Salmonella actively modulates TFEB in murine macrophages in a growth-phase and time-dependent manner

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Summary

The transcription factor TFEB promotes lysosomal and autophagic capacity in response to stresses like amino acid depletion. Additionally, TFEB drives expression of immune-responsive and immuno-protective genes in response to LPS, phagocytosis, and bacteria such as *Escherichia coli* (*E. coli*) and *Staphylococcus aureus*. Consistent with a role for TFEB in promoting immunity and bactericidal activity, intracellular pathogens like *Mycobacterium* and *Salmonella* appear to repress TFEB, whereas compounds that promote TFEB production or activity enhance macrophage killing of *Salmonella*. Intriguingly, *Salmonella enterica* sv. Typhimurium (*S.* Typhimurium) was observed to actively stimulate TFEB, implying a benefit to *Salmonella* during infection. To better understand the relationship between *S.* Typhimurium infection and TFEB, we assessed if *S.* Typhimurium regulated TFEB in murine macrophages in a manner dependent on infection conditions. Here, we show that macrophages that engulfed late-logarithmic grown *Salmonella* accumulated and maintained nuclear TFEB, comparable to macrophages that engulfed *E. coli*. In contrast, stationary-phase *S.* Typhimurium infection of macrophages actively delayed TFEB nuclear mobilization over the first hour of infection. The delay in TFEB nuclear mobilization was not observed in macrophages that engulfed heat-killed stationary-phase *Salmonella*, or *Salmonella* lacking functional SPI-1 and SPI-2 type three secretion systems. *S.* Typhimurium mutated in the master virulence regulator *phoP* or the secreted effector genes *sifA*, and *sopD* also showed normal TFEB nuclear translocation. Interestingly, while *E. coli* survived better in *Tfeb*$^{-/-}$ macrophages, *S.* Typhimurium growth was similar in wild-type and *Tfeb*-/- macrophages. Yet, priming macrophages with phagocytosis enhanced the killing of *Salmonella* in wild-type, but not in *Tfeb*$^{-/-}$ macrophages. Collectively, *S.* Typhimurium seems to orchestrate TFEB in a manner dependent on infection conditions, while

conditions that disturb this context-dependent control of TFEB, such as forcing activation of TFEB seems to be detrimental to *Salmonella* survival.

Introduction

Macrophages are innate immune cells that hunt and engulf microbes through phagocytosis. As a corollary, following phagocytosis, microbes are quarantined within phagosomes, which are then transformed into hostile phagolysosomes. During this maturation process, phagosomes acquire a microbicidal milieu enriched in hydrolytic enzymes, antimicrobial peptides, and an acidic and oxidative lumen (Flannagan *et al.*, 2012; Levin *et al.*, 2016; Nguyen and Yates, 2021; Fountain *et al.*, 2021).

Phagosome maturation is critically dependent on the endolysosomal membrane system. Phagosomes fuse sequentially with endosomes and ultimately with lysosomes in a process that depends on various Rab GTPases, lipid mediators like phosphatidylinositol-3-phosphate, and motor and SNARE proteins (Bouvier *et al.*, 1994; Fratti *et al.*, 2001:7; Harrison *et al.*, 2003; Vieira *et al.*, 2003; Pei *et al.*, 2014; Kim *et al.*, 2014; Levin *et al.*, 2016; Nguyen and Yates, 2021; Fountain *et al.*, 2021; Fountain *et al.*, 2021). Interestingly, while phagosome-lysosome fusion has been known for decades (Brandes and Anton, 1969; Kielian and Cohn, 1980), it was generally assumed that lysosomes and their properties remained unchanged during phagocytosis, phagosome maturation, and interaction with microbes – though at least one study in 1970s suggested that phagocytosis in macrophages induced lysosomal enzyme levels (Axline and Cohn, 1970). However, we now understand that lysosomes are remodelled in response to various stresses, including interaction with microbes, microbe-associated molecular patterns (MAMPs),

and phagocytosis (Swanson *et al.*, 1987; Vyas *et al.*, 2007; Mrakovic *et al.*, 2012; Pastore *et al.*, 2016; Gray *et al.*, 2016; Brady *et al.*, 2018; Hipolito *et al.*, 2018; Sachdeva *et al.*, 2020; Najibi *et al.*, 2022). In part, this is achieved by activation of the transcription factor TFEB, which drives expression of endolysosomal, autophagy, and immunomodulatory genes (Settembre *et al.*, 2011; Visvikis *et al.*, 2014; Pastore *et al.*, 2016; Gray *et al.*, 2016; Brady *et al.*, 2018; Hipolito *et al.*, 2018).

TFEB is activated by numerous signals including amino acid deprivation, metabolic stress, mitochondrial stress, lipid metabolism and several immune stimuli such as phagocytosis of IgG-coated particles, several types of bacteria, and pattern recognition receptors that bind MAMPs (Settembre *et al.*, 2011; Settembre *et al.*, 2012; Roczniak-Ferguson *et al.*, 2012; Tsunemi *et al.*, 2012; Settembre *et al.*, 2013a; Settembre *et al.*, 2013b; Visvikis *et al.*, 2014; Nezich *et al.*, 2015; Najibi *et al.*, 2016; Pastore *et al.*, 2016; Gray *et al.*, 2016; Inpanathan and Botelho, 2019; Hipolito *et al.*, 2019). TFEB activation increases intracellular killing of microbes like *Escherichia coli*, *Mycobacterium tuberculosis*, *S.* Typhimurium, *Staphylococcus aureus* and *C. albicans.* (Gray *et al.*, 2016; Rao *et al.*, 2020; Ammanathan *et al.*, 2020; Westman *et al.*, 2020). Overall, TFEB activation is thought to favour immune function and repress microbe survival (Brady *et al.*, 2018; Hipolito *et al.*, 2018; Irazoqui, 2020). However, there is evidence for some microbes actively manipulating TFEB function.

Macrophages that engulfed living *S.* Typhimurium displayed nuclear TFEB translocation relative to macrophages that internalized non-viable *S.* Typhimurium in a process dependent on PLC-PKD pathway (Najibi *et al.*, 2016). A more recent study by the same authors indicate that dead *Salmonella* can also activate TFEB, but more slowly (Najibi *et al.*, 2022). Nevertheless, the intentional stimulation of TFEB by invading microbes is peculiar since TFEB stimulation is

typically thought to benefit the host. Indeed, additional studies suggest that ectopic activation of TFEB with compounds like VX-765, acacetin and 4-octyl itaconate can limit *Salmonella* survival (Rao *et al.*, 2020; Ammanathan *et al.*, 2020; Zhang *et al.*, 2022). Collectively, these observations suggest a complex interplay between TFEB and *S.* Typhimurium that requires further investigation.

S. Typhimurium is a microbe that hijacks mammalian cells through a complex virulence program involving two type III secretion systems (T3SS), SPI-1 and SPI-2, that collectively inject ~50 bacterial effector proteins into infected host cells that manipulate the behaviour of both epithelial and immune cells (Portaliou *et al.*, 2016; Jennings *et al.*, 2017; Lou *et al.*, 2019). The SPI-I T3SS is usually associated with invasion of epithelial cells, a bacteria-driven process that remodels the epithelial cell plasma membrane to ruffle and internalize the bacteria (Lundberg *et al.*, 1999; Terebiznik *et al.*, 2002; Raffatellu *et al.*, 2005; Patel and Galán, 2005; Bruno *et al.*, 2009; Lou *et al.*, 2019). *S.* Typhimurium can also infect macrophages during macrophage-driven phagocytosis (Cirillo *et al.*, 1998; Drecktrah *et al.*, 2006). Here, *S.* Typhimurium does not have to be in an invasive state *per se*, but the SPI-2 T3SS is essential to usurp, remodel and even escape the *Salmonella*-containing vacuole to promote its replication within the host macrophage (Cirillo *et al.*, 1998; Shea *et al.*, 1999; Drecktrah *et al.*, 2006; Jennings *et al.*, 2017). Importantly, the behaviour of *Salmonella* within a host cell may depend on both bacterial and host cell conditions including entry mode, growth and metabolic state, and time post-infection (Deiwick *et al.*, 1999; Lundberg *et al.*, 1999; Drecktrah *et al.*, 2006; Ibarra *et al.*, 2010; Lathrop *et al.*, 2015; Kurtz *et al.*, 2020). In fact, the growth state and nutritional conditions of *Salmonella* appear to modulate the entry mode, where late-log/early stationary

phase grown *Salmonella* favour SPI-1 expression, while late stationary growth primes SPI-2 expression (Song *et al.*, 2004; Bustamante *et al.*, 2008; Ibarra *et al.*, 2010).

Given this, we postulated that *Salmonella* and TFEB activation may depend on the invasive state of *Salmonella* and/or bacterial growth conditions. Here, we provide evidence that *Salmonella* grown to stationary-phase actively and transiently represses TFEB in macrophages. In comparison, *Salmonella* grown to late log-phase promotes early translocation of TFEB to the nucleus. Moreover, while TFEB is needed to suppress *E. coli* survival in macrophages, its deletion had no apparent effect on *Salmonella* growth in macrophages. Yet, pre-activation of TFEB by phagocytosis increased killing of both bacterial species. Overall, we reveal a complex interplay between TFEB and *Salmonella* infection conditions.

