Salmonella Typhimurium employ spermidine to exert protection against ROS-mediated cytotoxicity and rewires host polyamine metabolism to ameliorate its survival in macrophages

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Running Head: *Salmonella* mounts an antioxidative response using de-novo and host-acquired spermidine

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Abstract

Salmonella infection involves a cascade of attacks and defence measures. After breaching the intestinal epithelial barrier, *Salmonella* is phagocytosed by the macrophages, inside which, the bacteria face multiple stresses and, consequently, employ appropriate countermeasures. We show that, in *Salmonella*, the polyamine spermidine activates a stress response mechanism by regulating critical antioxidant genes. *Salmonella* Typhimurium mutants for spermidine transport and synthesis cannot mount an antioxidative response, resulting in high intracellular ROS levels. These mutants are also compromised in their ability to be phagocytosed by macrophages. Furthermore, it regulates a novel enzyme in *Salmonella*, Glutathionyl-spermidine synthetase (GspSA), which is known to prevent the oxidation of proteins in *E.coli*. Moreover, the spermidine mutants and the GspSA mutant show significantly reduced survival in the presence of hydrogen peroxide *in vitro*, and lesser organ burden in the mouse model of *Salmonella* infection. Conversely, in macrophages isolated from *gp91phox*$^{-/-}$ mice, we observed a rescue in the attenuated fold proliferation previously observed upon infection. Interestingly, *Salmonella* upregulates polyamine biosynthesis in the host, which also solves the mystery of the attenuated proliferation observed in spermidine transport mutants. Thus, inhibition of this pathway in the host abrogates the proliferation of *Salmonella* Typhimurium in macrophages. From a therapeutic perspective, inhibiting host polyamine biosynthesis using an FDA-approved chemopreventive drug, D,L-α-difluoromethylornithine (DFMO), reduces *Salmonella* colonization and tissue damage in the mouse model of infection, while enhancing the survival of infected mice. Therefore, our work provides a mechanistic insight into the critical role of spermidine in stress resistance of *Salmonella*. It also reveals a strategy of the bacteria in modulating host metabolism to promote their intracellular survival and shows the potential of DFMO to curb S*almonella* infection.

Significance

A substantial duration of the infection cycle of *Salmonella* involves the macrophages, which present a very hostile environment to the bacteria. However, *Salmonella* is able to survive and proliferate within host macrophages and utilizes it to disseminate to secondary sites of infection. Our study identifies a novel strategy employed by *Salmonella* Typhimurium to counteract oxidative and nitrosative stress within the host. We demonstrate that spermidine is a critical regulatory molecule in *Salmonella* that regulates multiple antioxidative pathways along with a novel antioxidative enzyme (GspSA) in *Salmonella,* to prevent oxidative damage and assist in its virulence in mice. It further rewires host polyamine metabolism to prevent nitric oxide production and enhance its survival. In the era of anti-microbial resistance, this study further recognizes an FDA -approved chemopreventive drug, DFMO, which inhibits the host-polyamine metabolism, as a prospective antidote to treat *Salmonella* infection.

Introduction

A condition with ample nutrients, and desired temperature, pH, oxygen concentration, and osmolarity is usually considered optimal for the growth of microbes. An imbalance or any alteration in these parameters impedes the growth and survival of the microbes, considered as stress conditions. However, these parameters keep fluctuating in nature [1]. Hence, to persist and survive in the natural environment, with unforeseen stressful or lethal conditions, they must be adept in sensing, responding and adapting to them [2]. In the case of food-borne pathogens, they face an array of various stresses in multiple environments in which they dwell, such as from the natural habitat to commercial settings and inside the host system [3-5]. *Salmonella* is a food-borne pathogen that lives in aquatic bodies and soil, and enters the host system through contaminated food and water. In the host system, as it traverses to the intestine, *Salmonella* faces multiple

stressful conditions such as low pH, nutrient deprivation, bile salt stress, competition with the resident microbes of the gut and immunoglobulins, etc. Once it breaches the epithelial barrier, it is taken up by the macrophages at the lamina propria, through which it disseminates throughout the host system. Macrophages are phagocytic immune cells where *Salmonella* encounters a very hostile environment. Entry of the pathogen into the cell cytoplasm leads to a burst of reactive oxygen species (ROS) and reactive nitrogen species (RNS). In macrophages, *Salmonella* resides in a specialised niche called the *Salmonella*-containing vacuole (SCV), which presents multiple other stresses to the bacteria, such as acidification, nutrient limitation and attack by the antimicrobial peptides. However, *Salmonella* employs numerous weapons from its arsenal to counteract the stresses it faces within the host macrophages.

Polyamines are a group of primordial stress response molecules in prokaryotes [6]. Multiple research groups have elucidated the link between polyamines and bacterial stress response. In *Shigella spp.* the polyamine spermidine accumulates during its infection into macrophages, which increases the expression of *katG* and helps in bacterial antioxidant response [7]. Moreover, spermidine localises to the surface of *Pseudomonas aeruginosa* and bind to the lipopolysaccharide (LPS) to protect the cells from oxidative damage [8]. In the Gram-positive bacteria, *Streptococcus pyogenes,* extracellular spermidine enhance the survival of the bacteria by upregulating oxidative response genes [9]. A research group has shown that polyamines are vital in resistance against nitrosative stress in *Salmonella* Typhimurium. Further, the group showed that spermidine is required for the systemic infection of *Salmonella* Typhimurium in mice [10]. Thus, it is conceivable that *Salmonella* Typhimurium utilises polyamines, such as spermidine, as a stress response molecule; however, the mechanism remains elusive.

91 Here, we show that spermidine biosynthesis and transport mutants of *Salmonella* Typhimurium
92 exhibit reduced survival upon infection in RAW264.7 cells. This diminished proliferation is also
93 observed in mice models of *Salmonella* infection, which is rescued in *gp91phox*$^{-/-}$ mice. We
94 demonstrate that spermidine orchestrates the various arms of antioxidative response and aids in
95 tightly regulating intracellular ROS levels. We further identify a novel antioxidative enzyme, Gsp,
96 in *Salmonella* Typhimurium, which is regulated by spermidine. The fascinating question that arises
97 is why the transporter mutant shows reduced survival. To this we find that *Salmonella*
98 Typhimurium harnesses the host polyamine biosynthesis for its survival. Furthermore, for the first
99 time, we show that an FDA-approved chemopreventive and anti-African Human Trypanosomiasis
100 drug that inhibit the polyamine biosynthesis in the host, is able to curb *Salmonella* infection in
101 mice models.

102 **Material and methods**

103 **Bacterial strains and growth condition**

104 *Salmonella enterica* serovars Typhimurium (STM WT) wild type strain ATCC 14028 was used in
105 all experiments which was a kind gift from Prof. Michael Hensel, Abteilung Mikrobiologie,
106 Universität Osnabrück, 273 Osnabrück, Germany. The bacterial strain was cultured in Luria broth
107 (LB-Himedia) with constant shaking (175rpm) at 37°C orbital-shaker. Kanamycin,
108 Chloramphenicol and Ampicillin antibiotics (Kanamycin-50µg/ml, Cholramphenicol-20µg/ml and
109 Ampicillin-50µg/ml) were used wherever required. (Bacterial strain list in Supplementary table **S-**
110 **Table 1**)

111 **Bacterial gene knock-out and strain generation**

112 The generation of gene knock-out in bacteria was done using the One-step chromosomal gene
113 inactivation protocol [11]. Briefly, the Kanamycin resistance gene and the Chloramphenicol
114 resistance gene amplified products were purified using chloroform-ethanol precipitation.
115 Followed by electroporation into the STM WT cells (expressing PKD-46 plasmid which provides
116 the λ-Red recombinase system) by a single pulse of 2.25 kV separately for the Kan^R and $Chlm^R$.
117 The transformant colonies were selected and patched on fresh plates and confirmed for knock-out
118 using PCR with primers designed corresponding to the ~100bp upstream and downstream of the
119 genes (knocked out) for the knock-out strains to observe a difference in PCR product size upon
120 STM *ΔpotCD* and STM *ΔspeED* knockout genration. For the generation of the double knock-out
121 strain (STM *ΔpotCDΔspeED*), the STM *ΔpotCD* (resistant to Kanamycin) was first transformed
122 with the plasmid pKD46 which provides the λ-Red recombinase system. To this transformed strain,
123 the purified PCR product to knock-out *speED* was electroporated to generate the STM
124 *ΔpotCDΔspeED* (resistant to Kanamycin and Chloramphenicol). For the generation of STM *Δgsp,*
125 Kanamycin resistance gene was amplified from pKD4 plasmid, and a similar protocol was used,
126 followed by selection on Kanamycin containing LB agar plates. (Knockout generation Primer list
127 in Supplementary table **S-Table 2**)

128 **Cell culture and maintenance**

129 RAW264.7 cells (murine macrophage cell line) were cultured in DMEM - Dulbecco's Modified
130 Eagle Medium (Lonza) supplemented with 10% FBS (Gibco) and 1% Penicillin-streptomycin
131 (Sigma- Aldrich) at 37°C humidified chamber (Panasonic) with 5% CO_2. For each experiment, the
132 cells were seeded onto the appropriate treated cell culture well plate at a confluency of 80% for
133 intracellular survival assay, and expression studies.

134 **Gentamicin protection assay**

The cells were infected with STM WT, STM *ΔpotCD*, STM *ΔspeED*, STM *ΔpotCDΔspeED*, STM *Δgsp* and STM *ΔkatG* at MOI of 10 (for intracellular survival assay) and MOI 25 (for qRT-PCR). After infecting the cell line with STM WT and the mutants, the plate was centrifuged at 700-900 rpm for 10 minutes to facilitate the proper adhesion. The plate was then incubated for 25 minutes at 37°C humidified chamber and 5% CO_2. Then the media was removed from the wells and washed with 1X PBS. Fresh media containing 100 µg/mL gentamicin was added and again incubated for 60 minutes at 37°C and 5% CO_2. The media was then removed, cells were washed with 1X PBS twice, and fresh media containing 25µg/mL gentamicin was added. The plate was incubated at 37°C and 5% CO_2 till the appropriate time. For the intracellular survival assay, two time points were considered 2 hours and 16 hours, and for qRT-PCR three time points were considered 2 hours, 6 hours and 16 hours. For phagocytosis assay upon opsonisation, all the strains were washed with 1XPBS and incubated at 37°C with mouse complement sera for 1 hour before gentamicin protection assay

Intracellular survival assay and phagocytosis assay

At the appropriate time post-infection, the cells were lysed using 0.1% Triton X followed by addition of more 1X PBS and samples were collected. The collected samples were plated at the required dilutions on LB agar plates and kept at 37°C. 12 hours post incubation the Colony forming units (CFU) were enumerated for each plate.

