Salmonella Typhi survives in human macrophages by neutralizing the RAB32/BLOC-3 host-defence pathway

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ABSTRACT

Salmonella enterica **serovar Typhi (*S*. Typhi) causes typhoid fever, a bacterial disease killing hundreds of thousands of people annually. Unlike other Salmonella, *S*. Typhi only infects humans. The inability of *S*. Typhi to infect other animal species depends at least partially on a host-defence pathway regulated by the RAB32 GTPase. It was therefore assumed that a RAB32-associated pathway was absent or inactive as a host-defence pathway in humans. Here we show that RAB32 and its guanine-nucleotide exchange factor Biogenesis of Lysosome-related Organelle Complex-3 (BLOC-3) control *S.* Typhi replication in human macrophages, as inactivating RAB32 or removing BLOC-3 by CRISPR-Cas9 targeting increases *S.* Typhi replication. We also report that, to survive in human macrophages, *S.* Typhi actively counteracts the RAB32/BLOC-3 pathway through its *Salmonella* pathogenicity island-1-encoded type III secretion system. These findings indicate that the RAB32/BLOC-3 pathway is a general host-defence pathway protecting mammalian species from bacterial infections and that *S.* Typhi has evolved specific strategies to neutralize this pathway.**

INTRODUCTION

S. Typhi is the causative agent of typhoid fever, a disease that infects ≈26 million people, killing many thousands every year (Waddington *et al*., 2014). The spread of multidrug-resistant strains makes *S*. Typhi a global public health risk (Wong *et al*., 2015; Klemm *et al*., 2018). Like other *Salmonella enterica* (*Salmonella*) serovars, *S*. Typhi has evolved to establish an intracellular replicative niche, a process that is mediated by bacterial effectors delivered by two type III secretion systems encoded in Salmonella Pathogenicity Islands (SPI) 1 and 2 respectively (Galán *et al*., 2014; Jennings *et al*., 2017; Hume *et al*., 2017). However, in contrast to the majority of *Salmonella* serovars, which infect a broad-range of hosts, *S*. Typhi naturally only infects humans (Dougan and Baker, 2014). The inability of S. Typhi to infect mice depends at least in part on a trafficking pathway regulated by the RAB32 GTPase and its guanine nucleotide exchange factor BLOC-3 (Spanò *et al*., 2016; Spanò and Galán, 2012). This host-defence pathway is effectively neutralized by broad-host *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium)

through the delivery of two type III secretion effectors, GtgE and SopD2, that directly target RAB32 acting as a protease and a GTPase activating protein, respectively (Spanò *et al*., 2016). In contrast, *S.* Typhi, which lacks these two effectors, cannot neutralise the murine RAB32 pathway. Consequently, *S.* Typhi succumbs in murine macrophages and cannot cause a systemic infection in mice (Spanò and Galán, 2012; Spanò *et al*., 2016; Solano-Collado *et al*., 2016). These findings and the fact that *S.* Typhi is able to survive in human macrophages and cause a systemic infection in humans suggest that the RAB32/BLOC-3 host-defence pathway is not effective in human macrophages. However, the RAB32 and BLOC-3 genes are present in humans. In addition, genome-wide association studies showed that single nucleotide polymorphisms in the RAB32 untranslated regions are associated with increased susceptibility to leprosy, a human bacterial infection caused by the intracellular bacterium *Mycobacterium leprae* (Zhang *et al*., 2011; Liu *et al*., 2015), suggesting that RAB32 could be part of a pathway critical to control some bacterial infection in humans. More precisely, two scenarios are possible: a) RAB32/BLOC-3 are not part of a host-defence pathway in humans or b) a RAB32/BLOC-3 pathway is active in humans as an anti-microbial pathway but *S.* Typhi has evolved molecular strategies to evade it. Therefore, to assess if the RAB32/BLOC-3 host-defence pathway is active as an antimicrobial pathway in humans, we investigated the requirement of RAB32 and BLOC-3 in controlling bacterial growth in human macrophages.

RESULTS

To test if RAB32 has a role in controlling bacterial infection in human macrophages we used an approach that removes RAB32 from infected cells, based on a *S.* Typhi strain that delivers the *Salmonella* Typhimurium type III secretion effector GtgE, a specific protease that cleaves the three RAB GTPases RAB32, RAB29 and RAB38 (Spanò and Galán, 2012; Spanò *et al*., 2011). We infected THP-1 macrophage-like cells with a *S.* Typhi wild-type isolate or an isogenic strain engineered to express GtgE (*S.* Typhi::*gtgE*) and performed an immune-blot analysis to confirm that the expression of GtgE results in the cleavage of RAB32 (Fig 1A). The results obtained indicate that GtgE is able to cleave endogenous human RAB32, in agreement with the previous observation that GtgE targets ectopically expressed human RAB32 (Spanò and Galán, 2012). As shown in Fig 1B, the strain expressing GtgE had a replication advantage in THP-1 cells,

suggesting that RAB32 or another RAB targeted by GtgE limits *S.* Typhi replication in human macrophages.