Results

Salmonella grown to stationary phase delays activation of TFEB after uptake by macrophages

Phagocytosis of IgG-coated particles, unopsonized *E. coli*, *M. smegmatitis, and S. aureus*, and invasive *Salmonella* all activate TFEB in macrophages (Visvikis *et al.*, 2014; Najibi *et al.*, 2016; Pastore *et al.*, 2016; Gray *et al.*, 2016; Najibi *et al.*, 2022). In most cases, TFEB activation is thought to boost the immunological response by stimulating lysosomal, autophagy and immune-protective genes (Visvikis *et al.*, 2014; Pastore *et al.*, 2016; Gray *et al.*, 2016; Brady *et al.*, 2018). However, in the case of invasive *Salmonella*, activation of TFEB has been reported to be driven by *Salmonella*, which implies a benefit to the microbe during invasion of macrophages (Najibi *et al.*, 2016). In apparent conflict with these observations, there are also observations showing that *Salmonella* represses TFEB and that TFEB stimulation increases *Salmonella* killing (Rao *et al.*,

2020; Ammanathan *et al.*, 2020). Given these discrepancies, we sought to better understand the relationship between *Salmonella* infection and TFEB activation.

We began by comparing the ability of *Salmonella* grown to stationary phase to elicit TFEB activation in macrophages by measuring the relative nuclear to cytosolic (N/C) ratio of TFEB. Stationary-phase *Salmonella* cultures are reported to be enriched in non-invasive *Salmonella*, and like *E. coli*, engulfment is thought to be driven by macrophage phagocytosis rather than by invasion (Drecktrah *et al.*, 2006; Gog *et al.*, 2012). As expected, we found that phagocytosis of non-pathogenic *E. coli* DH5α increased the N/C ratio of GFP-TFEB in RAW macrophages within 1 h of phagocytosis relative to resting macrophages (Fig. 1A, B). By contrast, the N/C ratio of GFP-TFEB in macrophages that engulfed stationary-phase *Salmonella* was similar to resting cells and significantly lower than macrophages that engulfed *E. coli* (Fig. 1A, B). Moreover, phagocytosis of heat-killed *Salmonella* previously grown to stationary-phase elicited nuclear mobilization of GFP-TFEB that was comparable to macrophages that engulfed *E. coli* (Fig. 1A, B). Collectively, these observations suggest that stationary-phase *S.* Typhimurium actively represses TFEB nuclear translocation in macrophages at 1 h post-infection. However, we also observed an increase over time in the N/C ratio of GFP-TFEB in macrophages that engulfed stationary-phase *Salmonella*; at 4 and 6 h post-engulfment, the N/C ratio of GFP-TFEB in these cells was higher than resting cells or those at 1 h post-infection, and comparable to macrophages infected with *E. coli* (Fig. 1C, D). Thus, stationary-phase *Salmonella* delays TFEB mobilization to the nucleus, rather than fully arresting it.

Salmonella growth conditions affect TFEB response in macrophages

The growth phase of *Salmonella* is thought to affect the expression of invasion effectors and modulate the mode of infection (Lundberg *et al.*, 1999; Drecktrah *et al.*, 2006; Ibarra *et al.*, 2010). Typically, *Salmonella* is grown to late log-phase before experimentation. We thus tested whether the growth phase of *Salmonella* affected the N/C ratio of TFEB in RAW cells. First, we observed that stationary- and log-phase *E. coli* similarly induced nuclear translocation of TFEB (Fig. 2A, B). Moreover, TFEB remained enriched in its nuclear distribution in macrophages 1 h or 4 h post-uptake of *E. coli* grown to either culture phase (Fig. 2A, C, D). *Salmonella* infected cells displayed a contrasting behaviour in terms of TFEB nuclear entry. While stationary-phase *Salmonella* failed to activate TFEB at 1 h post-infection, as illustrated before, macrophages infected with late-logarithmic *Salmonella* significantly mobilized TFEB into the nucleus within 1 h of uptake (Fig. 2A, B), consistent with observations by Najibi *et al* (Najibi *et al.*, 2016). We observed similar results when staining for endogenously expressed TFEB; stationary-phase live *Salmonella* was the only condition that failed to enrich TFEB in the nucleus after 1 h of uptake, while live and dead *E. coli*, live late log-phase *Salmonella*, and non-viable stationary-phase and late log-phase *Salmonella* all triggered accumulation of TFEB in the nucleus relative to resting cells (Fig. 3A-B).

We next tested if TFEB nuclear mobilization was subject to temporal modulation by *Salmonella* grown to either log- or stationary-phases by analysing the N/C ratio of TFEB at 1 or 4 h post-infection. As previously observed, macrophages infected with stationary-phase *Salmonella* displayed mostly cytosolic TFEB at 1 h post-infection and higher nuclear levels of TFEB at 4 h post-infection (Fig. 2A, C). In comparison, the N/C ratio of TFEB was similar between macrophages that engulfed log-phase *Salmonella* after 1 h or 4 h of infection, and both were statistically comparable to the counterpart macrophages that engulfed *E. coli* (Fig. 2A, 2D).

Collectively, *Salmonella* modulates TFEB nuclear localization in macrophages in a manner dependent on its growth conditions and stage of infection. Specifically, *Salmonella* grown to stationary phase actively delays TFEB mobilization relative to *E. coli* or *Salmonella* grown to log-phase.

The effect of Salmonella on TFEB phosphorylation

The cytosolic and nuclear distribution of TFEB can be regulated by the phosphorylation status at various residues of TFEB (Settembre *et al.*, 2012; Roczniak-Ferguson *et al.*, 2012:1; Martina *et al.*, 2012:1; Medina *et al.*, 2015; Puertollano *et al.*, 2018). To better understand how *Salmonella* might affect TFEB distribution, we used Phos-tag gels to determine the general phosphorylation of TFEB using whole cell lysates (Nagy *et al.*, 2018) and quantified the band migration front of TFEB. To analyse total levels, we employed a standard SDS-PAGE gel and Western blotting.

As expected, cell lysates from macrophages that engulfed *E. coli* for 1 h displayed a TFEB band pattern that migrated faster relative to lysates from resting, *i.e.*, phagocytosis of *E. coli* caused a significant net dephosphorylation of TFEB (Fig. 4A, B). Interestingly, this was more variable in macrophages 4 h post-phagocytosis of *E. coli*. In comparison, infection with *Salmonella* for either 1 or 4 h appeared to have an intermediate and heterogeneous effect on TFEB phosphorylation (Fig. 4A, B). This result was not expected and may reflect a change in phosphorylation sites without a net change in number of phosphorylated residues of TFEB and/or heterogeneous infection rates that is not resolvable by a population-based assay such as Western blotting (Puertollano *et al.*, 2018), or may suggest that TFEB is regulated through another mechanism such as the recently discovered itaconate modification of TFEB (Zhang *et al.*, 2022; Schuster *et al.*, 2022). Interestingly, the levels of total TFEB normalized to actin were

reduced in macrophages infected for 4 h with late-log-phase *Salmonella*, indicating that under specific conditions, *Salmonella* can repress TFEB expression or promote its degradation (Fig. 4A, C, D), consistent with *Salmonella* infection of murine bone marrow-derived primary macrophages (Rao *et al.*, 2020). Overall, TFEB control by *Salmonella* is subject to a temporal sequence of events that include control of its levels and likely a complex pattern of post-translational modifications.

T3SS-1 and T3SS-2 secretion systems are needed for stationary phase-grown* Salmonella *to delay TFEB nuclear mobilization

Our data show that stationary-phase *Salmonella* actively delays TFEB mobilization into the nucleus after macrophage engulfment. To test whether SPI-1 and SPI-2 T3SS were involved in this delay, we permitted macrophages to engulf stationary-phase and log-phase *Salmonella* carrying loss-of-function mutations in *invA* and *ssaR genes*, which are respectively required to express subunits and/or form the SPI-1 and SPI-2 complexes (Collazo and Galán, 1997; Cirillo *et al.*, 1998; Hensel *et al.*, 1998; Lundberg *et al.*, 1999). As before, wild-type *Salmonella* grown to stationary phase did not elicit nuclear accumulation of TFEB, unlike *Salmonella* grown to log-phase (Fig. 5A, B). However, macrophages that engulfed *invA ssaR Salmonella* grown to either growth conditions displayed higher levels of nuclear TFEB relative to stationary-phase wild-type *Salmonella* (Fig. 5A, B). We note that single *invA* and *ssaR* mutants had intermediate effect on TFEB localization, suggesting some redundancy towards TFEB manipulation (not shown). Thus, *Salmonella* SPI-1/SPI-2 double mutants fail to repress TFEB activation during phagocytosis and we predict that TFEB activation is now a host-driven process. In conclusion, our data suggest

that live *Salmonella* grown to stationary phase actively represses TFEB in a time-dependent and effector-dependent manner.

