The results are
481 from two independent experiments. Data shown is represented as mean ± sem (UI –
482 uninfected, UT– untreated). ** p value <0.01, *** p<0.005, ns – not significant.
483
484 **Figure 4: Induction of antimicrobial molecules in peritoneal cells from mice infected**
485 **with *S*. Typhi or *S*. Typhimurium.**
486 (**A**) Mice were infected intraperitoneally with $5x10^6$ CFU of *S*. Typhi or *S*. Typhimurium.
487 RNA was isolated from peritoneal cells collected after 4 h and 8 h of infection. mRNA
488 levels of Lipocalin, Ferroportin, Hepcidin and CRAMP, were determined using real time
489 PCR. Data are representative of three mice per group from two independent experiments.
490 (**B**) Mouse resident peritoneal macrophages were infected *in vitro* in triplicate with 10
491 MOI of *S.* Typhi or *S.* Typhimurium for 1 h. Cells were washed to remove extracellular
492 bacteria and incubated for another 24h in presence of gentamycin (100 μg/ml). CRAMP,
493 Lipocalin, Ferroportin and Hepcidin mRNA levels were determined using real time PCR.
494 Data representative of two independent experiments is shown as mean ± sem. ** p value
495 <0.01, *** p<0.005, ns – not significant.
496

Notes

Data Availability Statement: All relevant data are within the paper and its supporting information files.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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648

Figure 1

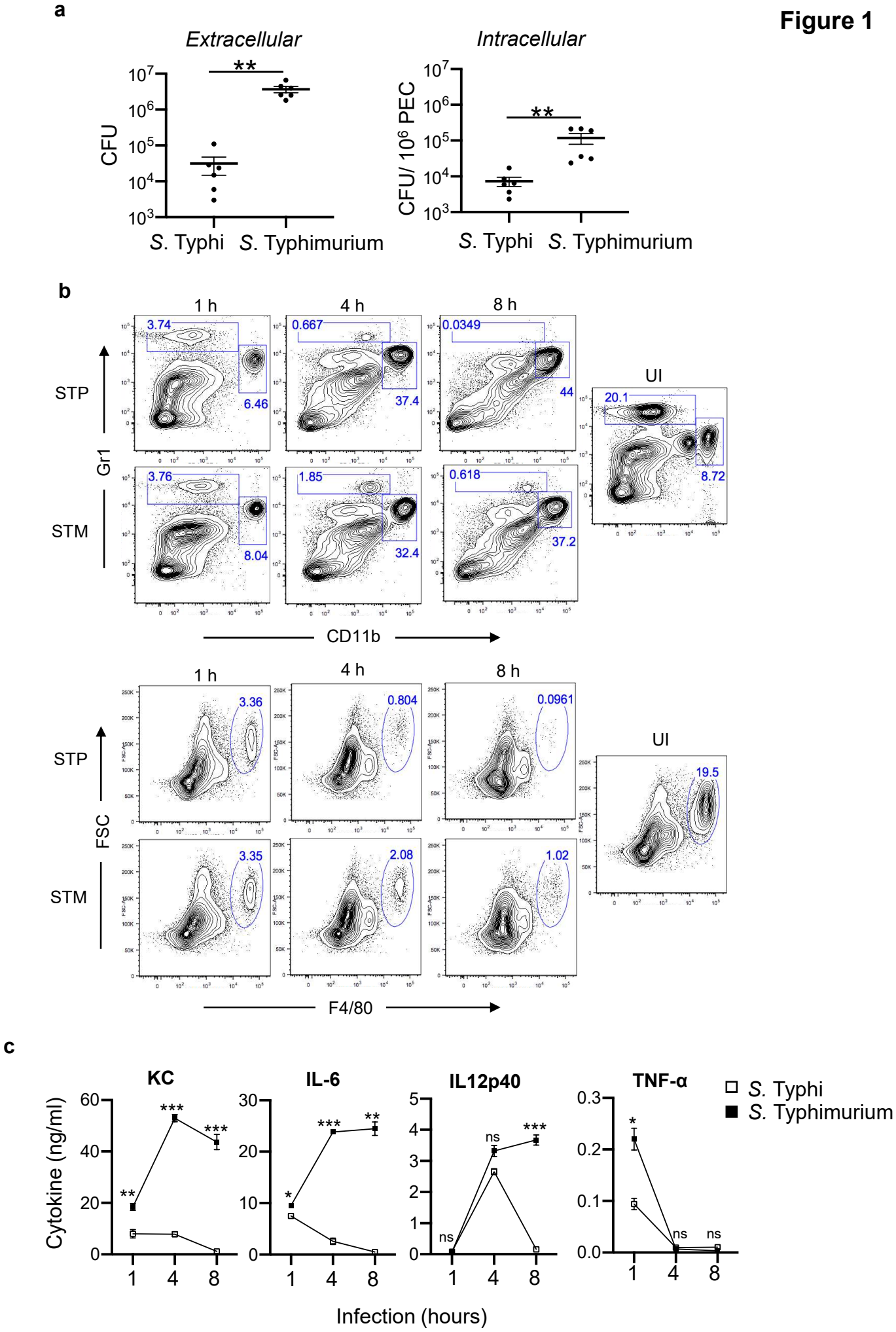
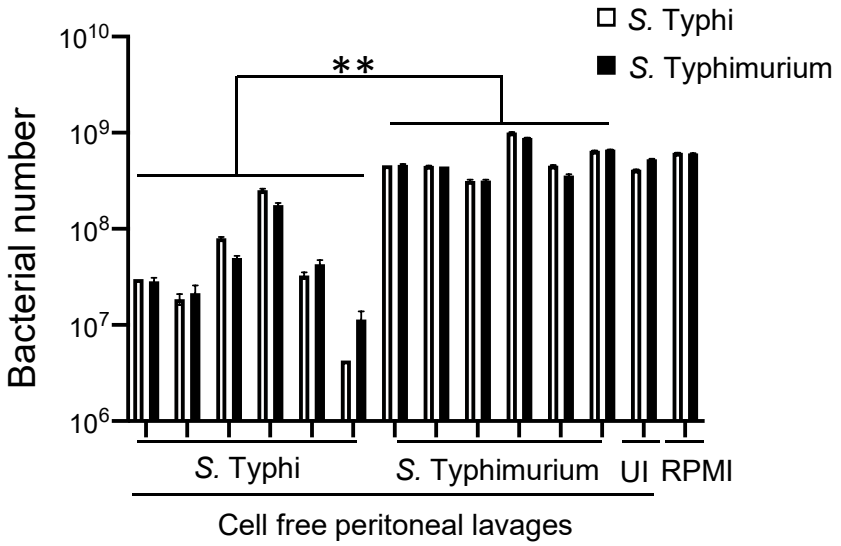