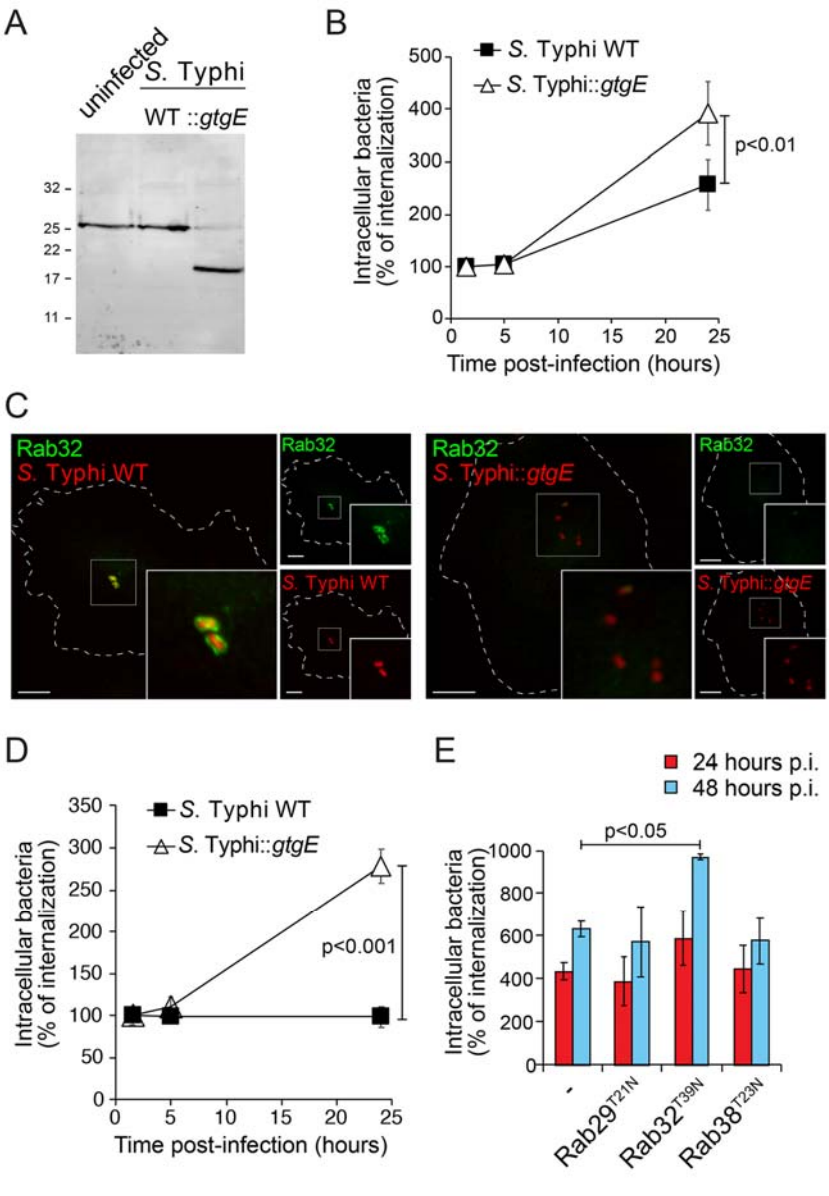

Fig 1. RAB32 inactivation results in *S.* Typhi over-replication in human macrophages. (A) and (B) PMA-differentiated THP-1 cells were left uninfected or infected with either wild-type *S.* Typhi (WT) or a *S.* Typhi strain expressing GtgE (::*gtgE*). Cells were lysed 2.5 hours post-infection (p.i.) and analyzed by western blot with a RAB32 specific antibody (A) or lysed at the indicated time points to measure intracellular colony-forming units (CFUs) (B). (C) Peripheral blood monocyte-derived macrophages were infected with either wild-type *S.* Typhi (WT) or a *S.* Typhi strain expressing GtgE (::*gtgE*), both carrying a chromosomal copy of the *mCherry* gene, fixed at 2.5 hours p.i. and analyzed by immunofluorescence with a RAB32 specific antibody. Scale bar=10 µm. (D) Peripheral blood monocyte-derived macrophages were infected with either wild-type *S.* Typhi (WT) or a *S.* Typhi strain expressing GtgE (::*gtgE*). Cells were lysed at the indicated time points to measure CFUs. (E) THP-1 cells were transduced with lentivirus expressing the indicated dominant negative RAB mutants, stimulated with PMA, infected with wild type *S.* Typhi and lysed at the indicated time points to measure CFUs. Results in (B), (D) and (E) are reported as percentage of CFUs measured at the first time point (1.5 hours p.i.). Values are means ± SEM of at least three independent experiments performed in triplicates. p-values were calculated using the Student's t-test and are indicated only when <0.05.

To confirm these findings we infected primary macrophages derived from human blood monocytes with wild-type *S*. Typhi or *S*. Typhi::*gtgE*. First, we examined the localization of RAB32 in these primary macrophages. We observed that RAB32 localises on the surface of the vacuoles containing wild-type *S*. Typhi, but it is mostly absent from the surface of the vacuoles containing *S*. Typhi::*gtgE* (Fig 1C). These results confirm that GtgE is able to cleave endogenous human RAB32 and eliminate it from the *S*. Typhi-containing vacuole. Next, we investigated if the removal of RAB32 from the bacterial vacuole resulting from GtgE expression has any effect on *S*. Typhi survival. We observed that GtgE expression confers *S*. Typhi a 3-fold replicative advantage at 24 hours post-infection (Fig 1D), suggesting that either RAB32 or another RAB GTPase targeted by GtgE controls *S*. Typhi intracellular survival in human macrophages. To determine which of the three RAB GTPases that are targeted by GtgE is responsible for *S*. Typhi growth control in human macrophages, we used a dominant negative approach to inhibit each of the three RAB GTPases. When THP-1 expressed a dominant negative mutant of RAB32, we observed a significantly increased replication of *S*. Typhi (Fig 1E). No change in replication was instead observed when THP-1 expressed dominant negative mutants of RAB29 and RAB38. Taken together these results indicate that RAB32 is critical to control *S*. Typhi infections in human macrophages.