TFEB repression depends on PhoP, SifA and SopD2

Stationary-phase *Salmonella* appear to actively delay TFEB nuclear migration during macrophage infection. To further determine if stationary-phase *Salmonella* actively repress TFEB, we examined TFEB nuclear mobilization in macrophages after engulfment of seven *Salmonella* mutants; these mutants were either disrupted in the master virulence regulator, PhoP (Δ*phoP*) or eliminated effectors of the *Salmonella* SPI-2 system known to alter intracellular signalling and membrane trafficking (Δ*sopD2*, Δ*sopA*, Δ*sifA*, Δ*pipB2*, Δ*sspH2*, Δ*slrP*, or Δ*sigD*); (Brumell *et al.*, 2003:2; Geddes *et al.*, 2005; Figueira *et al.*, 2013; Jennings *et al.*, 2017:2) These *Salmonella* mutants were grown to stationary phase since this is the bacterial growth condition that actively suppressed TFEB nuclear entry. Of these seven mutants, we identified three whose phagocytosis triggered significant activation of GFP-tagged and endogenous TFEB relative to wild-type *Salmonella*: *sopD2*, *sifA* and *phoP* (Fig. 6). These data further support a functional role for *Salmonella* in impeding early TFEB activation after phagocytosis by macrophages. We speculate that the PhoPQ two-component system is necessary to sense the intra-phagosomal environment of engulfed *Salmonella* eliciting expression of effectors that delay TFEB activation such as effectors of the SPI-II system (Deiwick *et al.*, 1999; Bijlsma and Groisman, 2005; Martin-Orozco *et al.*, 2006; Prost and Miller, 2008). In turn, SopD2 and SifA are known to alter intracellular signalling and trafficking, which may prevent activation of TFEB (Brumell *et al.*, 2003:2; Dumont *et al.*, 2010; McEwan *et al.*, 2015; D'Costa *et al.*, 2015:2). These observations

support a model whereby stationary-grown *Salmonella* actively repress TFEB, at least in the early stages of infection.

TFEB expression impacts E. coli and Salmonella survival differently in macrophages

Our observations indicate that TFEB distribution in macrophages during *Salmonella* uptake is dependent on *Salmonella* growth conditions, wherein stationary *Salmonella* transiently, but actively represses TFEB, while invasive *Salmonella* permits or elicits nuclear translocation of TFEB. In comparison, phagocytosis of non-pathogenic *E. coli* always triggers nuclear entry of TFEB in macrophages irrespective of the growth conditions of *E. coli* and over the observed period. This raises the question of whether TFEB helps repress *Salmonella* or enables *Salmonella* growth in macrophages.

To better understand this potential conundrum, we investigated the survival of stationary-grown *E. coli* and *Salmonella* within wild-type and *Tfeb*$^{-/-}$ RAW macrophages (Pastore *et al.*, 2016). To do this, we allowed macrophages to phagocytose bacteria for 1 h, followed by gentamicin treatment to kill external bacteria. Macrophages were then lysed or chased for an additional 4 h to respectively assess bacterial uptake and survival within macrophages. Lysates were plated and the number of colonies formed were scored and normalized against the initial uptake. For *E. coli*, we observed a significant increase in bacterial survival in *Tfeb*$^{-/-}$ RAW macrophages compared to wild-type RAW cells (Fig. 7A), consistent with previous results (Gray *et al.*, 2016). By contrast, we did not observe a difference in *Salmonella* survival infecting wild-type or *Tfeb*$^{-/-}$ RAW macrophages (Fig. 7B). TFE3 is a homolog of TFEB that may have redundant functions (Pastore *et al.*, 2016). Deletion of both TFEB and TFE3 in RAW cells (Pastore *et al.*, 2016) also produced equivocal effects on *Salmonella* survival regardless of time

of infection (4 vs. 20 h) and the bacterial growth phase (Fig. 7C). Overall, our evidence suggests that TFEB expression aids macrophages in killing *E. coli*, but there is an equivocal effect on *Salmonella* survival, which may reflect a dual role of TFEB towards *Salmonella* that depends on its tight regulation by both host and microbe.

Pre-activation of TFEB by previous IgG-phagocytosis boosts Salmonella killing in macrophages

Our work suggests that *Salmonella* may orchestrate a temporal sequence of TFEB repression and activation that suits the stage of infection within macrophages. We hypothesized that disrupting this timed sequence may be detrimental to *Salmonella*. To test this, we employed an initial round of phagocytosis of IgG-coated beads, which we previously showed activates TFEB and enhances degradation of lysosomes and killing of *E. coli* (Gray *et al.*, 2016). We pre-treated wild-type and *Tfeb*$^{-/-}$ RAW macrophages with vehicle or IgG-coated beads, followed by phagocytosis of stationary phase *E. coli* or *Salmonella*, and then chased for 1 h to determine uptake or for 4 h to determine killing. The number of colonies formed was normalized to 1 h condition. As before, we observed increased *E. coli* survival in *Tfeb*$^{-/-}$ macrophages relative to wild-type macrophages without priming (Fig. 8A; percent survival: 84% ± 5%, and 130% ± 11% in wild-type and in *Tfeb*$^{-/-}$ macrophages, respectively). In comparison, *Salmonella* survived about the same in both genotypes (Fig. 8B; 160 ± 15% vs. 187% ± 19% in wild-type and in *Tfeb*$^{-/-}$ macrophages, respectively). When wild-type macrophages were primed with IgG-coated beads, we observed reduced survival of 57% ± 9% for *E. coli*, compared to 84% in unprimed wild-type macrophages (Fig. 8A). This drop in survival was eliminated in *Tfeb*$^{-/-}$ macrophages (Fig. 8A; 130%±11% vs. 137±`13%, unprimed vs. primed). Interestingly, priming wild-type macrophages decreased

Salmonella survival (Fig. 8B; 94%±11% vs. 160±15%, primed vs. unprimed), which was lost in *Tfeb*$^{-/-}$ macrophages (Fig. 8B; 187% ± 19% vs. 196% ± 26%, unprimed vs. primed).

To complement these observations, we extended pre-activation of TFEB using apilimod, an inhibitor of PIKfyve that stimulates TFEB (Choy *et al.*, 2018). However, apilimod treatment had little effect in *Salmonella* survival in both wild-type and *Tfeb*$^{-/-}$ macrophages under our experimental conditions, which may reflect a more complex relationship between TFEB activation and PIKfyve-mediated membrane trafficking (Fig. 8C). Collectively, our observations with IgG-primed phagocytosis suggests that pre- or forced activation of TFEB can restrict *Salmonella* survival in macrophages.

Discussion

Recent work has highlighted the role of TFEB as a conserved immunoprotective transcription factor that promotes lysosomal and autophagic activities, antimicrobial peptide production, and modulation of cytokine secretion in response to various immune stimuli (Visvikis *et al.*, 2014; Pastore *et al.*, 2016; Gray *et al.*, 2016; Brady *et al.*, 2018; Najibi *et al.*, 2022). Previous research showed that *Salmonella* can actively stimulate TFEB in RAW macrophages (Najibi *et al.*, 2016; Najibi *et al.*, 2022), suggesting that TFEB activation provides an advantage to this microbe. However, this model appears inconsistent with observations that i) *Salmonella* represses TFEB to escape macrophage clearance and ii) acacetin-induced activation of TFEB in HeLa cells enhances killing of *Salmonella* by stimulating xenophagy (Rao *et al.*, 2020; Ammanathan *et al.*, 2020). Collectively, this implies that the relationship between *Salmonella* and TFEB is multi-layered and possibly dependent on infection conditions. Indeed, our observations support this

view since the effect on *Salmonella* on TFEB was affected by the bacterial growth conditions and time of infection, whereby stationary, but not late-log bacteria, delayed TFEB activation. Furthermore, inactivation of the SPI-1 and SPI-2 T3SS of *S.* Typhimurium abrogated the inhibition of TFEB-nuclear translocation. The same effect was also seen when the master regulator, *phoP*, or the secreted effectors *sopD2* or *sifA* were deleted. Moreover, while deleting TFEB in macrophages protected *E. coli* from phagocytic killing, there was no effect on *Salmonella* survival. In comparison, promoting TFEB before infection enhanced killing of both bacterial species.

Host cell-dependent upregulation of TFEB

TFEB is subject to a complex regulatory network involving phosphorylation and other post-translational modifications (Bao *et al.*, 2016; Puertollano *et al.*, 2018; Zhang *et al.*, 2022). In the context of phagocytosis, host cells engage a signalling axis that promotes reactive oxygen species, which activates CD38 and synthesis of the second messenger NAADP (Najibi *et al.*, 2022). This in turn seems to activate MCOLN1/TRPML1 to release intraphagosomal Ca^{2+} to stimulate calcineurin, which then dephosphorylates and promotes TFEB nuclear entry (Gray *et al.*, 2016; Najibi *et al.*, 2022). This process is clearly driven by the host cell in response to microbe engulfment and phagocytosis (Gray *et al.*, 2016; Najibi *et al.*, 2022). Additionally, host macrophages engage a TFEB-Irg1-itaconate signalling axis in response to *Salmonella*, whereby TFEB promotes Irg1-driven itaconate production. Itaconate then alkylates and promotes TFEB nuclear translocation, which helps restrict *Salmonella* survival (Zhang *et al.*, 2022; Schuster *et al.*, 2022). As described, the activation of TFEB is macrophage-driven. We propose that stationary *Salmonella* actively delays macrophage-driven TFEB stimulation because living

stationary *Salmonella* repressed TFEB nuclear translocation, but not non-viable stationary *Salmonella* or mutants in *invA ssrA*, *phoP*, *sifA*, and *sopD2*. We further propose that stationary *Salmonella* through specific effectors delays TFEB activation by either preventing NAADP-Ca^{2+} signalling or blocking itaconate action. In fact, *Salmonella*, as well as *Yersinia pestis* can degrade itaconate to enhance their survival in macrophages since itaconate compromises the glycoxylate cycle whereby bacteria can use host cell resources for energy production (Sasikaran *et al.*, 2014; Hersch and Navarre, 2020).