The fold proliferation and invasion were determined as follows

Fold Proliferation = (CFU at 16 hours post-infection)/(CFU at 2 hours post-infection)

Percentage Phagocytosis = [(CFU at 2 hours post-infection)/(CFU of the Pre-inoculum)]×100

RNA isolation and qRT-PCR

157 RNA isolation was performed from infected cells after appropriate hours of infection with STM
158 WT, STM *ΔpotCD,* STM *ΔspeED* by RNA isolation was performed using TRIzol (Takara) reagent
159 according to manufactures' protocol RNA was quantified using Thermo-fischer scientific Nano
160 Drop followed by running on 2% agarose gel for checking the quality. For cDNA synthesis, first
161 DNase I treatment with 3µg of isolated RNA was done at 37°C for 60 minutes, which was then
162 stopped by heating at 65°C for 10 minutes. Then RNA (free of DNA) was subjected to Reverse
163 transcription using Random hexamer, 5X RT buffer, RT enzyme, dNTPs and DEPC treated water
164 at 37°C for 15 minutes, followed by heating at 85°C for 15 seconds. Quantitative real-time PCR
165 was done using SYBR green RT-PCR kit in BioRad qRT-PCR system. A 384 well plate with three
166 replicates for each sample was used. The expression levels of the gene of interest were measured
167 using specific RT primers. Gene expression levels were normalised to 16SrDNA primers of *S.*
168 Typhimurium. Gene expression levels of eukaryotic gene of interest were normalised to beta-actin
169 of mouse/human as required. For expression studies in bacteria grown in LB media, the bacterial
170 samples were harvested at 3 hours, 6 hours, 9 hours and 12 hours post subculture in fresh LB media
171 in 1:100 ratio and 1mM H_2O_2 was added to the broth, to study the gene expression in the presence
172 of oxidative stress. Then similar protocol was used to isolate total RNA using TRIzol (Takara)
173 reagent according to manufactures' protocol. (Expression Primer list in Supplementary table **S-**
174 **Table 2**)

175 **Primary macrophages isolation and infection**

176 Primary macrophages were isolated from C57BL/6 mice . The mice were intraperitoneally injected
177 with 8% Brewer's Thioglycolate. Five days post injection the primary macrophages were
178 aseptically isolated by injecting ice-cold 1xPBS into the peritoneal cavity the peritoneal lavage

179 was collected. Any residual erythrocytes were lysed using RBC lysis buffer, and the isolated cells
180 were maintained in complete RPMI 1640 media for further experiments.

181 **Intracellular Reactive oxygen species determination using H2DCFDA staining**

182 Overnight cultures were sub-cultured in fresh LB media. Once the cultures reached OD 0.1 then
183 10^8 CFU/ml of each strain was incubated with 10µM of 2',7'-dichlorodihydrofluorescein diacetate
184 (H2DCFDA) in 1xPBS (pH 7.2) at 37°C for 30 minutes. The bacterial cells were centrifuged and
185 the cells were resuspended in 1xPBS (pH 7.2) with Hydrogen peroxide of different concentrations,
186 and incubated 37°C (orbital shaker) for 2 hours. The samples were transferred to a 96 well ELISA
187 plate and fluorescence was determined in Tecan-ELISA plate reader (Ex- 490nm/ Em- 520nm).

188 **Intracellular redox status determination**

189 The STM WT, STM *ΔpotCD,* STM *ΔspeED* and STM *Δgsp* were transformed with pQE60-Grx1-
190 roGFP2 (a gift from Prof. Amit Singh, CIDR, IISc). Overnight cultures were sub-cultured in fresh
191 LB media. Once the cultures reached OD 0.1 then 10^8 CFU/ml of each strain was incubated with
192 Hydrogen peroxide of different concentrations in 1xPBS (pH 7.2) with, and incubated 37°C
193 (orbital shaker) for 2 hours. The samples were centrifuged and resuspended in fresh 1xPBS (pH
194 7.2). And tubes were analysed for GFP fluorescence at 408nm and 488nm respectively using BD-
195 FACS Verse flowcytometer (total 10,000 events for each sample).

196 ***In vitro* sensitivity assays**

197 Overnight cultures were sub-cultured in fresh LB media. Once the cultures reached OD 0.1 then
198 10^8 CFU/ml of each strain was incubated with Hydrogen peroxide or sodium nitrite of different
199 concentrations in 1xPBS(pH 7.2) (1xPBS of pH 5.4 was used for nitrite sensitivity assay) with,

200 and incubated 37°C (orbital shaker) for 2 hours. The samples were plated on SS agar to enumerate
201 the CFU and the percentage survival was determined as:

202 Percentage Survival : [CFU/ml for treated with H_2O_2/ $NaNO_2$]/ [CFU/ml for untreated] ×100

203 **Transfection for knockdown**

204 RAW 264.7 cells were seeded at a confluency of 50-60% 12 hours prior to transfecting using either
205 PEI (1:2 -DNA: PEI). We used two different constructs for knock-down of Odc1, A7 and F8 and
206 both as a mixed construct (A7:A8 at 1:1 ratio) and similarly for Srm , E9 and F1 and both as a
207 mixed construct (E9:F1 at 1:1 ratio) from the Sigma Mission shRNA library. 400ng of plasmid
208 DNA/well (ratio 260/280 ~1.8- 1.9) was used for transfection in a 24well plate. Cells were then
209 incubated for 8 hours at 37°C in a humidified incubator with 5% CO2; after that, the media
210 containing transfecting DNA and reagents were removed, and cells were further incubated for 48
211 hours in complete media DMEM +10% FBS. Cells were harvested for further analysis or infected
212 with the required MOI. (shRNA sequence list in Supplementary table **S-Table 3**)

213 **Immunofluorescence**

214 After the appropriate incubation time, the media was removed, and the cells were washed with 1X
215 PBS. The cells were then fixed with 3.5% Paraformaldehyde for 10 minutes. The cells were then
216 washed with 1X PBS and incubated with the required primary antibody (anti- *Salmonella* LPS) in
217 a buffer containing 0.01% saponin and 2% BSA and incubated at room temperature for 45-60
218 minutes. The primary antibody was then removed by washing with 1X PBS and then incubated
219 with the appropriate secondary antibody tagged to a fluorochrome. The coverslips were then
220 washed with PBS and mounted on a clean glass slide using mounting media containing an anti-
221 fade reagent. The coverslips were sealed with clear nail polish and observed under the confocal

microscope. For studying histopathology samples Zeiss 880 microscope 40X oil immersion 2x319 3x zoom was used.

Intracellular RNS determination

For determination of intracellular RNS, a cell permeable nitric-oxide probe, 4, 5-diaminofluorescein diacetate (DAF2A) was used. The protocol has been followed as Roy Chowdhury A et. al. [12]. Briefly, After 16 hours of infection of RAW264.7 cells(transiently knock down of *Odc1*) at MOI 10 with STM WT, the cells were incubated with fresh DMEM containing 5µM of DAF2DA. The cells were incubated at 37°C in a humidified incubator with 5% CO_2 for 30 minutes. The media containing dye was removed and the cells were washed with 1xPBS and the cells were acquired immediately for flow-cytometry (BD FACS Verse) (Ex-491nm/Em- 513nm).

Intracellular Glutathione determination

The intracellular reduced Glutathione (GSH) concentration was determined by modification of a pubmished protocol [13]. Briefly, a standard curve with known concentration of GSH was prepared. Reaction mixture for each contained 600µL of phosphate buffer (0.1M, pH7), 40µL of 0.4% w/v 5,5-dithiobis(2-nitrobenzoic acid) (DTNB, from Sigma), 100 µL of the standard solutions of GSH (0mM-1mM range) and autoclaved MilliQ water to make up the volume to 1000µL.The mixture was incubated at room temperature for 5 minutes and absorbance was measured at 412nm using tecan plate reader. The bacterial strains were subcultured in fresh LB media and grown till OD 0.1 (exponential phase) and washed with buffer (Tris, Sodium chloride and EDTA) and lysed using sonication. The supernatant after sonication was used as the sample for GSH detection. As previously described for standard curve. From the standard curve the intracellular concentrations were interpolated.

In silico analysis

The *in silico* protein structure determination was performed using the SWISS-MODEL software (https://swissmodel.expasy.org/), where we supplied the protein sequence of GspSA of *Salmonella* Typhimurium from UniProt. We analysed the model with highest sequence identity and maximum coverage with the Gsp from *E.coli*. The structure depicts a Homo-dimer with a GMQE of 0.93 and QMEANisCo Global of 0.88 ± 0.05.

In vivo animal experiment

5-6weeks old C57BL/6 mice were infected by orally gavaging 10^7 CFU of STM WT, STM *ΔpotCD,* STM *ΔspeED,* STM *ΔpotCDΔspeED,* STM *Δgsp* and STM *ΔkatG*. To study the colonisation in organs, the intestine (Peyer's patches), MLN, spleen and liver were isolated aseptically, 5 days post-infection, and the CFU was enumerated on differential and selective SS agar by serial dilution. For intraperitoneal infection 5-6weeks old C57BL/6 mice were infected by intraperitoneally injecting 10^3 CFU of STM WT, STM *ΔpotCD,* STM *ΔspeED,* STM *ΔpotCDΔspeED,* STM *Δgsp* and STM *ΔkatG.* To study the colonisation in organs, spleen and liver were isolated aseptically 3 days post-infection. Blood was isolated by heart puncture 3 days post-infection. The CFU was enumerated on differential and selective SS agar by serial dilution. Organs were stored in 3.5%PFA before histopathogical sample preparation.

For inhibitor treatment, 5-6weeks old C57BL/6 mice were infected by orally gavaging 10^7 CFU of STM WT. The inhibitor DFMO was intraperitoneally injected every alternate day from day 1 at two doses 2mg/kg and 1mg/kg of body weight of mice. To study the colonisation in organs, the intestine (Peyer's patches), MLN, spleen and liver were isolated aseptically, 5 days post-infection, and the CFU was enumerated on differential and selective SS agar by serial dilution. For survival assay of mice 5-6weeks old C57BL/6 mice were infected by orally gavaging 10^8 CFU of STM

268 WT. The inhibitor DFMO was intraperitoneally injected every alternate day from day 1 at two
269 doses 2mg/kg and 1mg/kg of body weight of mice. The survival was monitored till 10 days.
270 Likewise, organs were stored in 3.5% PFA before histopathogical sample preparation

271 **2.2.12 Statistical Analysis**

272 Statistical analyses were performed with GraphPad Prism software. The Student's t-test was
273 performed as indicated. The results are expressed as mean ± SD or mean ± SEM. Group sizes,
274 experiment number, and p values for each experiment are described in figure legends.