As BLOC-3 is a guanine nucleotide exchange factor for RAB32 (Gerondopoulos *et al*., 2012) and mutations as well as knock-down of BLOC-3 subunits prevent *S*. Typhi killing in mouse macrophages (Spanò and Galán, 2012), it was important to investigate the role of BLOC-3 in controlling *S*. Typhi infections in human macrophages. To this purpose we used macrophages derived from human-inducible pluripotent stem cells (hiPSCs), a recently established model for the study *Salmonella enterica* infection (Alasoo *et al*., 2015; Hale *et al*., 2015). After seven-days treatment of monocyte precursors with macrophage colony-stimulating factor, we obtained around 90% of Cluster of Differentiation 68 (CD68) positive cells (Fig 2A and B). First, we confirmed that GtgE expression confers an advantage to *S*. Typhi in hiPSC-derived macrophages (Fig 2C), similar to that observed in THP-1 and primary macrophages. Then, we used CRISPR/Cas9 technology to generate hiPSCs deficient for HPS4, one of the two subunits of BLOC-3.

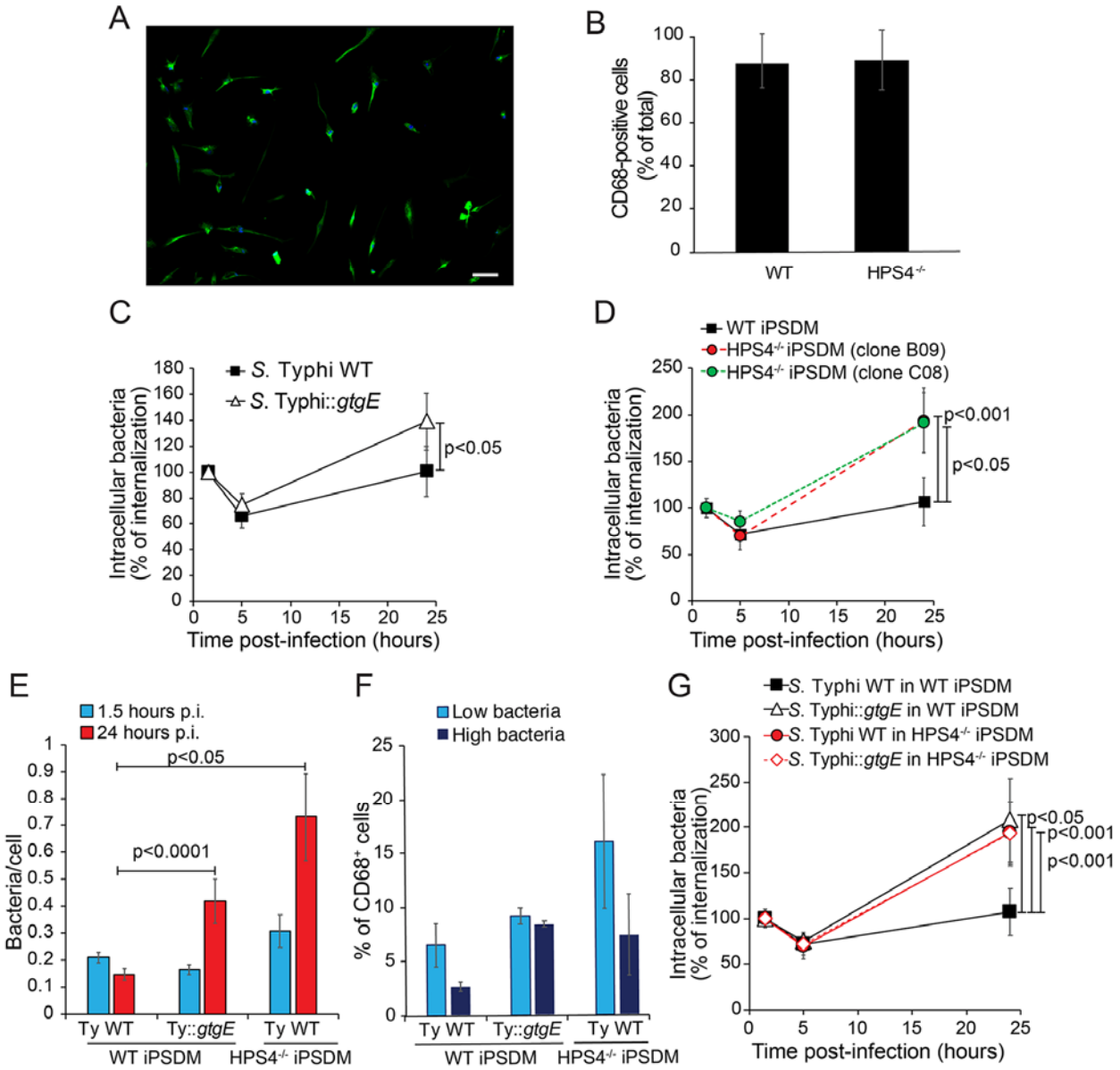

Fig 2. BLOC-3 knock-out results in *S.* Typhi over-replication in human induced pluripotent stem cells-derived macrophages

(A) Human macrophages derived from hiPSCs were analyzed by immunofluorescence with a CD68-specific antibody (green) and DAPI (blue). Scale bar=50 µm. (B) Human macrophages derived from WT or HPS4$^{-/-}$ hiPSCs, were treated as in (A) and manually scored for their CD68 expression. At least 270 cells were counted in two independent experiments. (C) Human macrophages derived from hiPSCs were infected with either wild-type *S.* Typhi (WT) or a *S.* Typhi strain expressing GtgE (::*gtgE*) and lysed at the indicated time points for counting of intracellular CFUs. (D) Human macrophages derived from WT or HPS4$^{-/-}$ hiPSCs and infected with wild-type *S.* Typhi. Cells were lysed at the indicated time points for counting of intracellular CFUs. (E) Human macrophages derived from WT or HPS4$^{-/-}$ hiPSCs were plated on glass coverslips, then infected with wild-type *S.* Typhi or a *S.* Typhi strain expressing GtgE (::*gtgE*)