Salmonella-dependent upregulation of TFEB

Viable *Salmonella* was observed to more readily activate TFEB relative to non-viable *Salmonella* by engaging a conserved PC-PLC-PKD pathway (Najibi *et al.*, 2016; Najibi *et al.*, 2022). This is in contrast to observations that *Salmonella* represses and reduces TFEB levels (Rao *et al.*, 2020). In our hands, while stationary *Salmonella* actively delays TFEB nuclear translocation, we did observe that late logarithmic-phase *Salmonella* triggers TFEB early during infection, which is consistent with Najibi *et al.* (Najibi *et al.*, 2016). However, contrary to their observations, dead *Salmonella* still activated TFEB in RAW macrophages – we do not know the reason for this discrepancy. It is also not unknown if any benefit exists for late-log *Salmonella* to promote or permit TFEB engagement; for example, it may be that specific temporal activation of TFEB may aid Salmonella in remodelling phagosomes or *Salmonella*-containing vacuoles. Perhaps aligning with the idea that the stage of infection is critical for what TFEB might do, we observed that late-log *Salmonella* reduced TFEB levels after 4 h of infection, consistent with reduced TFEB expression and lysosomal activity observed by Rao *et al.* (Rao *et al.*, 2020).

Mechanistic control of TFEB by Salmonella

How might stationary-phase *Salmonella* delay TFEB activation? Control of the expression of the SPI-1 and SPI-2 T3SS and their effectors depends on numerous regulatory inputs, including the phase of *Salmonella* growth (Portaliou *et al.*, 2016; Jennings *et al.*, 2017; Lou *et al.*, 2019). Specifically, late logarithmic phase growth promotes expression of SPI-1 and epithelial cell invasion, while stationary phase-growth promotes SPI-2 expression (Song *et al.*, 2004; Bustamante *et al.*, 2008; Ibarra *et al.*, 2010). We note that single mutants SPI-1 (*invA*) and SPI-2 (*ssaR*) had intermediate effects on TFEB nuclear entry relative to wild-type and *invA ssaR* strains (not shown) – this may represent redundancy between T3SSs relative to TFEB modulation or adaptation of the single mutant bacteria by upregulating other T3SSs. On the other hand, *invA ssaR* double mutants grown to stationary phase failed to delay TFEB nuclear translocation, suggesting that *Salmonella* effectors are required for this. Given that *phoP*, *sifA*, and *sopD2* mutants also failed to delay TFEB nuclear translocation, this suggests that the PhoPQ two-component system and alteration of membrane and intracellular signalling are involved in delaying TFEB nuclear entry. SopD2 is proposed to be a GTPase activating protein for the Rab7 GTPase, while SifA interacts with motor proteins to sculpt *Salmonella*-containing vacuoles into *Salmonella*-induced filaments (Brumell *et al.*, 2003:2; Dumont *et al.*, 2010; D'Costa *et al.*, 2015).

We do not know how these factors interfere with TFEB, but we propose several non-mutually-exclusive possibilities. First, BioID analysis of SopD2 and SifA identifies LAMTOR subunits as potential interactors (D'Costa *et al.*, 2019), which may allow *Salmonella* to modulate mTORC1, responsible for phosphorylation and repression of TFEB. While we did not see a significant change in TFEB phosphorylation by Phos-tag gels in *Salmonella*-infected cells, this

could reflect a different phosphorylation pattern without a change in net phosphate number. Second, stationary *Salmonella* may blunt reactive oxygen species (ROS), thus preventing NAADP-Ca^{2+} activation of TFEB after phagocytosis in macrophages (Najibi *et al.*, 2022). In fact, *Salmonella* appears to manipulate ROS in host cells (van der Heijden *et al.*, 2015; Kim *et al.*, 2022), though it is not clear if this depends on growth phase and time of infection. Third, as indicated above, stationary *Salmonella* may blunt itaconate action, thus reducing TFEB activation (Sasikaran *et al.*, 2014; Hersch and Navarre, 2020; Zhang *et al.*, 2022; Schuster *et al.*, 2022).

Ectopic activation of TFEB enhances bacterial killing in macrophages

TFEB plays a protective role against intracellular bacteria (Visvikis *et al.*, 2014; Gray *et al.*, 2016; Irazoqui, 2020; Zhang *et al.*, 2022; Schuster *et al.*, 2022). In fact, we previously showed that pre-activation of TFEB by stimulating Fcγ receptors with aggregated IgG, enhanced *E. coli* killing in macrophages (Gray *et al.*, 2016). More recently, pre-activation of TFEB by acacetin also promoted killing of *Salmonella* and *S. aureus* (Rao *et al.*, 2020; Ammanathan *et al.*, 2020). This suggests that strategies that obligately activate TFEB, thus bypassing any spatiotemporal manipulation of TFEB by *Salmonella*, may be detrimental to this microbe. Indeed, we observed that phagocytosis of IgG-coated beads, which stimulates TFEB, enhanced killing of both *E. coli* and *Salmonella* in wild-type but not in *Tfeb*$^{-/-}$ RAW macrophages. Thus, while it may be that *Salmonella* benefits from TFEB activation under specific conditions, treatments that erode control of *Salmonella* on TFEB appear detrimental to the microbe.

Overall, our work sheds additional light into the interactions between *Salmonella* and TFEB in host macrophages. Depending on bacterial growth conditions and stage of infection, we

find that *Salmonella* can either delay TFEB activation, or permit, or even elicit TFEB activation. This raises the possibility that TFEB presents a double-edged sword for *Salmonella* – depending on infection stages, TFEB may be detrimental or beneficial to *Salmonella*. Importantly, forcibly promoting TFEB can override the spatio-temporal control of TFEB imposed by *Salmonella*, enhancing the killing capacity of macrophages. Thus, therapeutics that promote TFEB and related proteins may be useful to enhance killing of intracellular pathogens

Materials and Methods

Macrophage Culture, Plasmids and Transfection

The RAW 264.7 macrophage-like murine cell line was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Wisent, QC) at 37 ˚C and 5% CO_2 up to passage 20. Before plating, cells were washed with sterile phosphate-buffered saline (PBS, Wisent) and released by scraping in 5 mL of DMEM media. Cells were seeded into 6-well or 12-well plates at a confluency of 40% and used the following day. Using the same growth conditions, we also used RAW 264.7 cells that were deleted for *TFEB* gene (RAW *Tfeb*$^{-/-}$ cells) or double-deleted for *TFEB* and *TFE3* genes (RAW *Tfeb*$^{-/-}$ *Tfe3*$^{-/-}$ cells) using CRISPR-Cas9 technology. As control cells, we used RAW cells subject to non-targeting CRISPR-Cas9 process (wild-type RAW cells). These cell lines were obtained from Dr. Rosa Puertollano (National Institutes of Health, MD and were previously described (Pastore *et al.*, 2016).

To express GFP-TFEB fusion protein, we used a plasmid provided by Dr. Shawn Ferguson (Yale University School of Medicine, CT) and previously described (Roczniak-

Ferguson *et al*., 2012). RAW cells were seeded at a 20-30% confluency in a 12-well plate the day before, and then transfected with the plasmid encoding GFP-TFEB using FuGene HD transfection reagent (Promega, WI), following manufacturer's instructions. The transfection mix was left on cells for 5 h followed by removal and addition of new media. Cells were used the following day.

Bacterial Strains and Culture

In this study, we used *Escherichia coli* DH5α, wild-type *Salmonella enterica sv.* Typhimurium (SL1344), and *S.* Typhimurium strain deleted for the *phoP* gene (Δ*phoP)* (Mulder *et al.*, 2012). *S. typhimurium* mutants (Δ*sopD2*, Δ*sifA*, Δ*slrp*, Δ*sigD (*Δ*sopB*), Δ*sspH2*, Δ*sopA*, Δ*pipB2*) were obtained from Dr. John H. Brumell at the Hospital for Sick Children (Toronto, ON). All bacterial strains were grown overnight in lysogeny broth (LB) at 37°C with shaking. To use stationary cultures, bacteria were pelleted, washed once with PBS, and resuspended in DMEM at OD600 ~0.8 and immediately used for infection. To prepare log-phase growth bacteria, an overnight culture was sub-cultured in the morning at 1:10 in fresh LB and then grown at 37°C for 3.5 h until reaching late-logarithmic phase (OD_{600} ~ 0.8), before pelleting, washing in PBS and resuspending in DMEM at OD_{600} ~0.8.

Phagocytosis and Infection Assays

For phagocytosis/invasion assays, RAW macrophages plated at 1-2x10^6 cells/well were exposed to either stationary-phase or late log-phase bacteria at a 100:1 multiplicity of infection (MOI). Phagocytosis was synchronized through centrifugation of the culture plates at 500 xg for 5 min at room temperature. Macrophages were allowed to phagocytose for 1, 4, or 6 h, unless stated

otherwise. Depending on the application, cells were then subject to 50 µg/mL gentamicin to kill extracellular bacteria for 30 min, followed by either lysis with Laemmli buffer for Western blot analysis or fixation with 4 % (v/v) paraformaldehyde (PFA) in PBS for 20 min and processing for imaging as described below.