a
Extracellular
Intracellular
CFU
CFU/ 10^6 PEC
**
S. Typhi
S. Typhimurium
b
1 h
4 h
8 h
UI
STP
STM
Gr1
CD11b
3.74
6.46
0.667
37.4
0.0349
44
20.1
8.72
3.76
8.04
1.85
32.4
0.618
37.2
FSC
F4/80
3.36
0.804
0.0961
19.5
3.35
2.08
1.02
c
KC
IL-6
IL12p40
TNF-α
S. Typhi
S. Typhimurium
Cytokine (ng/ml)
Infection (hours)
ns

Figure 2

a



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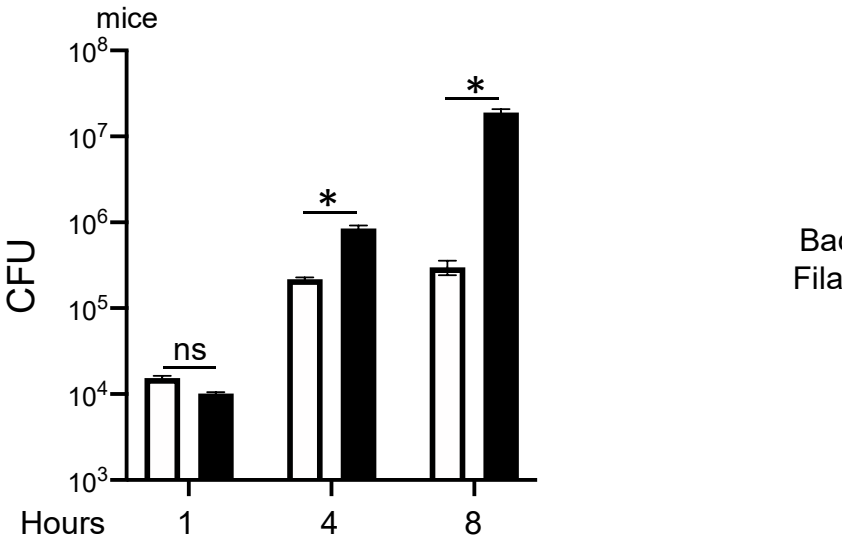