and fixed at 1.5 and 24 hours p.i. Differentiated macrophages were identified by CD68 staining, bacteria in CD68-positive cells were counted and the average value plotted. At least 120 cells were counted for each timepoint in two independent experiments. (F) Human macrophages derived from WT or HPS4$^{-/-}$ hiPSCs, were infected with *S*. Typhi *glmS::Cm::mCherry* or *S*. Typhi::*gtgE glmS::Cm::mCherry*, fixed at 24 hours p.i. and analyzed by flow cytometry. CD68+ cells were sub-gated in two population containing respectively low or high mCherry signal (i.e. bacterial content). (G) Human macrophages derived from WT or HPS4$^{-/-}$ hiPSCs, were infected with either wild-type *S*. Typhi (WT) or a *S*. Typhi strain expressing GtgE (::*gtgE*) and lysed at the indicated time points for counting of intracellular CFUs. Results in (C), (D) and (G) are reported as the percentage of CFUs measured at the first time point (1.5 hours p.i.). Values are means ± SEM of at least three independent experiments performed in triplicates. p-values were calculated using the Student's t-test and are indicated only when <0.05.

Macrophages derived from wild-type and HPS4-deficient hiPSCs were infected with *S*. Typhi and its intracellular replication assessed. As shown in Fig 2D, macrophages derived from two independent clones of HPS4-deficient hiPSCs have a significant increased number of *S*. Typhi intracellular CFUs at 24 hours post-infection, indicating that BLOC-3 is important to limit *S*. Typhi growth in human macrophages. To obtain further insights into the effects of GtgE expression in *S*. Typhi or the knock-out of BLOC-3 in these macrophages, we analyzed *S*. Typhi replication in single cells by immunofluorescence (Fig 2E) or flow cytometry analysis (Fig 2F). These experiments confirmed that removal of either RAB32, obtained through GtgE delivery, or BLOC-3 results in increased percentage of host cells showing high numbers of bacteria, indicating that both RAB32 and its guanine nucleotide exchange factor are required for control of bacterial replication in human macrophages. We also observed that although *S*. Typhi has a replicative advantage in the absence of BLOC-3, the expression of GtgE does not confer any significant additional advantage (Fig 2G), in agreement with the model that RAB32 and BLOC-3 are components of the same pathway. The results of these experiments indicate that the RAB32/BLOC-3 pathway is active as a host-defence pathway in human macrophages and can limit *S*. Typhi replication.

The previous results indicated that *S*. Typhi survives better in macrophages deficient for the RAB32/BLOC-3-dependent pathway. However, *S*. Typhi is not cleared by wild type human macrophages during infection, and this is in contrast to other pathogens that are effectively killed

by human macrophages (Fig 3A). Therefore, we hypothesized that *S.* Typhi actively counteracts the RAB32/BLOC-3 pathway. Since the broad-host *Salmonella* Typhimurium neutralizes this pathway through the action of effectors delivered by type III secretion systems, we tested if *S.* Typhi survival in wild-type human macrophages is dependent on *S.* Typhi type III secretion systems. First, we observed that the ability of *S.* Typhi to survive in macrophages derived from hiPSCs depends on its SPI-1 type III secretion system (Fig 3B), but not on its SPI-2 type III secretion system (supplementary Fig 1). Indeed, a SPI-1 type III secretion system mutant of *S.* Typhi (*S.* Typhi Δ*invA*) was unable to survive in macrophages derived from hiPSCs (Fig 3B), in agreement with published results (Forest *et al*., 2010). Interestingly, a *S.* Typhi Δ*invA* survived much better in HPS4-deficient macrophages (Fig 3B), suggesting that *S.* Typhi can counteract this pathway through the action of this secretion system. To confirm this hypothesis, we infected HPS4-deficient macrophages with pathogenic *E. coli* O157. This pathogen is not able to survive in either wild type or HPS4-deficient macrophages (Fig 3C). These results indicate that *S.* Typhi is able to target the human RAB32/BLOC-3 pathway likely through effectors delivered by the SPI-1 type III secretion system.