Bacterial Survival Assays

Prior to engulfment of unopsonized *E. coli* or *S.* Typhimurium, RAW macrophages remained untreated or were pre-activated with IgG-opsonized beads for 1 h, followed by a 3 h chase. Treated or untreated macrophages were then infected with stationary-phase bacteria (*E. coli* and *S.* typhimurium) at a final $OD_{600} = 1.0$. Uptake was synchronized through centrifugation at 400 *g* for 5 min. At 1 h post-infection, macrophages were washed 3x with PBS and replaced with DMEM media containing 50 µg/ml gentamicin to kill extracellular bacteria for 30 min. After, cells were washed 3x with PBS and either lysed immediately (uptake) with 200 µL 1% Triton X-100 for 5 min or the media was replaced with new DMEM supplemented with 10% FBS, containing low concentration of gentamicin (12 µg/ml), and incubated for 3 h at 37˚C to allow time for phagosome maturation and bacteria killing. Prior to lysing, cells were washed multiple times with PBS and replaced with DMEM media containing 50 µg/ml gentamicin to kill extracellular bacteria for 30 min. They were then washed 3x with PBS and lysed with 200 µL 1% triton X-100 for 5 min and scraped to release bacteria. Cell lysates were re-suspended in 800 µL PBS and subjected to serial dilutions in LB broth, followed by plating of 10 µL in LB plates, and incubated overnight at 37 ºC. Colony numbers were counted and recorded to determine CFU/mL relative to uptake.

For *Salmonella* survival in RAW^{NT} and RAW *tfeb*$^{-/-}$ macrophages, the above was done with some changes. Cells were plated in 12-well plates and used at a density of $1x10^6$ cells/well and infected with *S.* Typhimurium grown to stationary or late-logarithmic phase at an MOI of 100:1. Cells were lysed immediately after 1 h of phagocytosis followed by 30 min incubation in DMEM with 50 µg/ml gentamicin to estimate bacterial uptake or lysed at 4 or 20 h post-infection to estimate killing. Lysates were then resuspended in 500 µL of PBS, serially diluted and 10 µL were plated on LB plates, and these were incubated overnight. Colony numbers were counted and used to determine bacterial survival relative to uptake.

For pre-activation of TFEB by apilimod, macrophages were treated with vehicle or 20 nM apilimod (Toronto Research Chemicals, Toronto, ON) for 1 h, followed by bacterial infection with the drugs present. Cells were then lysed immediately to determine uptake of bacteria or chased for 4 h post-infection with the drug to determine survival.

Immunofluorescence and Fluorescence Microscopy

To visualize external bacteria in TFEB-GFP transfected cells, immunostaining was conducted by briefly washing cells 3x with 0.5% Bovine Serum Albumin (BSA) and then incubating cells for 30 min with either rabbit anti-*E. coli* antibodies (BioRad, ON) or rabbit anti-*Salmonella* O antiserum group B antibodies (Fisher Scientific, ON) at 1:500. Cells were then washed 3x with 0.5% BSA in PBS, followed by secondary donkey anti-rabbit antibodies conjugated to Dylight355 (Bethyl Laboratories, TX) for 30 min at 1:500 in 0.5% BSA in PBS. To visualize internal bacteria, cells were permeabilized with 1% triton X-100 in PBS, followed by incubation with the same primary antibodies above, but followed with donkey anti-rabbit antibodies

conjugated to Dylight 550 (Bethyl Laboratories, TX). Cells were washed 3x with 0.5% BSA and mounted onto glass slides with fluorescent mounting media (DAKO) for imaging.

To stain for endogenous TFEB, cells were fixed with 4 % PFA (v/v) at room temperature for 20 min and 0.1 M glycine in PBS for 10 min, before being washed 3x with 0.5% BSA and permeabilized with 0.1% triton for 10 min or using ice cold methanol for 5 min. Cells were blocked for 30 min with 0.5% BSA in PBS, before incubating for 30 min with 1:250 dilution of rabbit anti-TFEB antibodies (Bethyl Laboratories, TX) in 0.5% BSA. Cells were then washed 3x with 0.5% BSA, incubated with 1:500 dilution of Dylight 488-conjugated donkey anti-rabbit antibodies (Bethyl Laboratories, TX) for 30 min. In some experiments, cells were also incubated for 5 min with 0.4 µg/ml 4',6-diamidino-2-phenylindole (DAPI), washed, and mounted onto glass slides with fluorescent mounting media (DAKO) for imaging.

Fluorescently labelled samples were imaged with one of two microscopy systems. For epifluorescence, we used a 60x oil immersion objective mounted on an Olympus IX83 Inverted Microscope linked to a Hamamatsu ORCA-Flash 4.0 digital camera (Olympus, ON). Images were either used as is or subjected to deconvolution using CellSens Dimension Module (Olympus, ON) with the advanced maximum likelihood estimation algorithm (ADVMLE). Alternatively, confocal imaging was done using a Quorum DisKovery spinning disc confocal microscope system equipped on a Leica DMi8 microscope, with a 63x 1.4 NA oil-immersion objective, and linked to an iXON 897 EMCCD camera. The system was controlled by the Quorum Wave FX powered by MetaMorph software (Quorum Technologies, Guelph, ON).

Image analysis

To quantify nuclear to cytosol TFEB intensity, images were acquired either through single plane acquisition or by acquiring a Z-stack. Epifluorescence Z-stacks were deconvolved to reduce out of focus light and improve contrast and resolution, while single slice images remained untouched. For confocal imaging, background correction was employed. Images were analyzed using ImageJ/FIJI (NIH, MD) or Volocity. For nuclear to cytosolic TFEB ratio, regions of interest were drawn around the nucleus and the cytosol, and the intensities within these regions were then measured. After background subtraction, the ratio of nuclear to cytosol fluorescence was calculated.

Cell lysates, SDS-PAGE, and Western blotting analysis

After specific cell treatments, whole cell lysates were prepared using 2x Laemmli buffer containing phosSTOP and protease inhibitor cocktail (Roche, Mississauga, ON). Lysates were passed through a 27-gauge needle ten times, then heated at 65 °C for 15 min. For total TFEB levels, lysates were resolved by SDS-PAGE using a 10% acrylamide resolving gel. Proteins were transferred to a PVDF membrane, blocked with 5% BSA, incubated with primary rabbit anti-TFEB antibodies (Bethyl Laboratories, Montgomery, Texas) or primary rabbit anti-β-actin antibodies (Cell Signaling Technology, Danvers, MA) at a dilution of 1:1000, followed by HRP-conjugated goat anti-rabbit secondary antibodies (Cell Signaling Technology) at 1:5000 dilution. Protein levels were detected via Clarity® enhanced chemiluminescence reagent (Bio-Rad Laboratories, Mississauga, ON) visualized by a ChemiDoc Touch Imaging system (Bio-Rad). Blot intensity quantification was accomplished using ImageLab software (Bio-Rad) and its volumetrics and background subtraction functions. Total TFEB protein levels were normalized to β-actin.

Phos-tag-PAGE and Western blotting analysis

For analysis of protein phosphorylation, lysates were prepared as described above. Lysates were then run on a 7.5% acrylamide resolving gel containing 50 µM SuperSep Phos-tag (Wako Chemicals, Richmond, Virginia). Proteins were separated at 15 mA and the gel was then washed as per manufacturer's instructions. Proteins were transferred onto a PVDF membrane and visualized as described above. Phosphorylated proteins experience decreased mobility in Phos-tag gels relative to less phosphorylated isoforms. To analyse changes in phosphorylation of TFEB, we marked the migration front of TFEB in resting cells, then measured the volume of TFEB signal upstream and downstream of this front for each condition using ImageLab (Bio-rad). We then measured the ratio of the downstream volume (less phosphorylated) to the upstream volume (more phosphorylated) to assess the degree of TFEB phosphorylation in each condition.

Statistical analyses

All experiments were repeated independently at least three times. For bacterial survival, each condition had two or three technical replicates. For microscopy analysis, the number of individual cells scored is indicated in corresponding figure legend. The mean of independent experiments is indicated along with either standard deviation or standard error of the mean, as appropriate. As indicated, sometimes data were normalized to a control condition to account for experimental variation. Unless otherwise stated, data were assumed to be normally distributed. Unless otherwise stated, data were subjected to analysis of variance (ANOVA) followed by

Tukey's or Sidak's post-hoc test, unless otherwise stated. A *p*-value of <0.05 was considered significant.

Figure Legends

Figure 1: Stationary-grown *Salmonella* delays TFEB nuclear translocation after phagocytosis. A. Epifluorescence micrographs of fixed RAW macrophages transiently expressing GFP-TFEB and that were given no bacteria (resting) or incubated for 1 h with living *E. coli*, or with living or heat-killed *Salmonella* grown to stationary phase. Samples were stained for external (before permeabilization) and all bacteria (after permeabilization) using anti-*E. coli* or anti-*Salmonella* antibodies. Arrows indicate internalized bacteria. Scale bar = 10 µm. **B.** The nuclear to cytosolic (N/C) fluorescence ratio of GFP-TFEB in macrophages was quantified for the indicated conditions after 1 h of uptake. Means ± SEM were tested using one-way ANOVA test and post-hoc Tukey's test, where *** indicates $p<0.001$. **C** and **D**. GFP-TFEB transfected RAW macrophages engulfed live stationary *E. coli* or *Salmonella* for 1, 4, or 6 h. The N/C fluorescence ratio of GFP-TFEB was then quantified. C and D are different representations of the same data. Data are shown as mean ± SEM from three independent (B) or four independent (C, D) experiments, scoring at least 50 cells for each condition per experiment. For C and D, mean ± SEM are shown and tested using two-way ANOVA test and post-hoc Tukey's test, where *, **, and *** indicate *p*-values of 0.05-0.01, *p*-values of 0.01-0.001, or $p<0.001$, respectively.