275 **Results**

276 **Loss of spermidine transporter and biosynthesis genes in *Salmonella* Typhimurium**
277 **compromises its ability to be phagocytosed by the macrophages**

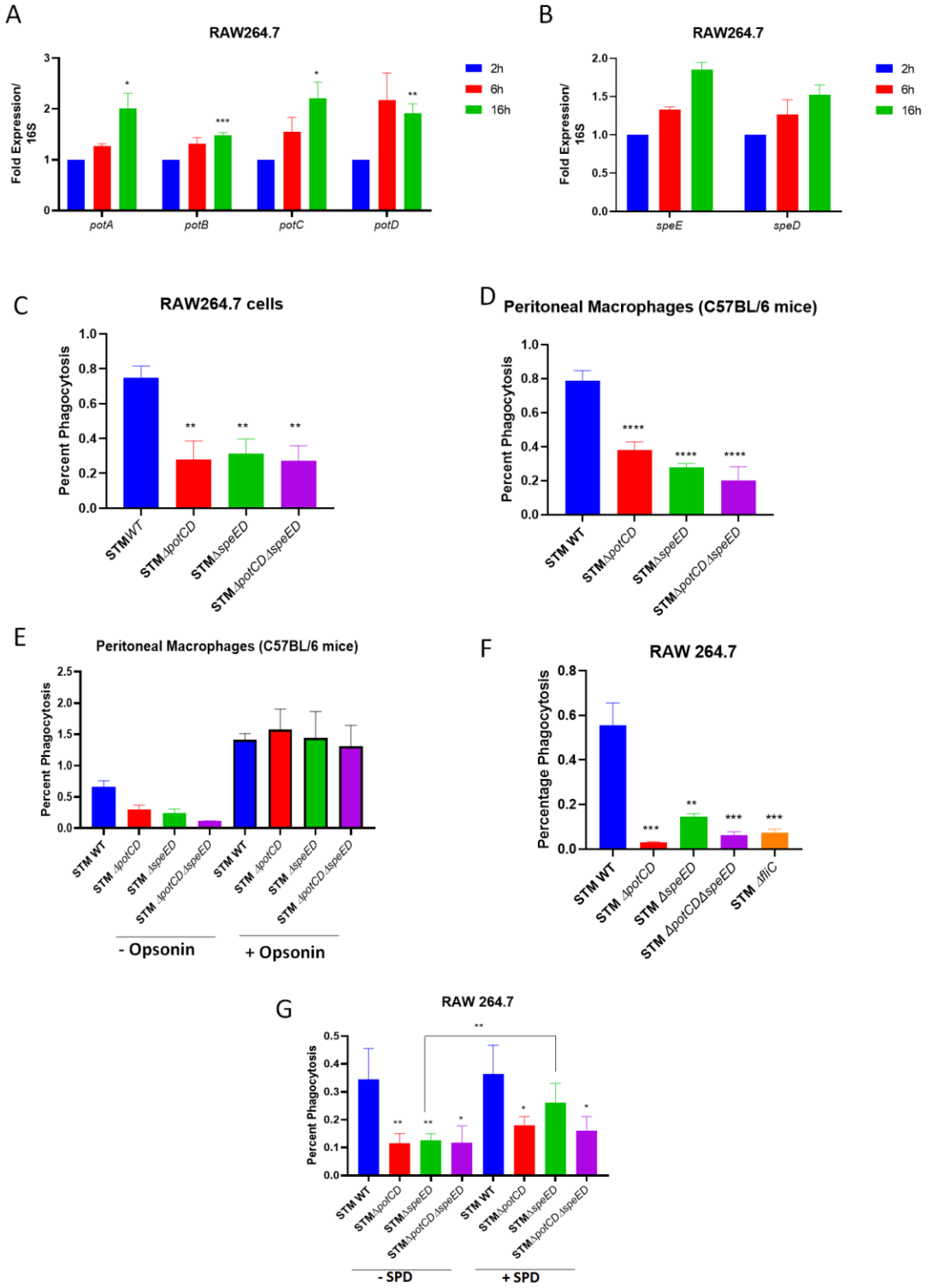
278 The pathoadaptation in *Salmonella* involves multiple players, which counteracts the stressful
279 condition it encounters in the host macrophages. Polyamines being a group of well-studied stress
280 response molecules, we were interested in determining the expression of the spermidine transporter
281 and biosynthesis genes in *Salmonella* Typhimurium. Our previous study shows that *Salmonella*
282 upregulates the spermidine transporter genes (*potA, potB, potC* and *potD*) and the biosynthesis
283 genes (*speE* and *speD*) during the log phase of growth *in vitro.* Here we assessed the mRNA levels
284 of *potA, potB, potC* and *potD* in STM WT upon infection into the RAW264.7 macrophage cell
285 line. We noted that all the genes showed a 1.5-2 fold upregulation post 6 hours of infection into
286 macrophages till 16 hours **(Fig 1 A)**. Further, our results showed that the spermidine biosynthesis
287 enzymes *speE* and *speD* were upregulated 1.5 to 2 folds post 6 hours to 16 hours post-infection
288 into macrophages **(Fig 1 B)**. These results indicate that *Salmonella* Typhimurium enhances its
289 intracellular spermidine biosynthesis and imports from the extracellular milieu. It might be a

290 strategy of the pathogen to increase the intracellular pool of stress response molecules like the
291 polyamine spermidine, as it encounters the hostile environment of host macrophages.

292 We then determined how the spermidine transporter and biosynthesis mutants (STM Δ*potCD*, STM
293 Δ*speED* and STM Δ*potCD*Δ*speED*) behave upon infection into RAW264.7 macrophages. We
294 infected STM WT, STM Δ*potCD*, STM Δ*speED* and STM Δ*potCD*Δ*speED* into RAW264.7 cells
295 and observed that STM Δ*potCD*, STM Δ*speED* and STM Δ*potCD*Δ*speED* showed reduced
296 phagocytosis by the macrophages compared to the wild type (**Fig 1 C**). Interestingly, STM
297 Δ*potCD*, STM Δ*speED* and STM Δ*potCD*Δ*speED* showed a compromised ability to proliferate
298 intracellularly in the RAW264.7 cells further validating a previous study [10](**Fig S1 A)**. To further
299 validate our results, we assessed the behaviour of the spermidine transporter and biosynthesis gene
300 mutants in primary macrophages isolated from the peritoneal lavage of C57BL/6 mice. Likewise,
301 the mutants showed attenuated proliferation and uptake by phagocytosis into the peritoneal
302 macrophages (**Fig 1 D and S1 B)**. Collectively these results suggest that spermidine is a critical
303 molecule in *Salmonella* Typhimurium to infect and survive in macrophages. To further investigate
304 the reason behind the reduced ability to be taken up by macrophages upon loss of spermidine
305 biosynthesis and transport, we treated STM WT, STM Δ*potCD*, STM Δ*speED* and STM
306 Δ*potCD*Δ*speED* with mouse complement-sera. Mouse complement-sera acts as an opsonin and
307 thus potentiates the interaction of the bacteria with the macrophages. Upon pre-treatment with
308 mouse complement-sera, we noted a rescue in the reduced uptake of the mutants by peritoneal
309 macrophages isolated from C57BL/6 mice (**Fig 1 E)**. A study on *Salmonella* Typhimurium
310 revealed that the aflagellate and non-motile *Salmonella* collide less frequently with macrophages
311 and gets merest time to maintain contact with the macrophages, thereby showing decreased
312 phagocytosis [14]. Our group previously showed that loss of spermidine production and import in

313 *Salmonella* Typhimurium results in the loss of flagella formation on the bacterial cell surface.
314 Thus, we determined the percentage phagocytosis for a flagellin-deficient strain of *Salmonella*
315 Typhimurium, STM Δ*fliC* and observed that STM Δ*potCD*, STM Δ*speED*, STM Δ*potCD*Δ*speE*
316 and STM Δ*fliC* exhibited a significantly decreased ability to be taken up by the RAW264.7
317 macrophages (**Fig 1 F**). Furthermore, we observed a rescue of the attenuated percentage
318 phagocytosis and fold proliferation of only STM Δ*speED* upon supplementation of spermidine
319 (100μM) during the growth of bacteria prior to infection (**Fig 1 G and S1C**). Hence, the plausible
320 explanation is that the reduced ability to form flagella in the spermidine mutants causes less
321 frequent interaction with the macrophages and provides minimal contact time for infection, leading
322 to reduced phagocytosis by the macrophages.

Figure 1: Loss of spermidine transporter and biosynthesis genes in *Salmonella* Typhimurium compromises its ability to be phagocytosed by the macrophages



323

324 **Figure 1: Loss of spermidine transporter and biosynthesis genes in *Salmonella* Typhimurium**
325 **compromises its ability to be phagocytosed by the macrophages**

326 A.The mRNA expression of spermidine transporter genes *potA, potB, potC* and *potD* in STM WT
327 upon infection into RAW264.7 cells, B. The mRNA expression of spermidine biosynthesis genes
328 *speE* and *speD* in STM WT upon infection into RAW264.7 cells, C. The percentage phagocytosis
329 of the spermidine mutants in RAW264.7 cells, D. The percentage phagocytosis in primary
330 macrophages isolated from peritoneal lavage of C57BL/6 mice, E. The percentage phagocytosis
331 upon pre treatment with mouse-complement sera which act as an opsonin, F. The percentage
332 phagocytosis in RAW264.7 cells with flagellin mutant (STM *ΔfliC*), G. The percentage
333 phagocytosis in RAW264.7 cells with the spermidine mutants grown in media supplemented with
334 100μM spermidine (SPD). Student's t-test was used to analyze the data; p values ****<0.0001,
335 ***<0.001, **<0.01, *<0.05. Two-way Anova was used to analyze the grouped data; p values
336 ****<0.0001, ***<0.001, **<0.01, *<0.05.

337 **Spermidine provides stress resistance in *Salmonella* Typhimurium by regulation of the**
338 **expression of numerous antioxidative enzymes**

339 The loss of spermidine transport and biosynthesis function in *Salmonella* Typhimurium renders it
340 incapable of proliferation and survival in macrophages. In the host macrophages, the bacteria
341 encounter numerous threats, of which the foremost is the rapid oxidative burst mediated by the
342 NOX2. The reactive oxygen species superoxide radical can easily diffuse through the bacterial
343 membrane and pose a major threat to the pathogen. ROS acts on multiple molecules such as nucleic
344 acids, proteins and lipids, thus damaging the cell membranes, DNA and proteins within the bacteria
345 [15]. Spermidine has been linked to stress response against oxidative stress and protects bacteria

346 in *E. coli* and *Pseudomonas aeruginosa.* Thus, we were intrigued to understand the role of
347 spermidine in antioxidative response in *Salmonella* Typhimurium. We examined the survival of
348 STM WT, STM Δ*potCD*, STM Δ*speED*, STM Δ*potCD*Δ*speE* upon exposure to the oxidative agent
349 hydrogen peroxide *in vitro.* We noticed that at high concentrations of hydrogen peroxide, 5mM
350 and 10mM, the STM Δ*potCD*, STM Δ*speED* and STM Δ*potCD*Δ*speE* showed significantly lesser
351 survival than STM WT(**Fig 2 A**). We further determined the expression of *potA, potB, potC, potD*,
352 *speE* and *speD* in STM WT upon exposure to 1mM hydrogen peroxide. There was a 2-3 fold
353 upregulation in the mRNA expression of the transporter genes over the untreated during the early
354 log phase of growth (6 hours) and the late log phase of growth (12 hours) *in vitro* (**Fig S2 A-D**).
355 Similarly, the biosynthesis genes *speE* and *speD* were 4-6 fold upregulated in their corresponding
356 mRNA expressions during their early log phase of growth (6 hours) and the late log phase of
357 growth (12 hours) *in vitro* (**Fig S2 E and F**). Our results show that *Salmonella* Typhimurium
358 upregulates spermidine transport and biosynthesis upon oxidative stress suggesting that
359 spermidine mounts a protective function in such a stressful condition to aid bacterial survival.