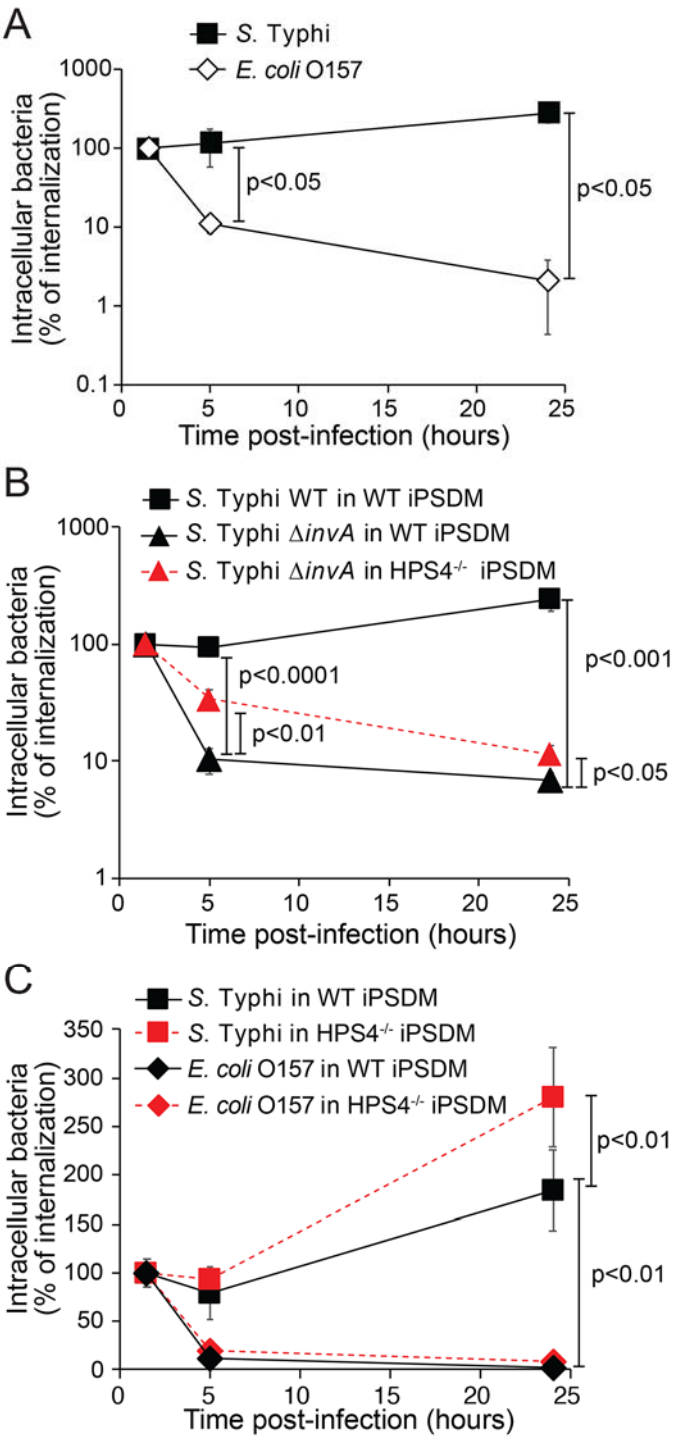

Fig 3. *S*. Typhi counteracts the RAB32/BLOC-3 pathway through the SPI-1 type III secretion system.

(A) Human macrophages derived from hiPSCs were infected with either wild-type *S*. Typhi (WT) or *E*. *coli* O157 and lysed at the indicated time points to measure intracellular CFUs. (B) Human macrophages derived from WT or HPS4$^{-/-}$ hiPSCs were infected with either wild-type *S*. Typhi (WT) or *S*. Typhi Δ*invA* (Δ*invA*). Cells were lysed at the indicated time points to measure intracellular CFUs. (C) Human macrophages derived from WT or HPS4$^{-/-}$ hiPSCs were infected with either wild-type *S*. Typhi (WT) or *E*. *coli* O157. Cells were lysed at the indicated time points to measure intracellular CFUs. All results are reported as percentage of CFUs measured at the first time point (1.5 hours p.i.). Values are means ± SEM of at least three independent experiments performed in triplicates. p-values were calculated using the Student's t-test and are indicated only when <0.05.

DISCUSSION

In this manuscript we outlined an unexpected role for the RAB32/BLOC-3 pathway in controlling *S.* Typhi infections in humans and showed that the SPI-1 type III secretion system underlies the ability of *S.* Typhi to evade the antimicrobial potential of this pathway. The molecular mechanisms beneath this adaptation of *S.* Typhi in humans are still mostly unknown, but it is believed that its ability to survive in macrophages, once obtained access to the lamina propria, plays a critical role in the establishment of typhoid fever (Dougan and Baker, 2014). Our findings showed that both RAB32 and its guanine nucleotide exchange factor BLOC-3 are required to limit *S.* Typhi replication in human macrophages. These results, firstly, establish the importance of the RAB32/BLOC-3 trafficking pathway in controlling infection in humans. The pathway is shown here to be active as an important antimicrobial pathway in humans, and not only, as previously shown, in mice (Spanò *et al*., 2016; Spanò and Galán, 2012). In macrophages that lack either RAB32 or BLOC-3, *S.* Typhi replicate more efficiently, indicating that the inactivation of this pathway renders the intracellular vacuole more permissive for bacterial growth. We also used single cell analysis to show that the bacteria undergo replication within macrophages defective for the RAB32/BLOC-3 pathway, indicating that this pathway is able to limit the bacterium's ability to grow in its intracellular niche, potentially by generating a hostile environment for bacterial replication. An adverse environment could possibly results from the RAB32-mediated delivery of an antimicrobial factor to the *Salmonella*-containing vacuole. Secondly, our results indicate that *S.* Typhi utilizes its SPI-1 type III secretion system to evade this antimicrobial pathway in human macrophages, suggesting that it delivers bacterial effectors that target RAB32, BLOC-3 or other components of this pathway to inactivate it. As *S.* Typhi cannot infect mice, we hypothesize that these effectors are unable to target the mouse RAB32/BLOC-3 pathway. The inability of *S.* Typhi to inhibit the mouse RAB32/BLOC-3 pathway suggests that there are significant differences between the murine and human RAB32-dependent pathways. This idea is supported by the observation that the RAB32 amino acid sequence is significantly divergent in human and mice (Spanò and Galán, 2012). Further investigations will be required to identify the exact differences in mouse and human specific components of the RAB32/BLOC-3 trafficking pathway, and the component of the human

RAB32/BOC-3 pathway targeted by *S*. Typhi. It will also be important to identify the SPI-1 type III secretion effector(s) that sanctions *S*. Typhi to evade this antimicrobial pathway.

S. Typhimurium has evolved to deliver the protease GtgE and the RAB GAP SopD2, which act in a partially redundant manner to inactivate the murine RAB32/BLOC-3 trafficking pathway. In contrast, *S*. Typhi appears to have evolved a unique strategy to target the pathway, which does not require GtgE or SopD2. Indeed, the gene encoding for GtgE is absent from *S*. Typhi, and may not have been acquired during evolution, while the gene encoding for SopD2 became a pseudogene. One possible reason for evolving a different strategy could rely on the fact that GtgE also targets Rab29, which is required for the efficient delivery of typhoid toxin from *S*. Typhi-infected cells (Spanò *et al*., 2011). Therefore, we speculate that GtgE, although able to neutralize the human RAB32, would not confer overall an advantage to *S*. Typhi because it would interfere with other pathogenic features of this bacterium. However, similar to *S*. Typhimurium, *S*. Typhi may have evolved a number of redundant effectors with different activities to target the RAB32/BLOC-3 pathway. In addition to indicate a critical role for RAB32/BLOC-3 pathway in controlling infection in humans, our findings represent a fundamental step toward the identification of these *S*. Typhi virulence factors, which will be essential to understand the molecular mechanisms underlying typhoid fever and can help to identify novel therapeutic targets to treat this bacterial infection.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