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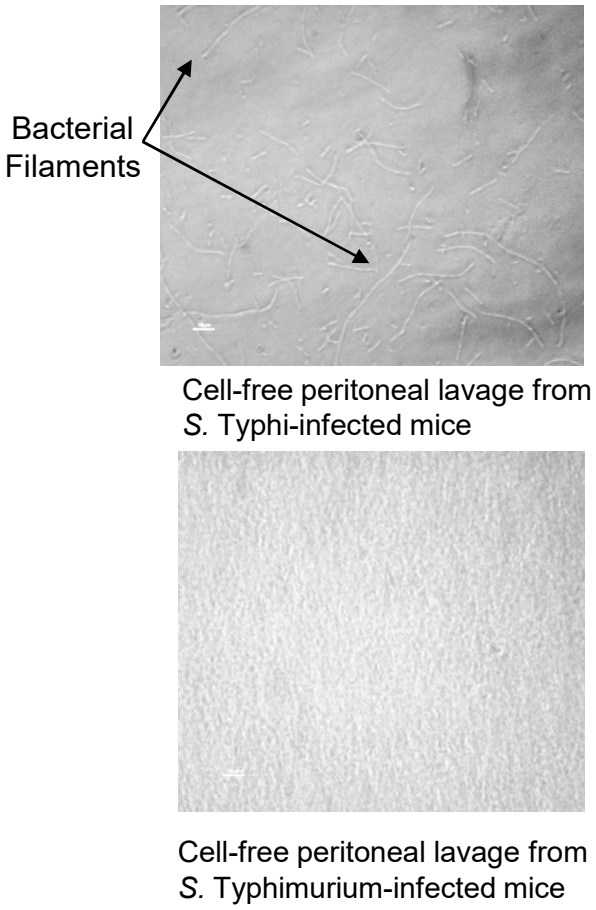

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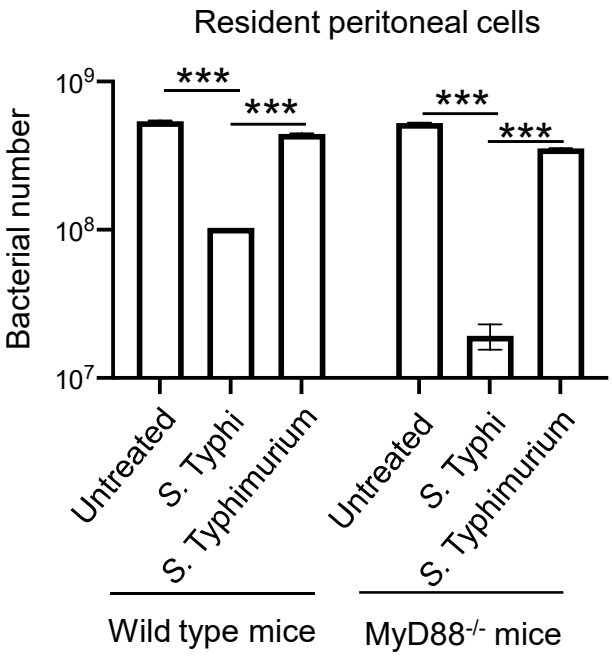