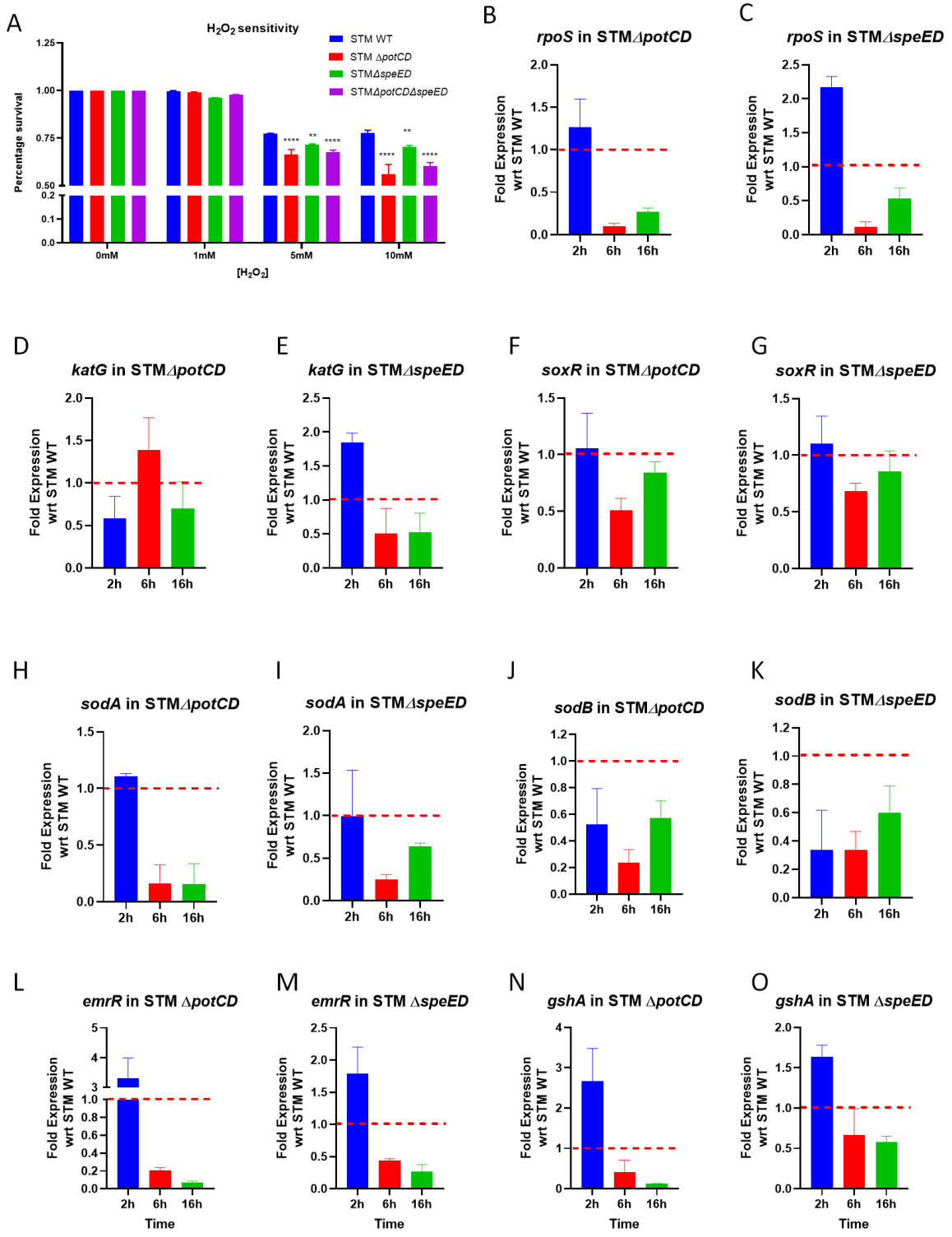
360 Bacteria sense the environmental changes and cues to respond and adapt to the altered
361 environment. They use the two-component systems, transcriptional activators and repressors to
362 alter gene expression in response to a stimulus [16]. Polyamines in *E. coli* regulate multiple genes
363 at the transcription and translation together, referred to as the "Polyamine modulon". These involve
364 the numerous mRNAs, tRNAs, sigma factors, translational factors and two-component systems
365 during the bacterial growth as well as in stress conditions [17-19]. *Salmonella* harbors multiple
366 antioxidative enzymes to detoxify the ROS intracellularly [20]. Our study so far show that STM
367 Δ*potCD*, STM Δ*speED* and STM Δ*potCD*Δ*speE* is attenuated in survival under *in vitro* oxidative
368 stress than STM WT. To gain mechanistic insight into the attenuated survival of *Salmonella*

369 Typhimurium we determined the mRNA expression of the critical transcription factor *rpoS*, which
370 activates the expression of the catalase enzymes (*katG and katE*) in order to detoxify hydrogen
371 peroxide to water in the bacteria enzymatically. In both STM Δ*potCD* and STM Δ*speED* the mRNA
372 expression of *rpoS* is significantly down regulated from 6 hours post infection in RAW264.7 cells
373 (**Fig 2 B and C, S3 A**). Further, its downstream target *katG* was also down regulated in STM
374 Δ*potCD* and STM Δ*speED* from 6 hours post infection into RAW264.7 cells (**Fig 2 D and E, S3**
375 **B**). We further assessed the mRNA expression of the transcription factor *soxR*, which regulates the
376 expression of superoxide dismutases (*sodA* and *sodB*). Upon infection in RAW264.7 cells,
377 expression of *soxR* was significantly downregulated in STM Δ*potCD* and STM Δ*speED* with
378 respect to STM WT (**Fig 2 F and G, S3 C**). Superoxide dismutases act on superoxide radicals, the
379 potent ROS encountered in macrophages, converting to hydrogen peroxide. The mRNA expression
380 of both *sodA* and *sodB* were likewise downregulated in STM Δ*potCD* and STM Δ*speED* upon
381 infection into RAW264.7 macrophages (**Fig 2 H-K, S3 D and E**). A major antioxidant in most
382 living organisms is glutathione (GSH), which directly acts as a quencher of ROS. GSH is
383 synthesized by Glutathione synthase (GshA) which in turn is regulated by EmrR transcription
384 factor. We observed that the mRNA expression of *emrR* is downregulated in STM Δ*potCD* and
385 STM Δ*speED* upon infection into RAW264.7 macrophages (**Fig 2 L and M, S3 F**). Similarly, *gshA*
386 transcript expression is downregulated as well (**Fig 2 N and O, S3 G)**. To further validate the down
387 regulation of the glutathione synthesis arm in spermidine transport and biosynthesis mutants, we
388 determined the intracellular GSH levels and noted the in STM Δ*potCD,* STM Δ*speED* and STM
389 Δ*potCD*Δ*speED* the levels were significantly less (**Fig S3H and I).** Taken together these results
390 indicate that spermidine regulates the transcription of multiple transcription factors involved in
391 oxidative stress response in *Salmonella* Typhimurium. Importantly, we found a mechanism of

392 oxidative stress resistance in *Salmonella* Typhimurium regulated by the spermidine, potentiating

393 the survival of the bacteria.

Figure 2: Spermidine provides stress resistance in *Salmonella* Typhimurium by regulation of the expression of numerous antioxidative enzymes



395 **Figure 2: Spermidine provides stress resistance in *Salmonella* Typhimurium by regulation of**
396 **the expression of numerous antioxidative enzymes**

397 A.The *in vitro* hydrogen peroxide sensitivity assay with the spermidine transport and biosunthesis
398 mutants, B. The mRNA expression of stress responsive transcription factor *rpoS* in STM *ΔpotCD*
399 upon infection into RAW264.7 cells, C. The mRNA expression of stress responsive transcription
400 factor *rpoS* in STM *ΔspeED* upon infection into RAW264.7 cells, D. The mRNA expression of
401 *katG* in STM *ΔpotCD* upon infection into RAW264.7 cells, E. The mRNA expression of *katG* in
402 STM *ΔspeED* upon infection into RAW264.7 cells, F. The mRNA expression of oxidative stress
403 responsive transcription factor *soxR* in STM *ΔpotCD* upon infection into RAW264.7 cells, G. The
404 mRNA expression of oxidative stress responsive transcription factor *soxR* in STM *ΔspeED* upon
405 infection into RAW264.7 cells, H. The mRNA expression of *sodA* in STM *ΔpotCD* upon infection
406 into RAW264.7 cells, I. The mRNA expression of *sodA* in STM *ΔspeED* upon infection into
407 RAW264.7 cells, J. The mRNA expression of *sodB* in STM *ΔpotCD* upon infection into
408 RAW264.7 cells, K. The mRNA expression of *sodB* in STM *ΔspeED* upon infection into
409 RAW264.7 cells, L. The mRNA expression of glutathione synthetase specific transcription factor
410 *emrR* in STM *ΔpotCD* upon infection into RAW264.7 cells, M. The mRNA expression of
411 glutathione synthetase specific transcription factor *emrR* in STM *ΔspeED* upon infection into
412 RAW264.7 cells, N. The mRNA expression of *gshA* in STM *ΔpotCD* upon infection into
413 RAW264.7 cells, O. The mRNA expression of *gshA* in STM *ΔspeED* upon infection into
414 RAW264.7 cells, Student's t-test was used to analyze the data; p values ****<0.0001, ***<0.001,
415 **<0.01, *<0.05.

416 **Spermidine controls a novel enzyme Glutathionyl-spermidine synthetase in *Salmonella***
417 **Typhimurium, and together mount an intracellular antioxidative response**

418 The spermidine synthesized from putrescine has two fates. It is either acetylated by the enzyme
419 SpeG or it is covalently conjugated to GSH to form Glutathionyl spermidine (GS-sp) catalysed by
420 the enzyme Glutathionyl-spermidine synthetase (Gss). Tabor (1974) first discovered the existence
421 of this enzyme in *E. coli* [21]. In *E. coli* GS-sp is generated at a higher level in the stationary phase
422 and very less in the late exponential phase. It also interacts and modifies the thiol containing
423 proteins under high H_2O_2-containing media leading to the formation of Gsp-thiolated proteins
424 (PSSGsp). Certain *in vitro* experiments with dehydro-ascorbate suggested that Gsp might have
425 higher antioxidant properties than GSH [22] and may be more effective in protecting against DNA
426 damage by free radicals [23]. However, in *E. coli* Gsp could not be linked to its pathogenicity [24].
427 Among *Enterobacteriaceae*, *Salmonella* was found to possess this unique enzyme. Our *in silico*
428 analysis suggested that the enzyme in *Salmonella* Typhimurium (GspSA, encoded by *gsp*) has 90%
429 identity with *E. coli gss* and the SWISS MODEL predicts it to be a homo-dimeric protein.(**Fig S4**
430 **A-C**). Also, the spermidine synthesised by SpeE in *Salmonella* Typhimurium is directly fed into
431 the pathway to synthesise GS-sp. We thus, investegated biological role of this novel enzyme in
432 *Salmonella* Typhimurium. We noted that the mRNA expression of *gsp* is significantly upregulated
433 at the late-log phase of growth of STM WT in LB media in the presence of H_2O_2 (**Fig S4 D**). Upon
434 infection into RAW264.7 cells, STM WT upregulates the mRNA expression of *gsp* at 6 hours and
435 16 hours post-infection into RAW264.7 cells (**Fig 3 A**). Further, the *Salmonella* Typhimurium
436 mutant of *gsp* (STM Δ*gsp*) showed attenuated proliferation in RAW264.7 cells similar to STM
437 Δ*katG,* which has reduced capability to detoxify ROS (**Fig 3 B)**. Thus, our results suggest that *gsp*
438 is a critical in *Salmonella* Typhimurium to survive and cope with the oxidative stress and hostile