The wild-type *Salmonella enterica* serovar Typhi strain ISP2825have been previously described (Galán and Curtiss, 1991). All the *S*. Typhi deletion strains were constructed by standard recombinant DNA and allelic exchange procedures as previously described (Kaniga *et al*., 1994) and are listed in Table S1. All the plasmids used in this study were constructed using standard recombinant DNA techniques and are listed in Table S2. *S*. Typhi *glmS::Cm::mCherry* and *S*. Typhi::*gtgE glmS::Cm::mCherry* that constitutively express *mCherry* from a single chromosomal copy at the *att*Tn7 site were generated by via P22 transduction using phages obtained from the *S*. Typhimurium SL1344 *glmS::Cm::mCherry* (a gift from Leigh Knodler; Knodler *et al*., 2014).

Table S1. List of *S*. Typhi strains used in this study

Strain	Genotype	References
ISP2825	wild type	Galán and Curtiss, 1991
SB2522	*::gtgE*	Spanò and Galán, 2012
SB2174	Δ*invA*	Spanò *et al*., 2011
SB1958	Δ*spiA*	Spanò *et al*., 2011
SBB001	*glmS::Cm::mCherry*	this study
SBB002	*::gtgE glmS::Cm::mCherry*	this study

Table S2. List of plasmids used in this study

Plasmid	Description	References
pVSVG	p*VSV-G*	Spanò *et al*., 2011
pGag/Pol	pMLV-*Gag-Pol*	Spanò *et al*., 2011
pSB4006	pLZRS-CFP-*Rab29*^{T21N}	This study
pSB4651	pLZRS-CFP-*Rab32*^{T39N}	This study
pSB4653	pLZRS-CFP-*Rab38*^{T23N}	This study

Cell culture

THP-1 cells were maintained in RPMI 1640 medium (Invitrogen), 10% Fetal Bovine Serum (FBS, Invitrogen), 2 mM Glutamine (Invitrogen), 1 mM Sodium Pyruvate (Invitrogen) and 10 mM Hepes (Invitrogen). The cells were maintained at a concentration between 0.1 and 1 million cell/ml. THP-1 differentiation was induced adding 100 nM Phorbol 12-Myristate 13-Acetate (PMA) for 24 hours before infection. HEK293T cells were maintained in DMEM high glucose 2 mM glutamax (Invitrogen), 10 % FBS.

Blood was collected from human healthy volunteers, according to procedures approved by the Life Science and Medicine College Ethics Review Board of the University of Aberdeen (CERB/2016/11/1299). Peripheral blood monocyte-derived macrophages were prepared as described in (Arnold *et al*., 2014) with some modifications. Briefly 13 ml of blood were collected from each donor, diluted to 35 ml with Hank's balance salt solution (HBSS, Invitrogen), then

loaded onto 15 ml of Lymphoprep™ (Stem Cell Technology) for the separation of the peripheral blood mononuclear cells. Isolated peripheral blood mononuclear cells were resuspended in DMEM containing 10% autologous human serum (freshly prepared from the same donor) and seeded on coverslips or tissue-treated plastic. Cells were plated at $5x10^5$/well in 24-well plates. After 24 hours the non-adherent cells were removed, fresh medium added, and the cells left for 7-9 days to differentiate.

Undifferentiated human induced pluripotent stem cells line (KOLF2-C1) was maintained on a monolayer of mitotically inactivated mouse embryonic feeder (MEF) cells in advanced Dulbecco's modified Eagles/F12 medium (DMEM/F12), supplemented with 20% knockout replacement serum (KSR, Invitrogen), 2 mM L-Glutamine, 0.055 mM β-mercaptoethanol (Sigma-Aldrich) and 8 ng/ml recombinant human FGF2 (RnD Systems), as described previously (Hale *et al*., 2015). These cells were differentiated into macrophages as described in a previously published method (Hale *et al.*, 2015).

CRISPR/Cas9 targeting of HPS4

Isogenic intermediate targeting vectors for HPS4 were generated using isogenic and haplotype specific DNA by PCR amplification of KOLF2-C1 gDNA. Firstly, a PCR fragment including homology arms and the critical exon of HPS4) was amplified from KOLF2-C1 gDNA using the following primers; f5F gccagtgaattcgatatacctgccttcttgaactgttttg and f3R tacgccaagcttgatttaaattgtgctctgtgtgttcctc. The first 15nt of each primer (underlined) served to mediate fusion with the intermediate targeting vector backbone, puc19_RV using the In-Fusion HD Cloning Plus kit (TAKARA). The HSP4 amplicon was purified and 75ng incubated with 50ng of EcoRV digested puc19_RV vector for 15 minutes at 50°C and transformed into Stellar competent cells (TAKARA). Positive clones were verified by Sanger sequencing. To replace the critical exon with the gateway R1-*pheS/zeo*-R2 cassette, sequence verified clones were electroporated with the pBABgbaA plasmid (Tate and Skarnes, 2011). This was then maintained in tetracycline (5 μg/ml) at 30°C. Early log phase cultures were induced to express the red operon following addition of 0.1% arabinose and incubation for 40 min at 37°C. From these cultures electro competent cells were prepared as previously described (Tate and Skarnes, 2011). The R1-*pheS/zeo*-R2 cassette was amplified using the following primers; U5