Figure 2: Stationary-grown *Salmonella* but not log-phase *Salmonella* delays TFEB activation in macrophages. A. Confocal micrographs of RAW cells expressing GFP-TFEB before, 1 h, or 4 h after engulfing living *E. coli* and *Salmonella* grown to stationary or late log-phase. After uptake, cells were fixed and stained with DAPI and with anti-bacteria antibodies to detect the nucleus and bacteria, respectively. **B**. The N/C fluorescence ratio of GFP-TFEB in

macrophages 1 h after engulfment of stationary or late-logarithmic bacteria. **C.** The N/C fluorescence ratio of GFP-TFEB in macrophages 1 h or 4 h after engulfment of stationary grown *E. coli* or *Salmonella*. **D.** The N/C fluorescence ratio of GFP-TFEB in macrophages 1h or 4 h after engulfment of log-phase grown *E. coli* or *Salmonella*. For B-D, data are shown as mean ± SEM from three independent experiments, scoring 70 cells for each condition per experiment. Means were tested using two-way ANOVA test and post-hoc Tukey's test, where *, **, and *** indicate p values of 0.05-0.01, p values of 0.01-0.001, or p<0.001, respectively.

Figure 3: The distribution of endogenous TFEB in macrophages after uptake of viable and non-viable *E. coli and Salmonella*. A. Confocal micrographs of RAW macrophages incubated with living or heat-killed stationary-grown *E. coli* or living or heat-killed *Salmonella* grown to either stationary or late-logarithmic phase. After 1 h, cells were fixed and stained with DAPI (blue) to identify the nucleus, anti-TFEB antibodies (green), and anti-LPS antibodies to identify all bacteria (red). Scale bar = 10 µm. **B.** N/C fluorescence ratio of endogenous TFEB in macrophages subject to indicated conditions. Data are shown as mean ± SEM from three independent experiments, scoring 80 cells for each condition and experiment. Means were tested using one-way ANOVA test and post-hoc Tukey's test, where * and ** indicate p values of 0.05-0.01 and of 0.01-0.001, respectively.

Figure 4: TFEB phosphorylation and protein levels in macrophages after *Salmonella* infection. A and **C.** Macrophages engulfed *E. coli* or *Salmonella* grown to stationary or log-phase for 1 or 4 h. Macrophages were then lysed and lysates separated on a Phos-tag SDS-PAGE (A) or standard SDS-PAGE (C), followed by blotting with anti-TFEB antibodies. The loading

control was β-actin for both types of electrophoresis. **B** and **D**. The migration front of TFEB separated in Phos-tag™ gels was measured as indicator of phosphorylation status of TFEB (B) and normalized total TFEB levels relative to β-actin (D). Shown is the mean ±SD analysed from five independent experiments. Data in B was analyzed by a one-way ANOVA and Dunnett post-hoc test, while data in D was tested by Friedman one-way ANOVA test and the Dunn's post-hoc test. * and ** indicates a *p*-value of <0.05 and <0.01, respectively.

Figure 5: SPI-I and SPI-II-deficient *Salmonella* do not stall TFEB activation in macrophages. A. Confocal images of RAW cells expressing GFP-TFEB (green) 1 h after engulfing wild-type or *invA ssaR Salmonella* grown to stationary or late log-phase. After uptake, cells were fixed and stained with DAPI (blue) and with anti-bacteria antibodies (red). **B**. The N/C fluorescence ratio of GFP-TFEB in macrophages after 1 h engulfment of *Salmonella* strains and grown to indicated phases. Data are shown as mean ± SEM from three independent experiments, scoring at least 70 cells for each condition per experiment. Means were tested using two-way ANOVA test and post-hoc Tukey's test, where * and ** indicate p values of 0.05-0.01 and 0.01-0.001, respectively.

Figure 6: Identification of *Salmonella* mutants that cannot suppress TFEB nuclear translocation in RAW macrophages. A. Epifluorescence micrographs of RAW cells expressing GFP-TFEB 1 h after engulfment of indicated bacteria strains. Cells were fixed and stained for external (cyan) and internal bacteria (red) bacteria with anti-*E. coli* or anti-*Salmonella* antibodies. Arrows point to specific internal bacteria, while full and dotted outlines indicate nucleus and individual cell boundaries. Scale bar represents 10 μm. **B.** N/C ratio of GFP-TFEB

in macrophages after engulfment of indicated bacterial strains. **C**. N/C ratio of endogenous TFEB in macrophages after engulfment of indicated bacterial strains. For both B and C, data is shown as mean ± SEM from four independent experiments with approximately 50 cells counted per condition per experiment. Means were tested using one-way ANOVA test and post-hoc Tukey's test, where *, **, and *** indicate p values of 0.05-0.01, p values of 0.01-0.001, or $p<0.001$, respectively.

Figure 7. *E. coli* and *Salmonella* survival in wild-type and *Tfeb*$^{-/-}$ macrophages. A and **B**. Wild-type and *Tfeb*$^{-/-}$ RAW macrophages were allowed to phagocytose live *E. coli* (A) or *Salmonella* (B) for 1 h, followed with gentamycin to kill external bacteria. One macrophage population was immediately lysed and bacteria were plated to count CFUs as an estimate of bacterial uptake. A second population of macrophages was incubated for 4 h with low gentamycin, then lysed, and bacteria plated to count CFUs as indicator of acute bacterial survival in macrophages. Data is shown as percent survival by taking the ratio of CFUs after 4 h of incubation relative to CFUs formed after 1 h incubation (uptake). **C.** Wild-type and *Tfeb*$^{-/-}$ *Tfe3*$^{-/-}$ RAW macrophages were allowed to phagocytose stationary and late-logarithmic grown *Salmonella*. Infection was followed for 4 and 20 h post infection. Percent survival was determined as described above. Data are shown as the mean ± SD from four independent experiments. Data in A and B were statistically analyzed by paired Student's t-test, while data in C was tested by two-way ANOVA and Šidák test. * indicates a $p<0.05$.

Figure 8: Ectopic activation of TFEB enhances *Salmonella* killing in macrophages in a TFEB-dependent manner. A and **B**. Wild-type and *Tfeb*$^{-/-}$ RAW macrophages were left

untreated or pre-activated with IgG-opsonized beads as described in methods and then allowed to engulf live *E.coli* (A) or *Salmonella* (B). Percent survival of *E. coli* (A) or *Salmonella* (B) was determined by comparing number of CFUs between initial uptake and 3 h post uptake. Shown is the average ± STD from n=8 independent experiments. **C**. Cells were pretreated with 20 nM apilimod or DMSO vehicle for 1 h and maintained through the infection with stationary or late-logarithmic *Salmonella*. Percent survival of bacteria was measured by comparing the number of CFU at the end of 4 h chase with CFUs from initial uptake (1 h) as described in methods. Shown is the mean and standard deviation from n=3 independent experiments. A-C: Data was analyzed by two-way ANOVA and Šidák's post-hoc test. Actual p values are shown for A and B.

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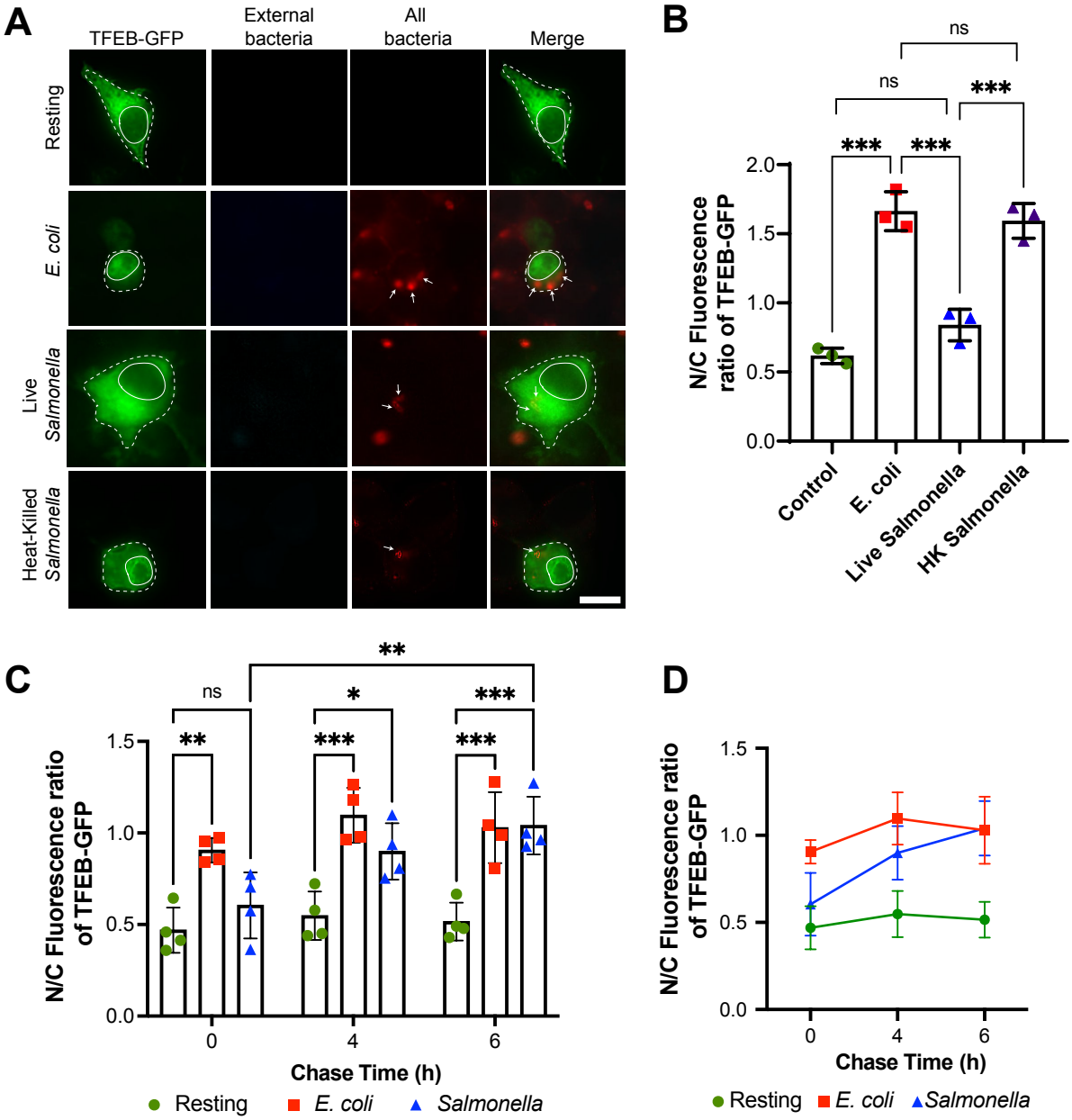