439 environment of macrophages. Interestingly, the mRNA expression of *gsp* was found to be
440 downregulated in both STM Δ*potCD* and STM Δ*speED* at 16 hours post-infection into RAW264.7
441 macrophages (**Fig 3 C and D)**. These findings show that spermidine maintains its intracellular
442 levels by regulating the flux of spermidine into other pathways, such as the GspSA pathway. Also,
443 it further potentiates the ability of *Salmonella* Typhimurium to mount an antioxidative response by
444 regulating *gsp* expression

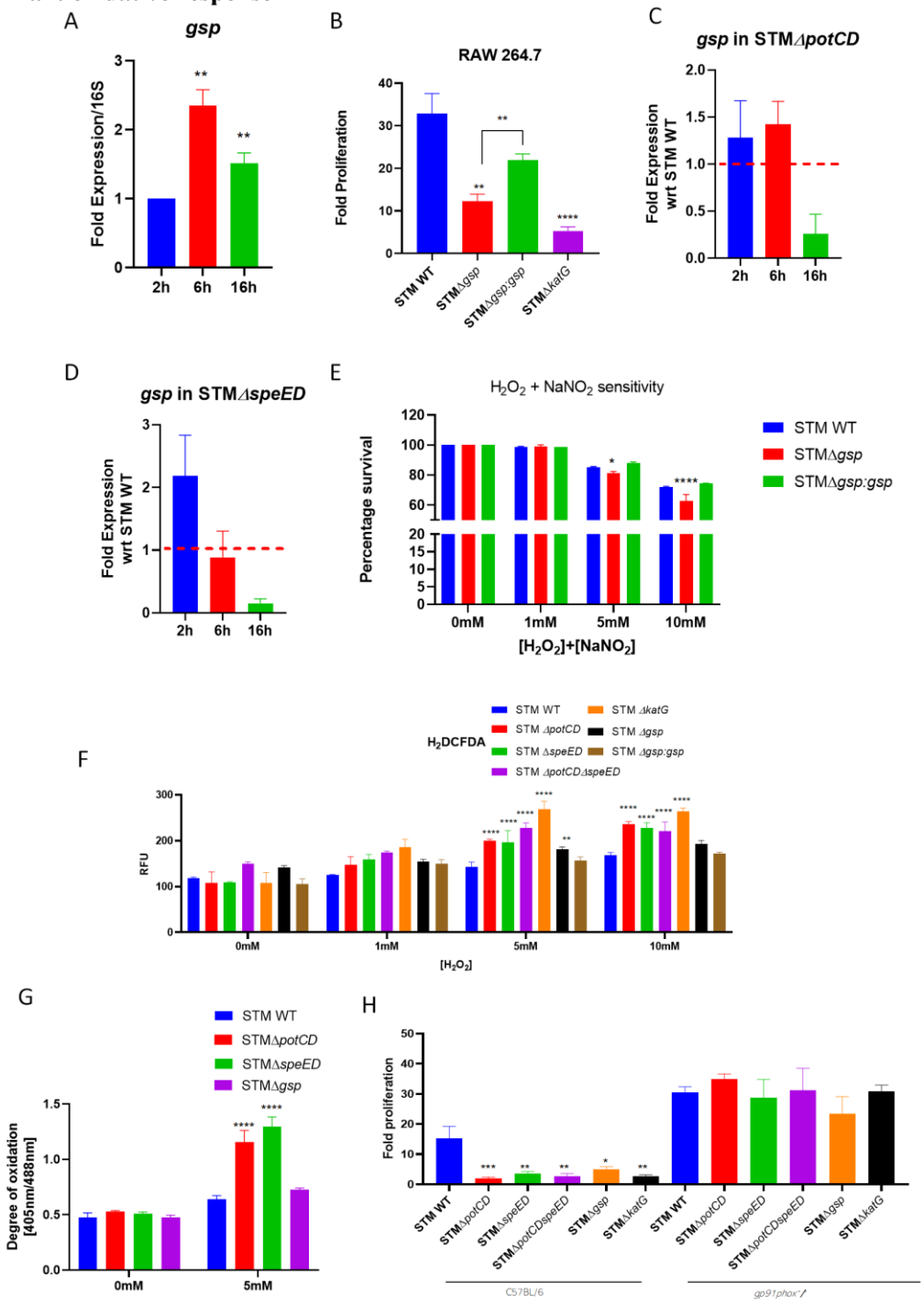
445 GspSA enzyme in Trypanosomes was named Trypanothione synthetase, while the conjugate is
446 called Trypanothione (TSH). In trypanosomes TSH is essential, and these organisms entirely rely
447 on TSH and possess no GSH reductase. Also, they have evolved to use TSH reductase instead of
448 GSH reductase, Glutaredoxins or Thioredoxins [25]. In *E. coli* Gsp forms bonds with the Cysteine
449 thiol groups of numerous proteins and protect them from oxidation under oxidative stress. Cysteine
450 thiol groups are highly prone to attack by ROS and get oxidised to sulphinic and sulphonic acids.
451 In *Salmonella* Typhimurium we observe that loss of *gsp* results in attenuated proliferation and
452 survival in macrophages. Thus, we investegated the survival of STM Δ*gsp* upon exposure to
453 hydrogen peroxide *in vitro*. STM Δ*gsp* exhibited reduced survival in the presence of high 5mM
454 and 10mM concentrations of H_2O_2 (**Fig S4 E**). Likewise, upon exposure to agents of oxidative
455 stress and nitrosative stress H_2O_2 and $NaNO_2$ together, we observe that STM Δ*gsp* exhibited
456 reduced survival at higher concentrations such as 5mM and 10mM (**Fig 4 E**). Thus, *gsp* is critical
457 in *Salmonella* Typhimurium to shield the bacteria from the action of ROS and RNS. As we
458 observed that spermidine transporter and biosynthesis mutants and *gsp* mutant of *Salmonella*
459 Typhimurium are compromised in their survival under oxidative stress and in macrophages thus,
460 we were interested to assess the intracellular ROS detoxification abilities of the strains. We
461 determined the intracellular ROS in STM WT, STM Δ*potCD*, STM Δ*speED*, STM Δ*potCD*Δ*speE,*

462 STM Δ*gsp* and STM Δ*katG*. Interestingly, STM Δ*potCD*, STM Δ*speED*, STM Δ*potCD*Δ*speE,*
463 STM Δ*gsp* and STM Δ*katG* showed significantly higher intracellular ROS levels when they were
464 exposed to 5mM and 10mM H_2O_2 (**Fig 3 F**). These suggest that lower intracellular levels of
465 spermidine correlate to higher intracellular ROS levels in *Salmonella* Typhimurium. To validate
466 our observed results, we utilised a genetically engineered tool to sense the redox status of the
467 bacterial cytosol, roGFP2 is a genetically modified form of GFP, and we use pQE60-Grx1-roGFP2
468 plasmid. The glutaredoxin (Grx1) fused to the roGFP2, reversibly transfers electrons between the
469 cytosolic pool of GSH/GSSG and the thiol group of roGFP2, and the ratio of fluorescence ratio at
470 408nm and 488nm determine the redox status of bacterial cytoplasm [12]. We observed that STM
471 Δ*potCD*, STM Δ*speED* harbouring the pQE60-Grx1-roGFP2 showed higher ratio of
472 405nm/488nm compared to STM WT in the presence of 5mM H_2O_2 (**Fig 3 G**). However, STM
473 Δ*gsp* did not show a significantly higher ratio of 405nm/488nm. Moreover upon supplementation
474 of the growth media with 100 μM spermidine, only in STM Δ*speED* we observed a lower
475 intracellular ROS and lesser 405nm/488nm in higher concentration of H_2O_2 (**Fig S4 F and**
476 **G)**.Thus, our results indicate that spermidine is critical in mounting an antioxidative response to
477 detoxify the intracellular ROS, by regulating multiple antioxidant genes in *Salmonella*
478 Typhimurium. To further dissect the role of spermidine in protection of *Salmonella* Typhimurium
479 from oxidative stress, we infected STM WT, STM Δ*potCD*, STM Δ*speED*, STM Δ*potCD*Δ*speE,*
480 STM Δ*gsp* and STM Δ*katG* in primary macrophages isolated from the peritoneal lavage of
481 *gp91phox -/-* mice. Gp91Phox is the major subunit of the NOX2 complex, that aids in the catalysis
482 of oxygen to superoxide radical. Interestingly we observe a rescue in the attenuated fold
483 proliferation of STM Δ*potCD*, STM Δ*speED*, STM Δ*potCD*Δ*speE,* STM Δ*gsp* and STM Δ*katG* in

484 peritoneal macrophages isolated from *gp91phox* -/- mice (**Fig 3 H**). Our results demonstrate the

485 vital role of spermidine in oxidative stress resistance in *Salmonella* Typhimurium.

Figure 3: Spermidine controls a novel enzyme Glutathionyl-spermidine synthetase in *Salmonella* Typhimurium, and together mount an intracellular antioxidative response



486

487 **Figure 3: Spermidine controls a novel enzyme Glutathionyl-spermidine synthetase in**
488 ***Salmonella* Typhimurium, and together mount an intracellular antioxidative response**