ttagtggtgtcagcagttctgagtatagagaggtagaatagtcccaagccaaggcgcataacgataccac and D3 agttgtgcagcaagggaatggggctggaagaaaggggtctggagttactccgcctactgcgactataga. Underlined sequences in each of these primers denotes 50nt of homology towards a region 5' (U5) or 3'(D3) of the critical exon. This amplicon was purified and 300ng electroporated into the recombination ready verified clones from the first step before selection in carbenicillin (50µg/ml) and zeocin (10 µg/ml). Positive clones were verified by Sanger sequencing. To generate the donor plasmid for precise gene targeting via homology directed repair, the intermediate targeting vectors were turned into donor plasmids via a Gateway exchange reaction. LR Clonase II Plus enzyme mix (Invitrogen) was used to perform a two-way reaction exchanging only the R1-*pheSzeo*-R2 cassette with the pL1-EF1αPuro-L2 cassette as previously described (Tate and Skarnes, 2011). The latter had been generated by cloning synthetic DNA fragments of the EF1α promoter and puromycin resistance cassette into a pL1/L2 vector (Tate and Skarnes, 2011).

As part of the primer design process, two separate guide RNAs (gRNAs) targeting within the same critical exon were selected. The gRNAs were identified using the WGE CRISPR tool (Hodgkins *et al.*, 2015) and were selected based on their off-target scores to minimise potential off target damage. gRNAs were suitably positioned to ensure DNA cleavage within the exonic region, excluding any sequence within the homology arms of the targeting vector. Plasmids carrying single guide (sg) RNA sequences were generated by cloning forward and reverse strand oligos into the BsaI site of either U6_BsaI_gRNA or p1260_T7_BsaI_gRNA vectors (kindly provided by Sebastian Gerety, unpublished). The CRISPR sequences as follows (PAM sequence); left CRISPR (CCA) GCGAGAATGTGAGGGCGAGCG and right CRISPR (CCT) TCAGCAACAACAGGGGCTCC (WGE IDs: 1181940311 and 1181940319 respectively).

To deliver plasmids expressing gRNA, donor templates and Cas9, 2 x 10^6 KOLF2-C1 cells were nucleofected (AMAXA nucleofector 2B) with 2 µg of donor plasmid, 4 µg hCas9 D10A (Addgene plasmid #41816) (Mali *et al.*, 2013) and 3 µg of gRNA plasmid DNA. Following nucleofection, cells were selected for up to 11 days with 0.25 µg ml^{-1} puromycin. Individual colonies were picked into 96-well plates, expanded and genotyped. Positive insertion of the cassette into the correct locus was confirmed by PCR using cassette-specific primers ER (gcgatctctgggttctacgttagtg) and PNFLR (catgtctggatccgggggtaccgcgtcgag). To determine the

presence of deleterious insertions or deletions (indels) around the CRISPR target site of the opposite allele, a PCR amplicon was generated using the primers PR (actagttctaacagctggtggatac) and PF (ttttgcagactgacaactattccag), purified and Sanger sequenced using SR1 (cttctggacaggcctccttg) and SF1 (atatttgccgaaccagccca). To minimize the potential for off-target effects, two independently derived clones, B09 and C08, with specific deletions of 47 and 29 base pairs, respectively, were isolated and used in this study.

Intracellular growth experiments

Overnight cultures of the different *S.* Typhi strains were diluted 1/20 in LB broth containing 0.3 M NaCl and grown for 2 hours and 45 minutes at 37ºC. Cells were infected with the different strains of *S*. Typhi in HBSS at the desired multiplicity of infections. One-hour p.i. cells were washed three times with HBSS and incubated in growth medium supplemented with 100 μg/ml gentamicin for 30 min to kill extracellular bacteria. Cells were then washed with HBSS, and fresh DMEM containing 5 μg/ml gentamicin was added to avoid cycles of reinfection. At the indicated time points the cells were washed twice in phosphate buffer saline (PBS, Sigma-Aldrich) and the intracellular bacteria recovered lysing the cells in 0.1% sodium deoxycholate (Sigma-Aldrich) in PBS and counted by plating serial dilutions on LB-agar plates.

Western blot

PMA-differentiated THP-1 cells were infected as described above and lysed in SDS-PAGE loading buffer 2.5 hours post-infection. Western blot analysis was performed using Odyssey infrared imaging system (LI-COR Biosciences). The following antibodies were used for Western blot analysis: rabbit polyclonal anti-Rab32 (GeneTex, 1:1,000 dilution); Donkey anti-rabbit IR Dye 800 (Li-COR, 1:10,000 dilution).

Expression of RAB mutants in THP-1

THP-1 were transduced with lentivirus expressing CFP-Rab29^{T21N}, CFP-Rab32^{T39N}, CFP-Rab38^{T23N}. Twenty hours post-transduction, they were treated with 100 nM PMA for 24 hours to induce differentiation and then infected with *S*. Typhi as described above.

Immunofluorescence

Human monocyte-derived macrophages and wild-type or HPS4 deficient iPSDM were plated on glass coverslips (#1 Thermo) infected with different *S.* Typhi strains and fixed at the indicated times p.i. with 4% paraformaldehyde (PFA) for 10 minutes. Cells were then permeabilized for 20 minutes by incubating in 0.02% Saponin (Sigma-Aldrich), 0.2% bovine serum albumin (BSA, Sigma-Aldrich), 50 mM NH_4Cl (Sigma-Aldrich) in PBS and incubated for 1 hour with monoclonal mouse anti CD68 (KP1 Invitrogen, 1:200 dilution). Alternatively, cells were permeabilized for 20 minutes by incubating in 0.2% Triton X-100 (Sigma-Aldrich), 0.2% bovine serum albumin (BSA, Sigma-Aldrich), 50 mM NH_4Cl (Sigma-Aldrich) in PBS and incubated for 1 hour with a rabbit polyclonal anti-Rab32 (GeneTex, 1:200 dilution). Cells were then stained using the appropriate Alexa Fluor® 488 or Alexa Fluor® 555-conjugated secondary antibodies (Invitrogen). Images were acquired either using in a Nikon (Eclypse Ti2) equipped with a CFI Plan Apochromat 100x objective and a Prime 95B 25 mm CMOS camera (Photometrics) or a PerkinElmer Spinning disk confocal equipped with an ORCA Flash 4.0 CMOS camera (Hamamatsu). Images were analyzed using the respective software (Nikon Elements or Volocity).