A
TFEB-GFP
External bacteria
All bacteria
Merge
Resting
E. coli
Live Salmonella
Heat-Killed Salmonella
B
ns
ns
***
***
***
N/C Fluorescence ratio of TFEB-GFP
2.0
1.5
1.0
0.5
0.0
Control
E. coli
Live Salmonella
HK Salmonella
C
**
ns
*
***
**
***
***
N/C Fluorescence ratio of TFEB-GFP
1.5
1.0
0.5
0.0
0
4
6
Chase Time (h)
Resting
E. coli
Salmonella
D
N/C Fluorescence ratio of TFEB-GFP
1.5
1.0
0.5
0.0
0
4
6
Chase Time (h)
Resting
E. coli
Salmonella

Figure 1

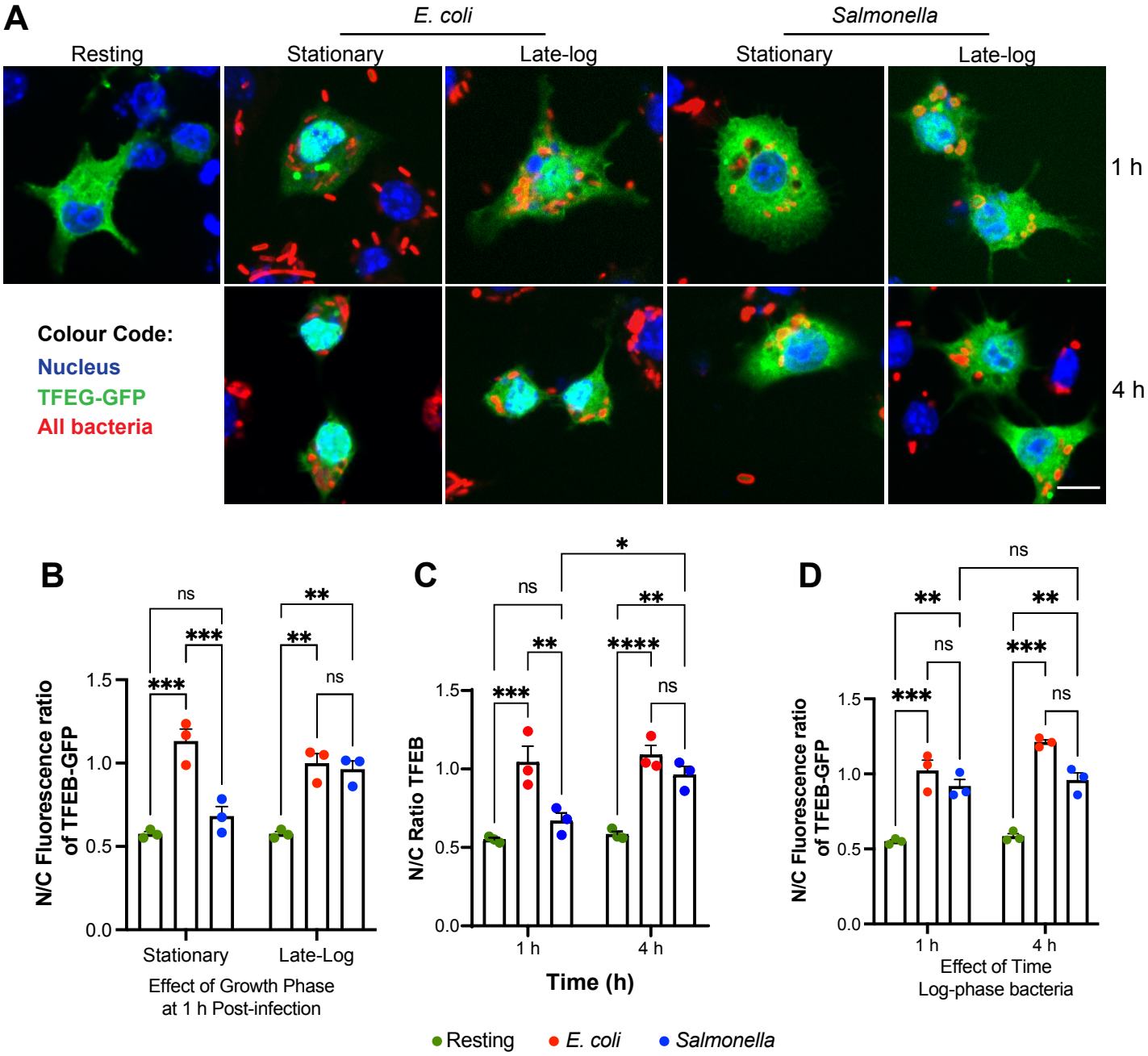

A
E. coli
Salmonella
Resting
Stationary
Late-log
Stationary
Late-log
1 h
4 h
Colour Code:
Nucleus
TFEG-GFP
All bacteria
B
N/C Fluorescence ratio of TFEB-GFP
1.5
1.0
0.5
0.0
ns
***
***
**
**
ns
Stationary
Late-Log
Effect of Growth Phase at 1 h Post-infection
C
N/C Ratio TFEB
*
ns
**
**
***
****
ns
1 h
4 h
Time (h)
D
N/C Fluorescence ratio of TFEB-GFP
ns
**
**
ns
***
***
ns
1 h
4 h
Effect of Time Log-phase bacteria
Resting
E. coli
Salmonella

Figure 2

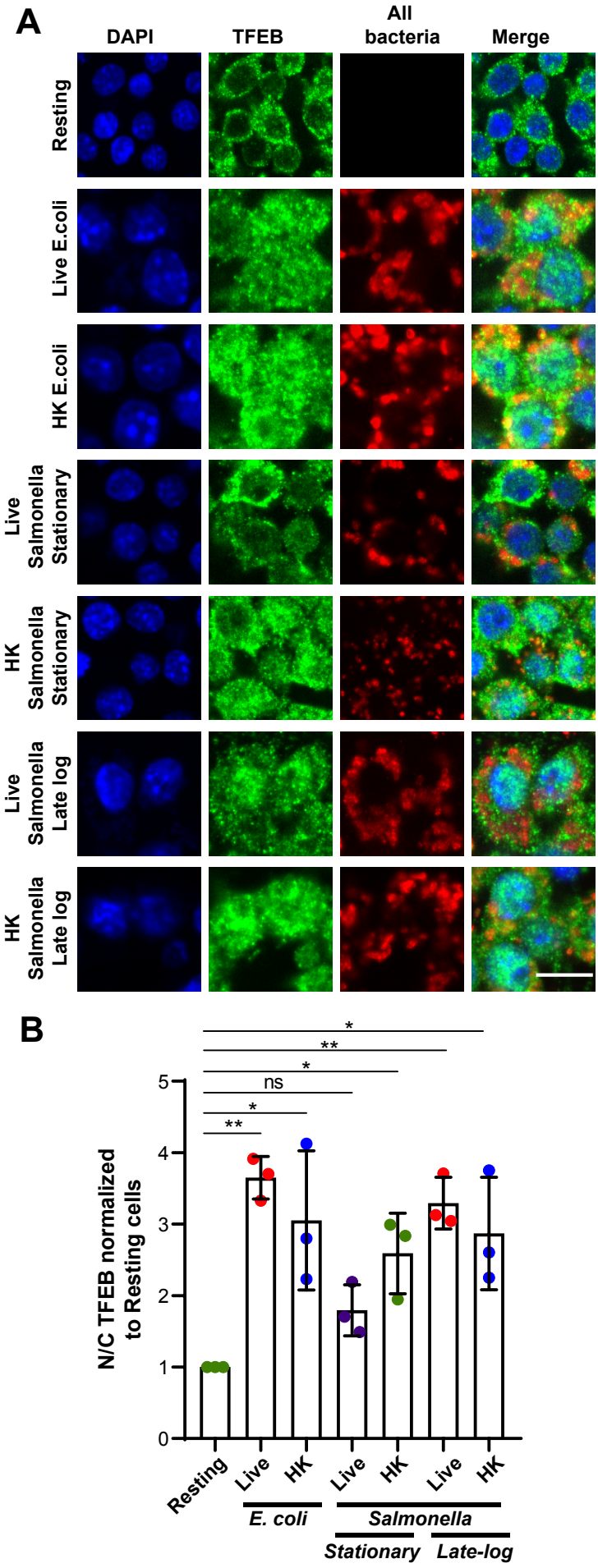

A
DAPI
TFEB
All bacteria
Merge
Resting
Live E.coli
HK E.coli
Live Salmonella Stationary
HK Salmonella Stationary
Live Salmonella Late log
HK Salmonella Late log
B
N/C TFEB normalized to Resting cells
0
1
2
3
4
5
*
**
*
ns
*
**
Resting
Live
HK
Live
HK
Live
HK
E. coli
Salmonella
Stationary
Late-log