Figure 3

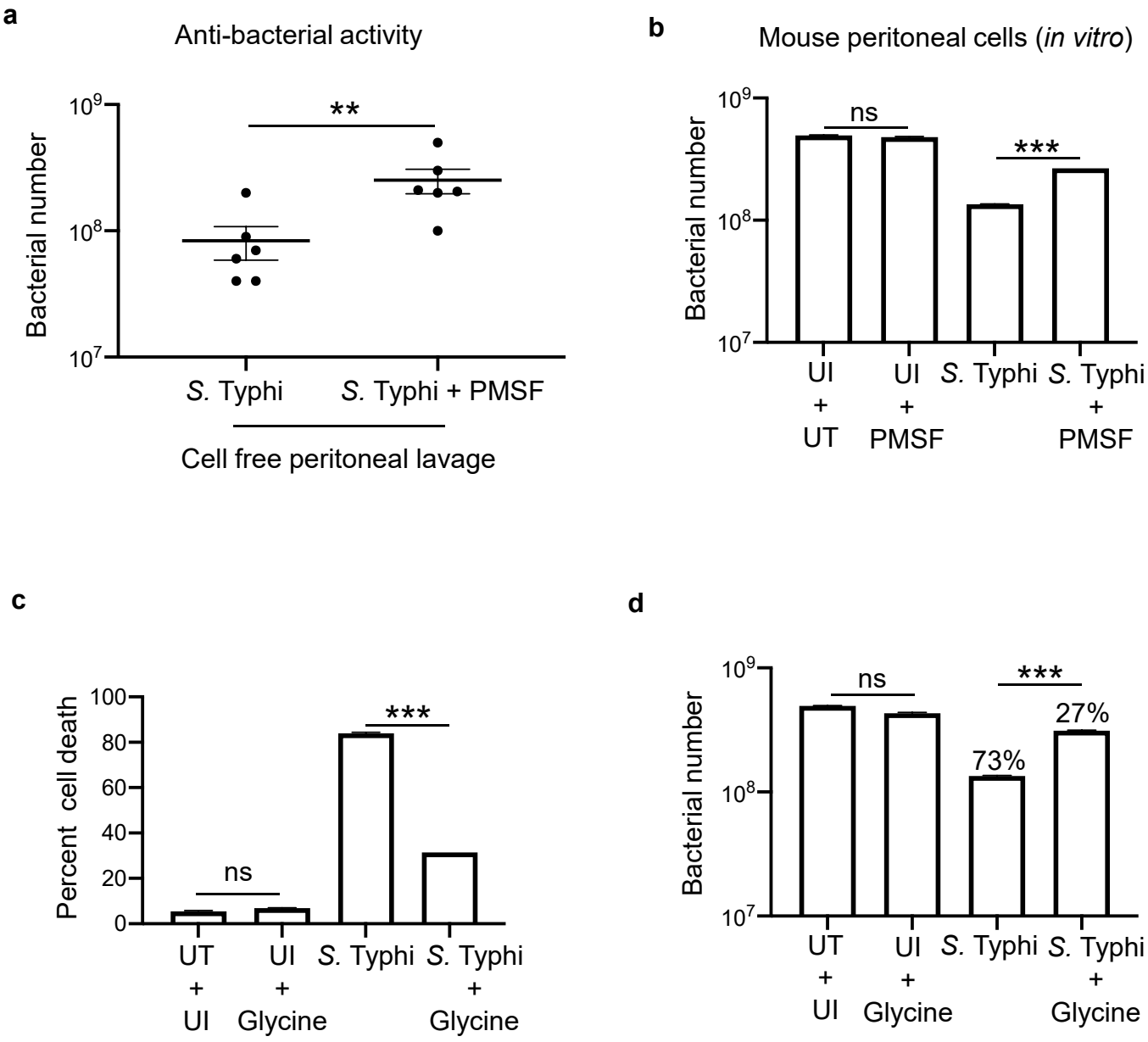
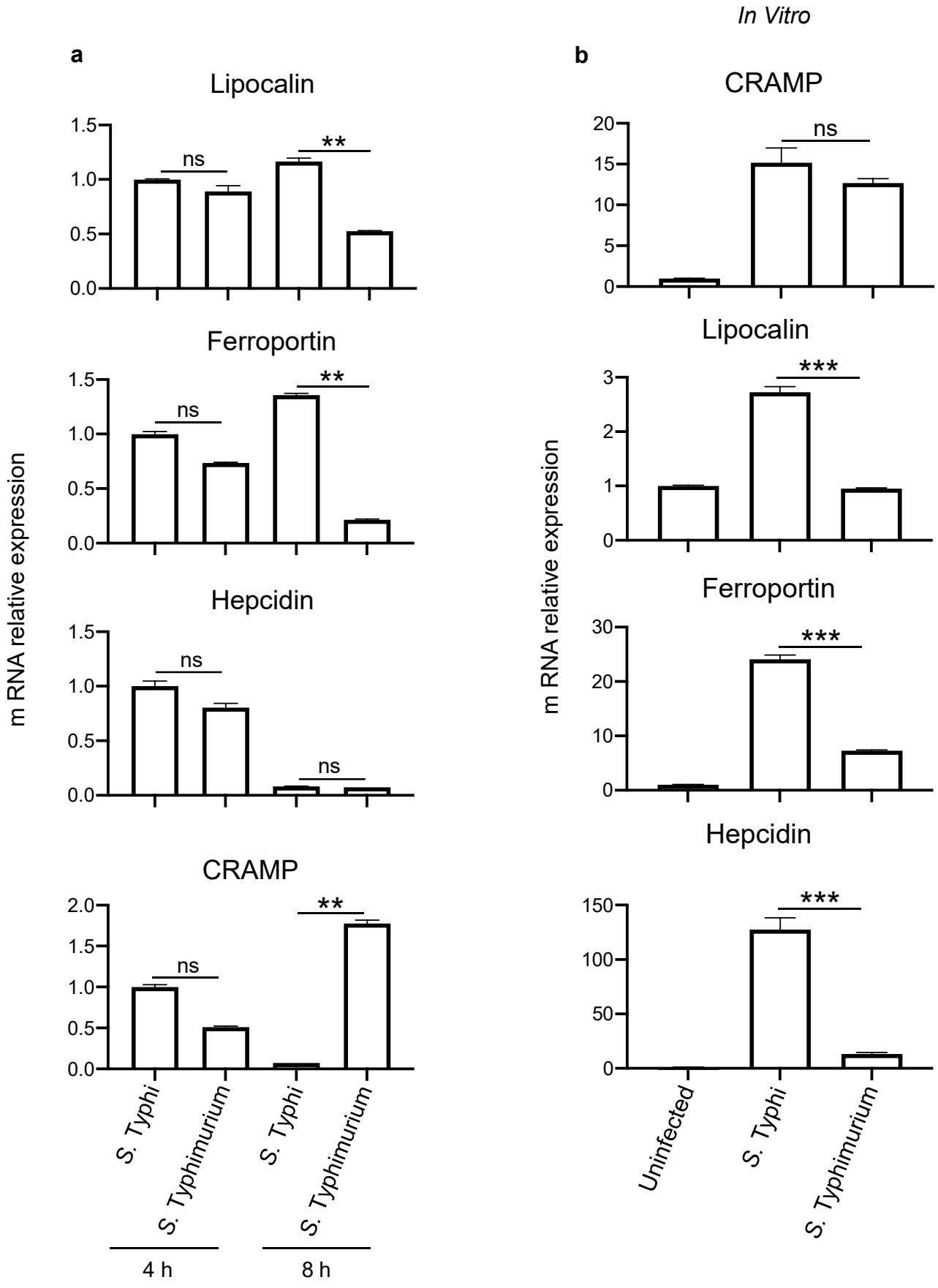

a
Anti-bacterial activity
Bacterial number
10^9
10^8
10^7
**
S. Typhi
S. Typhi + PMSF
Cell free peritoneal lavage
b
Mouse peritoneal cells (in vitro)
Bacterial number
10^9
10^8
10^7
ns
***
UI + UT
UI + PMSF
S. Typhi
S. Typhi + PMSF
c
Percent cell death
100
80
60
40
20
0
ns
***
UT + UI
UI + Glycine
S. Typhi
S. Typhi + Glycine
d
Bacterial number
10^9
10^8
10^7
ns
***
73%
27%
UT + UI
UI + Glycine
S. Typhi
S. Typhi + Glycine

Figure 4


In Vitro
a
b
Lipocalin
Ferroportin
Hepcidin
CRAMP
m RNA relative expression
ns
**
***
S. Typhi
S. Typhimurium
Uninfected
4 h
8 h