Flow cytometry

hiPSC-derived macrophages wild type or HPS4 deficient were plated on non-tissue culture treated 6-well plates (Thermo Fisher Scientific) and infected with *S.* Typhi *glmS::Cm::mCherry* or *S.* Typhi::*gtgE glmS::Cm::mCherry*. At the indicated time p.i. the cells were detached using 500 µl of Versene (Invitrogen) and mixed with an equal volume of 4% PFA for 5 minutes. Fixed cells were then centrifuged and resuspended in 4% PFA for 5 minutes. The cells were then transferred in flow cytometry tubes, permeabilized 15 minutes in PMZ-S, then incubated for 1 hour with anti-CD68 (1:200) and then with anti-mouse Alexa Fluor® 488. The samples were analyzed by flow cytometry (Fortessa, BD Biosciences) and FlowJo software.

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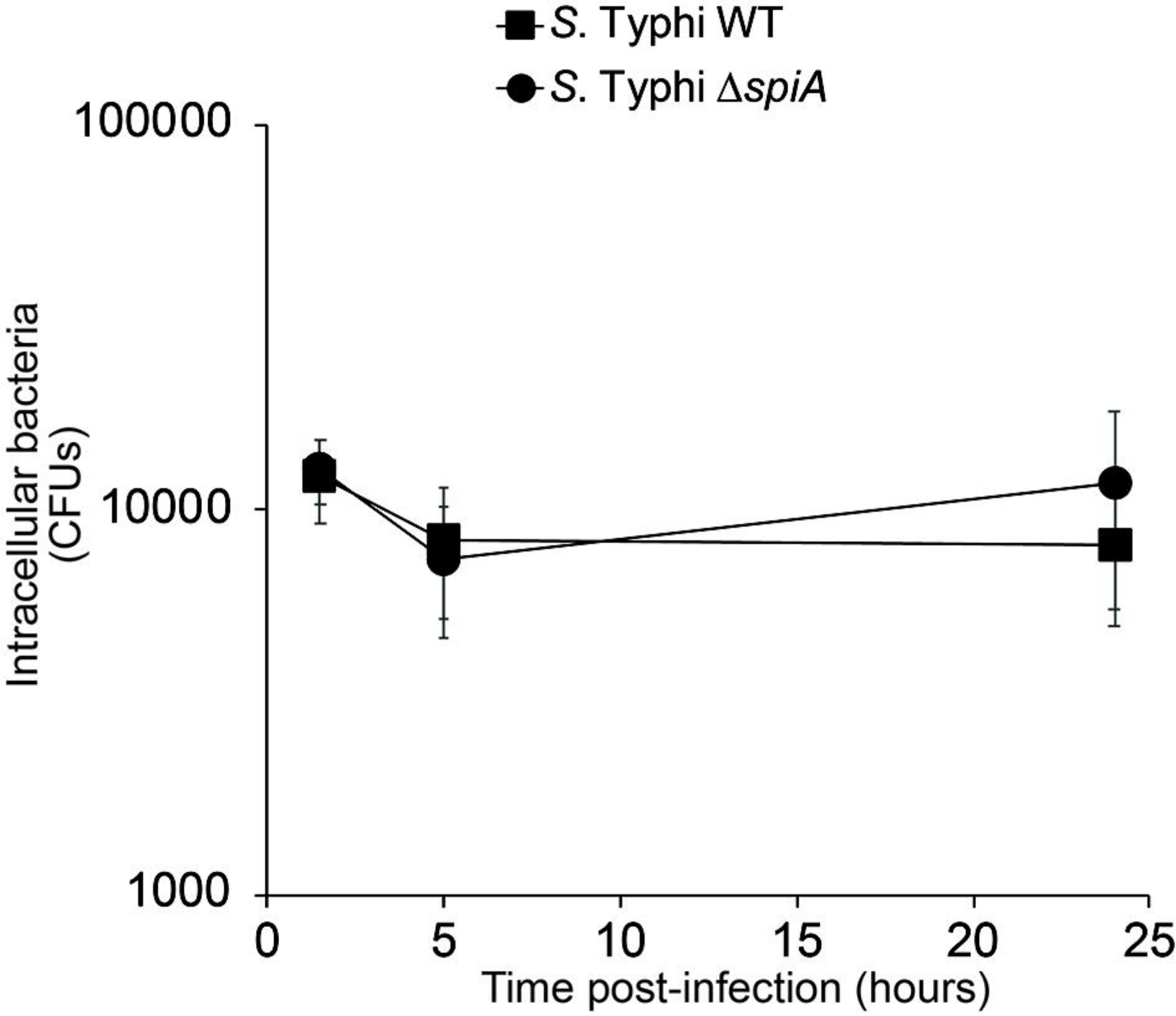
SUPPLEMENTARY FIGURE LEGEND

Fig S1. *S.* Typhi survival in macrophages does not depend on its SPI-2 type III secretion system.

Human macrophages derived from induced pluripotent stem cells were infected with either wild-type *S.* Typhi or *S.* Typhi Δ*spiA*. Cells were lysed at the indicated time points to measure intracellular CFUs.


S. Typhi WT
S. Typhi ΔspiA
100000
10000
1000
Intracellular bacteria (CFUs)
0
5
10
15
20
25
Time post-infection (hours)