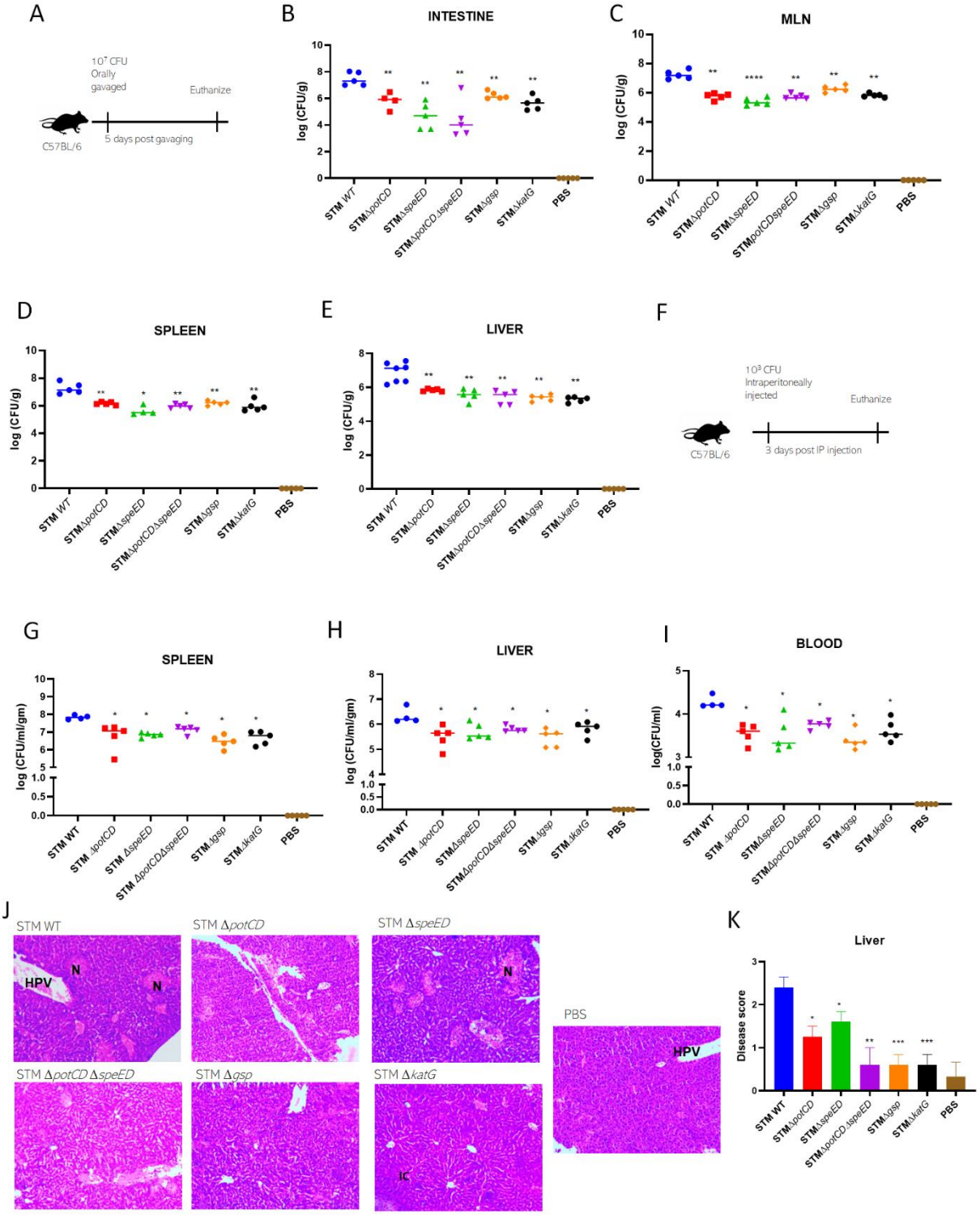
489 A.The mRNA expression of *gsp* in STM WT upon infection into RAW264.7 cells, B The fold
490 proliferation of STM WT, STM *Δgsp* and STM *Δgsp:gsp* in RAW264.7 cells, C. The mRNA
491 expression of *gsp* in STM *ΔpotCD* upon infection into RAW264.7 cells, D. The mRNA expression
492 of *gsp* in STM *ΔspeED* upon infection into RAW264.7 cells, E The *in vitro* Hydrogen peroxide
493 and nitic oxide sensitivity assay, F. Intracellular reactive oxygen species determination using the
494 cell permeable H2DCFDA dye, G. The intracellular degree of oxidation using pQE60-grx1-
495 roGFP2 construct, H. The fold proliferation in primary macrophages isolated from wild type
496 C57bl/6 mice and *gp91phox-/-* mice, Student's t-test was used to analyze the data; p values
497 ****<0.0001, ***<0.001, **<0.01, *<0.05. Two-way Anova was used to analyze the grouped
498 data; p values ****<0.0001, ***<0.001, **<0.01, *<0.05.

499 **Spermidine is critical for *Salmonella* Typhimurium to colonise the primary and secondary**
500 **sites of infection in mice**

501 *Salmonella* infects the host and breaching the epithelial cells at the Peyer's patches in the distal
502 ileum, it disseminates to the secondary sites of infection namely the Mesentric Lymph node
503 (MLN), spleen and liver. From the basolateral surface of the epithelial cells at the lamina propria,
504 *Salmonella* is taken by the macrophages and polymorphonuclear cells (PMN). We observed that
505 the spermidine transporter and biosynthesis mutants show attenuated survival in macrophages and
506 under oxidative stress *in vitro.* We were intrigued to study the behaviour of the mutants during *in*
507 *vivo* colonisation in the mouse model of *Salmonella* infection. We infected C57BL/6 mice by
508 orally gavaging with STM WT, STM Δ*potCD*, STM Δ*speED*, STM Δ*potCD*Δ*speE,* STM Δ*gsp* and

509 STM Δ*katG* at a CFU of 10^7 per mice. We noted that STM Δ*potCD*, STM Δ*speED*, STM
510 Δ*potCD*Δ*speE,* STM Δ*gsp* and STM Δ*katG* showed a significantly lower organ burden in Peyer's
511 patches, MLN, spleen and liver upon oral gavage (**Fig 4 A-E)**. Our previous study showed that the
512 spermidine transporter and biosynthesis mutants exhibit a poor invasiveness into IECs, which can
513 explain our *in vivo* colonisation upon oral gavage. Oral gavage mimics the physiological route of
514 *Salmonella* infection into its host. Hence, it requires to be able to breach the intestinal barrier
515 successfully. Incapability to invade the IECs in STM Δ*potCD*, STM Δ*speED*, STM Δ*potCD*Δ*speE*
516 and STM Δ*gsp* explains the diminished colonisation in the organs. To dissect the role of spermidine
517 in *in vivo* colonisation we infected C57Bl/6 mice intraperitoneally, by bypassing the entry by
518 breaching epithelial barrier. We observed that upon infecting intraperitoneally, STM Δ*potCD*, STM
519 Δ*speED*, STM Δ*potCD*Δ*speE,* STM Δ*gsp* and STM Δ*katG* exhibited reduced colonisation in
520 spleen and liver and less dissemination in blood compared to STM WT (**Fig 4 F-I)**. Also, the
521 histopathological sections show significantly less liver tissue damage with STM Δ*potCD*, STM
522 Δ*speED*, STM Δ*potCD*Δ*speE,* STM Δ*gsp* and STM Δ*katG* , which is validated by disease scoring
523 of the same(**Fig 4 J and K)**. Our results, thus show that spermidine aids in the *in vivo* pathogenesis
524 and virulence of *Salmonella* Typhimurium.

Figure 4: Spermidine is critical for *Salmonella* Typhimurium to colonise the primary and secondary sites of infection in mice



525

526 **Figure 4: Spermidine is critical for *Salmonella* Typhimurium to colonise the primary and**
527 **secondary sites of infection in mice**

528 A.The experimental protocol for organ burden in C57BL/6 mice by orally gavaging 10^7 CFU per
529 mice, B. The organ burden post 5 days of oral gavage in intestine, C. in Mesentric lymph node
530 (MLN), D. in spleen, E. in liver, F. The experimental protocol for organ burden in C57BL/6 mice
531 upon intraperitoneal (I.P) infection with 10^3 CFU per mice, G. The organ burden 3 days post I.P
532 infection in spleen, H. in liver, I. dissemination in blood, J. The hematoxylin and eosin staining of
533 the sections of liver 3 days post I.P infection of C57Bl/6 mice, K. Disease score from the
534 histopathogical sections of liver, Here, Small necrotic areas (N), Congestion and damage to the
535 endothelial lining of the central vein (C), Congestion of the hepatic portal vein and poor hepatic
536 architecture (HPV), Inflammatory immune cells (IC). The disease score is as :0 for normal
537 pathology, 1 for mild/ minor pathology, 2 for moderate pathology, and 3 for severe pathological
538 changes. Mann Whitney test was used to analyse organ burden in mice; p values ****<0.0001,
539 ***<0.001, **<0.01, *<0.05. Student's t-test was used to analyze the data; p values ****<0.0001,
540 ***<0.001, **<0.01, *<0.05.

541

542 ***Salmonella* rewires host polyamine metabolism to potentiate its survival within host**
543 **macrophages**

544 Most of the intracellular pathogens establish their persistence in the phagocytic cells and are often
545 found to be associated with different population of the macrophages. Like *Brucella abortus*
546 preferentially resides in the Alternatively activated macrophages (AAM), where it survives and
547 replicates by exploiting the host polyamines. A research group has shown that the metabolism of

548 AAM is shifted to increase polyamine biosynthesis by *Brucella abortus* and thereby promote the
549 bacterial survival [26]. Similarly, *Salmonella* also resides in macrophages, a way to lead chronic
550 infections. We were interested to know whether *Salmonella* exploits the host polyamines and leads
551 to a rewiring of host cell metabolism. To understand the host-pathogen relationship in depth, we
552 assessed the mRNA expression of Ornithine decarboxylase 1 (*mOdc1*) which catalyses the rate-
553 determining step of polyamine biosynthesis and Spermidine synthase (*mSrm*) that synthesises
554 spermidine from putrescine by transferring the aminopropyl group from decarboxylated-S in
555 RAW264.7 cells upon infection with STM WT. We observed that mRNA expression of *mOdc1*
556 and *mSrm* were upregulated at 6 and 16 hours post-infection (**Fig 5 A and B**). Further, the mRNA
557 expression of *mOdc1* and *mSrm* were enhanced in the spleen and liver of C57BL/6 mice 5 days
558 post oral gavage with STM WT (**Fig C- F**). Our results show that *Salmonella* Typhimurium upon
559 infection into the host enhances the expression of host polyamine biosynthesis genes. Moreover,
560 we have previously observed that the *Salmonella* Typhimurium that cannot import spermidine
561 cannot survive and proliferate as much as STM WT in macrophages. To further delve into the role
562 of host acquired polyamines, we knocked down *Odc1* in RAW264.7 cells (**Fig S5 A**). Upon
563 knockdown of *mOdc1*, *Salmonella* Typhimurium showed significantly attenuated proliferation in
564 RAW264.7 cells (**Fig 5 G**). However, the knockdown of *mOdc1* did not alter the percentage of
565 phagocytosis by the macrophages (**Fig 5 H)**. Similarly we knocked down *Srm* in RAW264.7 cells
566 and observed that knock-down of spermidine synthase in the host compromises the ability of STM
567 WT to proliferate and get phagocytosed by the macrophages (**Fig S5 B-D)**.

Figure 5: *Salmonella* rewires host polyamine metabolism to potentiate its survival within host macrophages

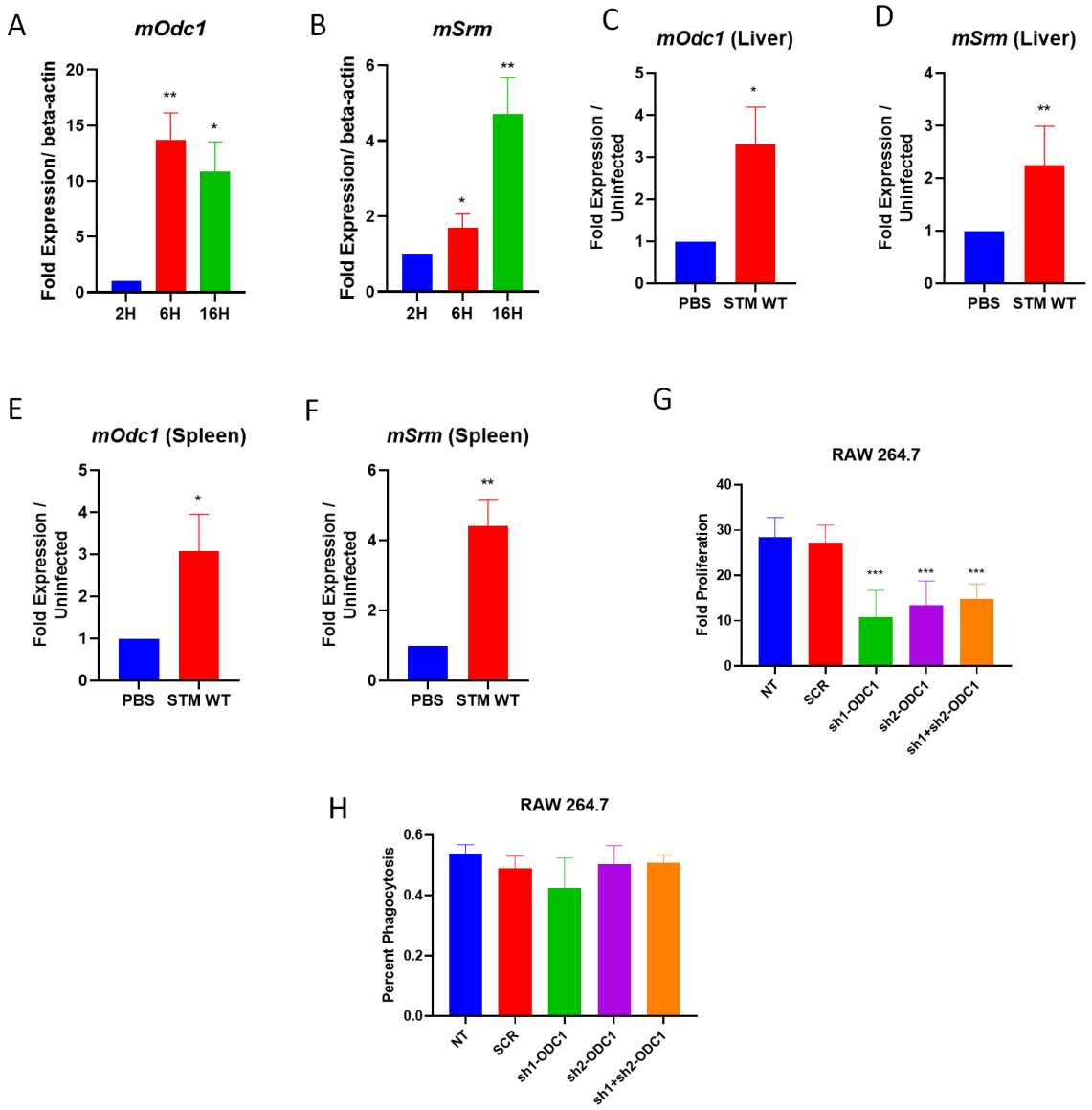


Figure 5: *Salmonella* rewires host polyamine metabolism to potentiate its survival within host macrophages

571 A.The mRNA expression of *mOdc1* (mouse ornithine decarboxylase) in RAW264.7 cells upon
572 infection with STM WT, B. The mRNA expression of *mSrm* (mouse spermidine synthase) in
573 RAW264.7 cells upon infection with STM WT, C. The mRNA expression of *mOdc1* (mouse
574 ornithine decarboxylase) in spleen of C57BL/6 mice 5 days post infection with STM WT by oral
575 gavage, D. The mRNA expression of *mOdc1* (mouse ornithine decarboxylase) in liver of C57BL/6
576 mice 5 days post infection with STM WT by oral gavage, E. The mRNA expression of *mSrm*
577 (mouse spermidine synthase) in spleen of C57BL/6 mice 5 days post infection with STM WT by
578 oral gavage, F. The mRNA expression of *mSrm* (mouse spermidine synthase) in liver of C57BL/6
579 mice 5 days post infection with STM WT by oral gavage, G. The fold proliferation of STM WT in
580 RAW264.7 cells upon transient knockdown of *mOdc1,* H. The percentage phagocytosis of STM
581 WT in RAW264.7 cells upon transient knockdown of *mOdc1.* Here SCR is Scrambled (no target
582 for knock-down), two different targeted shRNA were used for knock-down purposes, Sh1is
583 shRNA-1 for knock-down, Sh2 is shRNA-2 for knock-down, and Sh1+Sh2 indicates where both
584 the shRNAs were used to obtain the knock-down Student's t-test was used to analyze the data; p
585 values ****<0.0001, ***<0.001, **<0.01, *<0.05.

586 **The chemopreventive drug DFMO, reduces *Salmonella* Typhimurium burden in the host by**
587 **enhancing nitric oxide production**

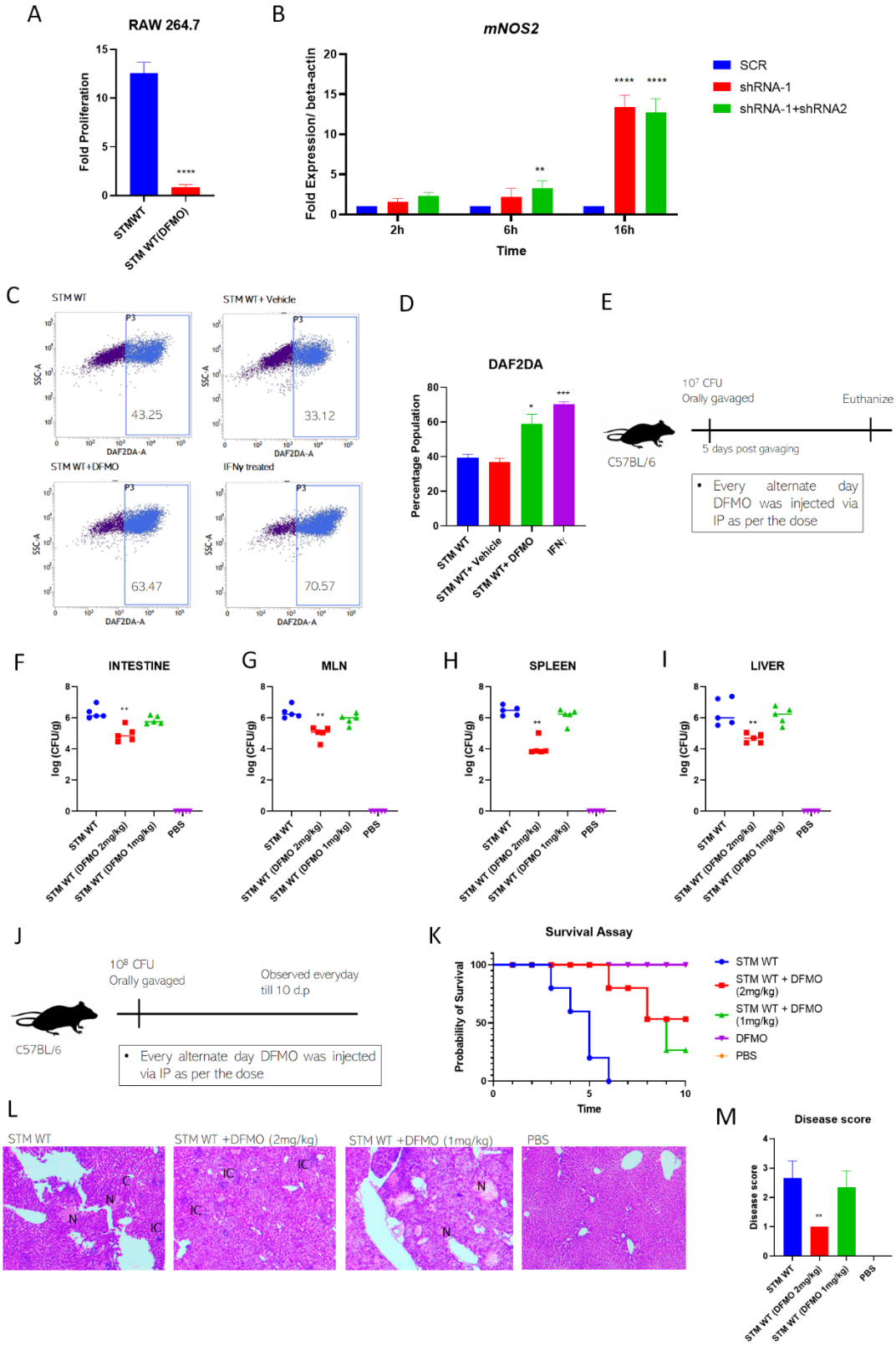
588 Polyamines are essential molecules in eukarotes, with multiple roles in differentiation,
589 proliferation and development. Many studies have shown that polyamine levels are upregulated in
590 cancer cells and elevated levels of polyamines are associated with breast cancer, neuroblastoma,
591 hepatocellular carcinoma, prostate cancer, lung cancer, colorectal cancer and lukemia [27-31].
592 D,L-α-difluoromethylornithine (DFMO) , an inhibitor of ODC was developed as a potent drug to
593 treat cancer in the year 1970 [32]. DFMO irreversibly binds to the active site of ODC and acts as

594 a suicide inhibitor, thereby reducing polyamine levels and having a cytostatic effect. As a single
595 therapeutic agent it was found to be effective only in neuroblastoma , and cinical trial for other
596 cancer type was unsatisfactory [33, 34]. However, DFMO has been successfully developed as a
597 chemopreventive drug, with FDA approval for treatment of Human African Trypanosomiasis
598 (HAT) [35, 36]. To test whether DFMO can be used as a therapeutic drug against *Salmonella*
599 infection, we treated RAW264.7cells with DFMO during the infection with *Salmonella*
600 Typhimurium, and observed a significant attenuation in the fold proliferation of STM WT (**Fig 6**
601 **A)**. Studies have shown that DFMO binds to ODC to prevent further production of putrescine from
602 ornithine and also acts on Arginase1 and reduces the available pool of ornithine for polyamine
603 biosynthesis [37, 38]. This, ensures the flux of arginine to be fed into the nitric oxide synthase
604 (NOS2) pathway and leads to elevated levels of nitric oxide in the cell, which in turn negatively
605 acts on ODC to further block polyamine synthesis [39].We assessed the mRNA expression of
606 *mNos2* upon knockdown of *mOdc1* in RAW264.7 cells followed by infection with STM. We
607 observed an upregulation of *mOdc1* mRNA levels at 6 hours and 16 hours post infection in
608 RAW264.7 cells (**Fig 6 B)**. Further we determined the levels of nitric oxide using a cell permeable
609 dye DAF-2DA, and noted that upon treatment with DFMO there was higher production of nitric
610 oxide (**Fig 6 C and D)**. Taken together our results demonstrate the role of DFMO in diminishing
611 the survival and proliferation of *Salmonella* Typhimurium by blocking Odc1 and enhanced
612 production of nitric oxide in murine macrophages.

613 To further address whether DFMO also act as potent anti-*Salmonella* drug in mice model of
614 infection, we infected C57BL/6 mice with 10^7 CFU per mice by oral gavage. Every alternate day
615 the mice were injected with DFMO in two different doses for two different cohorts (2mg/kg of
616 body weight of mice and 1mg/kg of body weight of mice). Five days post oral gavage we observed

617 that DFMO treatment of 2mg/kg body weight of mice significantly reduced the colonisation of
618 STM WT in intestine, MLN, spleen and liver than in the untreated mice (**Fig 6 E-I)**. Further, using
619 immunofluorescence we observed DFMO significantly reduced the colonisation of STM and and
620 the levels of spermidine in mouse ileum (**Fig S6 A-D).** Moreover, treatment of mice with DFMO
621 at a dose of 2mg/kg body weight of mice, increased the survival of mice upon infection with STM
622 WT (**Fig 6 J and K)**. Also, the weight reduction in mice treated with with DFMO at a dose of
623 2mg/kg body weight, was less upon infection with STM WT (**Fig S6 E)**. Next, we evaluated the
624 tissue damage upon infection of STM in mice liver. The results show that DFMO treatment of
625 mice at a dose of 2mg/kg of body weight significantly lowered the disease score, suggesting lesser
626 tissue damage compared to untreated (**Fig 6 L and M)**. Thus, DFMO serves as a potential drug to
627 treat *Salmonella* infection in mice by reducing the bacterial burden and tissue damage in mice and
628 enhancing the survival of mice upon infection with STM.

Figure 6: The chemopreventive drug DFMO, reduces *Salmonella* Typhimurium burden in the host by enhancing nitric oxide production



630 **Figure 6: The chemopreventive drug DFMO, reduces *Salmonella* Typhimurium burden in**
631 **the host by enhancing nitric oxide production**

632 A. The intracellular fold proliferation of STM WT in RAW264.7 cells upon treatment with
633 Difluoromethyl ornithine (DFMO) (5μM), quantified by Gentamicin protection assay, B. The
634 mRNA expression of *mNos2*(mouse Nitric oxide synthase) in RAW264.7 cells, upon transient
635 knockdown of *Odc1* followed by infection with STM WT, the expression is normalized to beta-
636 actin as an internal control, C. The representative scatter plot for DAF2DA dye positive population
637 of RAW264.7 cells, upon transient knockdown of *Odc1* followed by infection with STM WT, the
638 data is determined by flow-cytometry post staining the infected macrophages with the dye, D. The
639 quantification of (C), E. The experimental procedure to study the organ burden of STM WT in
640 C57BL/6 mice upon treatment with DFMO, F-I. The organ burden of STM WT in Intestine, MLN,
641 Spleen and Liver of C57BL/6 mice upon intraperitoneal treatment of DFMO (2mg/kg and 1mg/kg
642 of body weight) as mentioned in (E), J. The Experimental procedure to study the survival of
643 C57BL/6 mice upon infection with STM WT and treatment with DFMO, K.The survival of
644 C57BL/6 mice upon infection with STM WT and upon intraperitoneal treatment of DFMO
645 (2mg/kg and 1mg/kg of body weight) as mentioned in (J), L. Hematoxylin and eosin staining of
646 histopathological sections of liver upon DFMO treatment to C57BL/6 mice. Here, Small necrotic
647 areas (N), Congestion and damage to the endothelial lining of the central vein (C), Congestion of
648 the hepatic portal vein and poor hepatic architecture (HPV), Inflammatory immune cells (IC), M.
649 The disease score index for liver tissue damage upon STM WT infection in C57BL/6 mice with
650 DFMO treatment. The disease score is as :0 for normal pathology, 1 for mild/ minor pathology, 2
651 for moderate pathology, and 3 for severe pathological changes, Student's t-test was used to analyze

652 the data; p values ****<0.0001, ***<0.001, **<0.01, *<0.05. Two-way Anova was used to
653 analyze the grouped data; p values ****<0.0001, ***<0.001, **<0.01, *<0.05.

654

655 **Discussion**

656 A significant part of the life cycle of *Salmonella* during its pathogenesis involves its stay inside
657 the macrophages. The Gram-negative bacteria come across multiple host environmental stress
658 conditions inside the macrophages [40]. A key mechanism by which the host macrophages try to
659 limit the invading pathogen is by the rapid oxidative burst and ROS production. ROS includes
660 superoxide radicals, hydroxyl radicals, peroxy-nitrites, peroxy-chlorides and hydrogen peroxide.
661 ROS can pass through the bacterial cell wall and act on lipids, proteins and nucleic acids by
662 oxidising them and leading to cellular damage. *Salmonella* is often referred to as a smart pathogen.
663 Over the years, it has evolved to possess multiple strategies to combat the host-derived stresses
664 [41, 42]. To combat the oxidative burst generated NOX2 in macrophages, *Salmonella* possesses
665 multiple antioxidant enzymes such as the catalases KatE and KatG, the superoxide dismutases
666 SodA and SodB, the Alkyl hydroperoxide reductase, the glutaredoxins and thioredoxins and Hrg
667 transcriptional regulator [43]. Polyamines assist in *Salmonella* virulence and aid in stress
668 resistance. However, the mechanism behind the role of polyamines in *Salmonella* stress resistance
669 and virulence remains less appreciated.

670 Our study identifies spermidine as a stress-responsive regulatory molecule in *Salmonella*
671 Typhimurium. We show spermidine is critical for the survival and proliferation of STM in
672 macrophages and in the presence of oxidative stress *in vitro.* The spermidine transporter and
673 biosynthesis mutants show significant reduced capability to be phagocytosed by the macrophages.

674 Findings from our previous study (unpublished) showed that the intracellular level of spermidine
675 is significantly less in both the spermidine transport and biosynthesis mutants, which further
676 explains the attenuated proliferation of both the mutants in macrophages. The previous findings
677 also showed that in absence of the transport genes in *Salmonella* Typhimurium the synthesis genes
678 are down regulated and vice versa. The absence of spermidine transport and biosynthesis
679 diminishes the mRNA expression of multiple arms of oxidative stress response in *Salmonella.*
680 Multiple studies show the role of polyamines in the regulation of transcription of multiple genes
681 by interacting with DNA in eukaryotes. They bind to the DNA and change conformation as in C-
682 MYC transcription, and in other cases, enhance DNA-protein binding affinities like for Estrogen-
683 response elements [44, 45]. Thus, in *Salmonella* Typhimurium *rpoS, soxR* and *emrR* fall under the
684 "Polyamine modulon".

685 We further characterise a novel enzyme GspSA in *Salmonella* Typhimurium, which synthesises a
686 conjugated product of glutathione (GSH) and spermidine called GS-sp. GspSA is critical for
687 *Salmonella* Typhimurium to survive and proliferate in macrophages. Our study shows that the
688 absence of GspSA attenuates the survival of STM under oxidative stress conditions *in vitro,*
689 suggesting a vital role of Gsp in protecting STM from oxidative damage. Gsp in *E. coli* carries out
690 its function by modifying protein thiol groups under oxidative stress to protect the proteins from
691 getting oxidised and damaged. In *Salmonella* Typhimurium, we expect GspSA to generate GS-sp
692 under oxidative stress conditions and inside the macrophages, and likewise interact with cysteine-
693 thiol groups in proteins to shield them from oxidative damage. MK Chattopadhyay (2013) showed
694 that this enzyme (GspSA) is specifically present in two groups of organisms namely bacteria and
695 kineto-plastids respectively and completely absent in other organisms such as human, rats,
696 drosophila etc., among the bacteria group in all *enterobacteria* showed 27-100% homology and

697 >65% identity in around 50% of the species, with *E. coli* [24]. Thus, the absence of GspSA in
698 eukaryotes makes it a potent drug target for treatment of *Salmonella* infections. Thus, spermidine
699 exerts pleiotropic effects in *Salmonella*, by orchestrating the multiple arms of antioxidative
700 response. It strengthens the bacteria to cope with hostile host environments. Our studies in the *in*
701 *vivo* model of *Salmonella* Typhimurium infection further dissect the role of spermidine in assisting
702 in the pathogenesis by enhancing its virulence properties. Moreover, it is at the nexus of multiple
703 oxidative stress response arms in *Salmonella*, thereby assisting in mounting an antioxidative
704 response to promote its survival in macrophages.

705 Studies over the past years have given insights into the function of polyamines in the pathogenesis
706 of multiple virulent bacteria. Many human pathogenic bacteria have developed ways to exploit,
707 interfere and manipulate the polyamine metabolism of the host to enhance their fitness within the
708 niche. As in *Shigella* and *Vibrio spp.* polyamines produced by the bacteria are critical in
709 determining virulence [46, 47]. While bacteria such as *Legionella spp*., which does not synthesise
710 polyamines, depend on the host-acquired polyamines for their pathogenesis [48]. Another unique
711 mechanism is observed in *H. pylori* which activates polyamine oxidation, thereby dysregulation
712 the innate-immune response [49]. Also, in response to *H. pylori* infection, the host macrophages
713 increase the arginase activity and ornithine decarboxylase activity to produce polyamines [50]. A
714 recent study showed that SARS-Cov-2 hijacks the host polyamines for its reproduction and
715 infection [51].

716 Our findings also reveal that *Salmonella* Typhimurium enhances the polyamine production in the
717 host upon infection, which might be a strategy to hijack the host polyamines for its survival. This
718 further explains another reason for the reduced proliferation observed for the spermidine transport
719 mutant in macrophages. Also, the knockdown of host ornithine decarboxylase attenuates

720 *Salmonella* proliferation in macrophages. The upregulation of Odc1 activity manages to feed the
721 amino acid, L-arginine, into the polyamine biosynthesis and prevent nitric oxide production, which
722 otherwise would be detrimental for the pathogen. Polyamines have been a well-studied as a drug
723 target for treatment of multiple cancers. The oncogene MYC is upregulated in 70 percent of the
724 cancer types. Ornithine decarboxylase (Odc1) rate limiting enzyme of polyamine biosynthesis is
725 transcriptionally activated, directly by MYC, thereby increasing Odc1 levels in cancer [52]. RAS
726 is another essential factor in cell growth and cancer development, and it directly acts on Odc1 to
727 translationally activate Odc1 in cancer cells [53]. Polyamines biosynthesis is also associated with
728 multiple other oncogenes such as AKT1 and mTORC1 [54, 55]. We further show that upon using
729 Odc1 suicide inhibitor, DFMO, *Salmonella* proliferation could be diminished, and it reduces the
730 bacterial burden in mice. The FDA-approved chemopreventive drug, DFMO, for Human African
731 Trypanosomiasis treatment serves to be a potential drug to cure *Salmonella* infection. In the
732 devastating age of increasing antibiotic resistance, such a drug promises to effectively combat
733 deadly pathogens like *Salmonella.*

734 **Availability of data and materials**

735 All data generated and analyzed during this study, including the supplementary information files,
736 are incorporated in this article. The data is available from the corresponding author on request.

737 **Ethics statement**

738 All the animal experiments were approved by the Institutional Animal Ethics Committee, and the
739 Guidelines provided by National Animal Care were strictly followed during all experiments.
740 (Registration No: 435 48/1999/CPCSEA).

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Author contribution statement

AVN and DC conceived the study. AVN and DC designed experiments. AVN and AS performed experiments. RSR analysed for the disease score of tissue samples. AVN, analyzed the data, prepared the figures and wrote the manuscript draft. AVN, AS and DC reviewed and edited the manuscript. DC supervised the work. All the authors read and approved the manuscript.

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Conflict of Interest

The authors declare no conflict of interest.

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