Figure 3

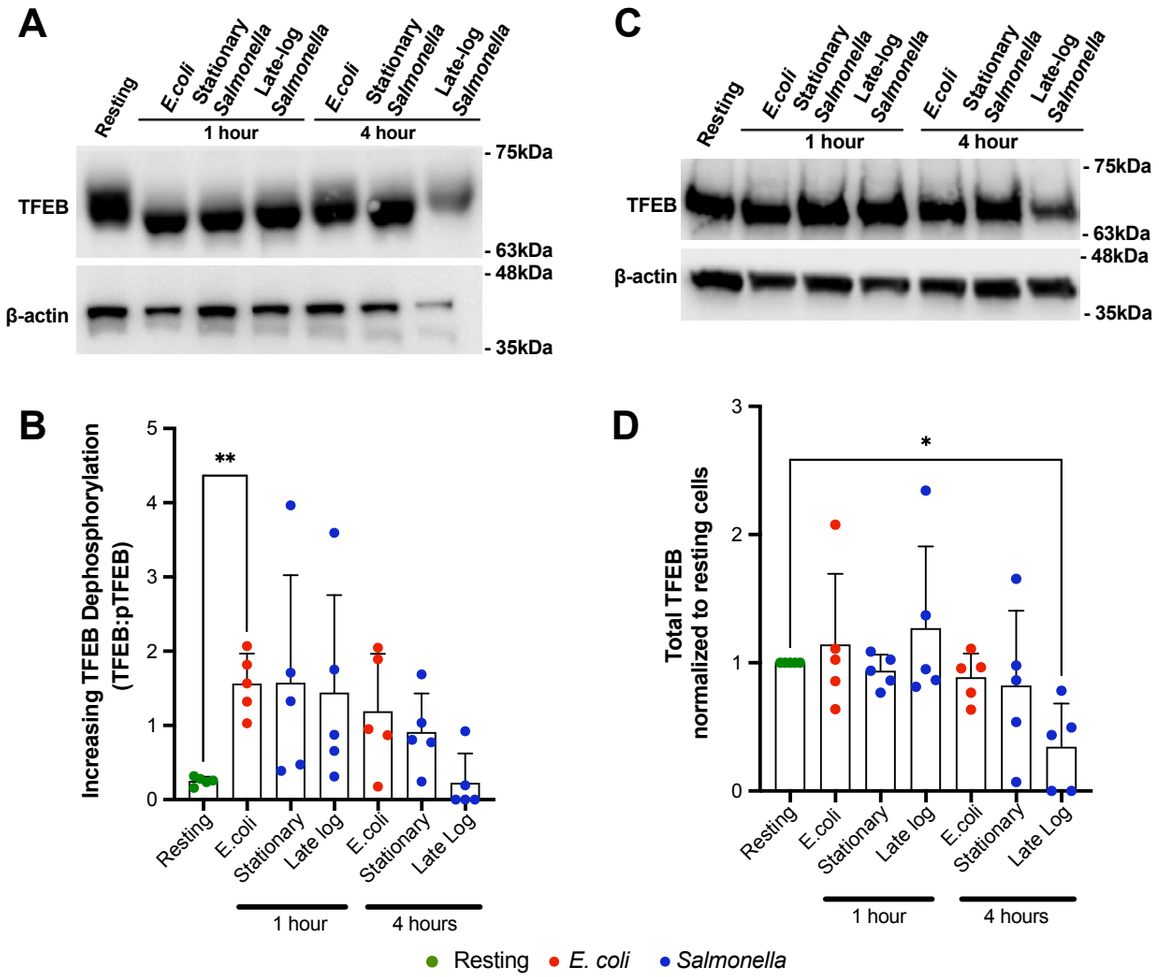

A
Resting
E.coli
Stationary Salmonella
Late-log Salmonella
1 hour
4 hour
TFEB
β-actin
- 75kDa
- 63kDa
- 48kDa
- 35kDa
C
B
Increasing TFEB Dephosphorylation (TFEB:pTFEB)
**
Resting
E.coli
Stationary
Late log
Late Log
1 hour
4 hours
D
Total TFEB normalized to resting cells
*
Resting
E. coli
Salmonella

Figure 4

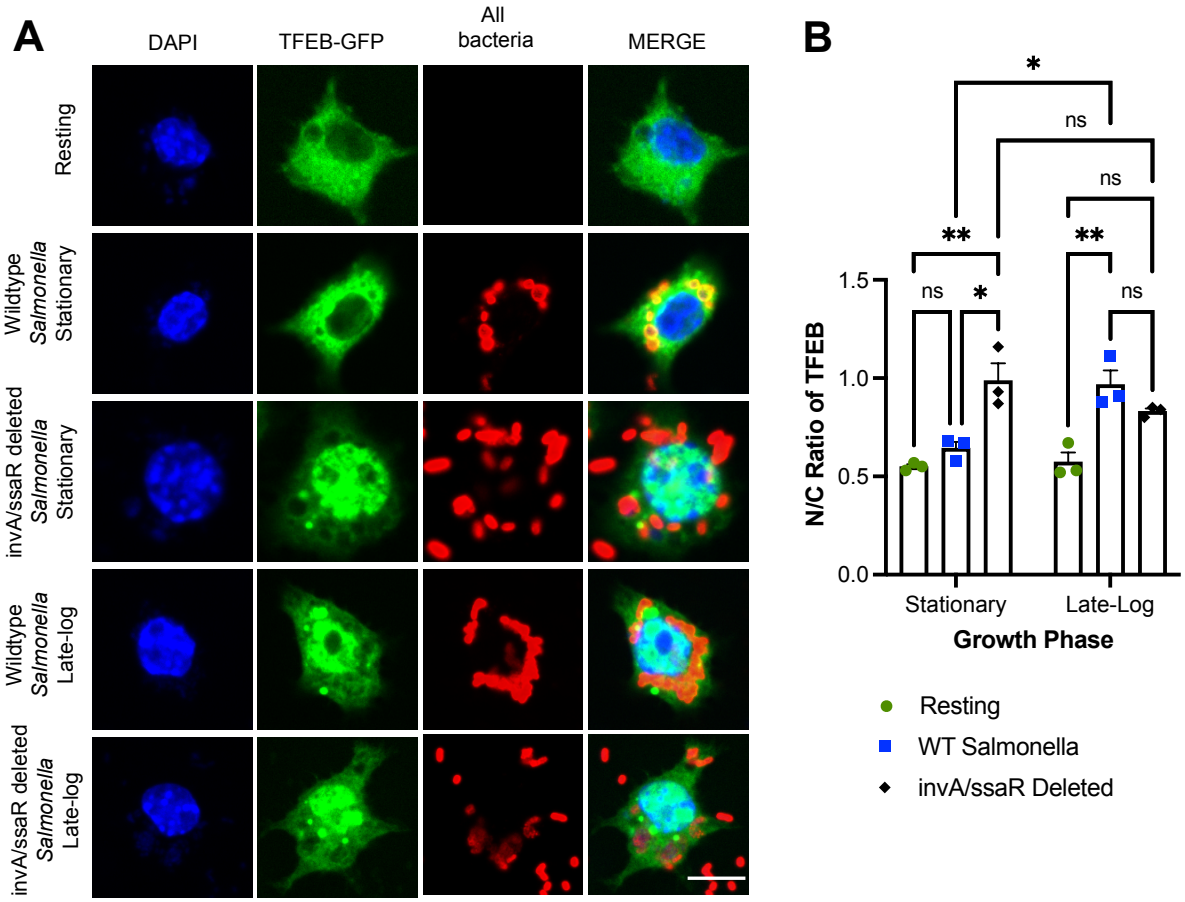

A
DAPI
TFEB-GFP
All bacteria
MERGE
Resting
Wildtype Salmonella Stationary
invA/ssaR deleted Salmonella Stationary
Wildtype Salmonella Late-log
invA/ssaR deleted Salmonella Late-log
B
N/C Ratio of TFEB
1.5
1.0
0.5
0.0
Stationary
Late-Log
Growth Phase
ns
*
**
Resting
WT Salmonella
invA/ssaR Deleted

Figure 5

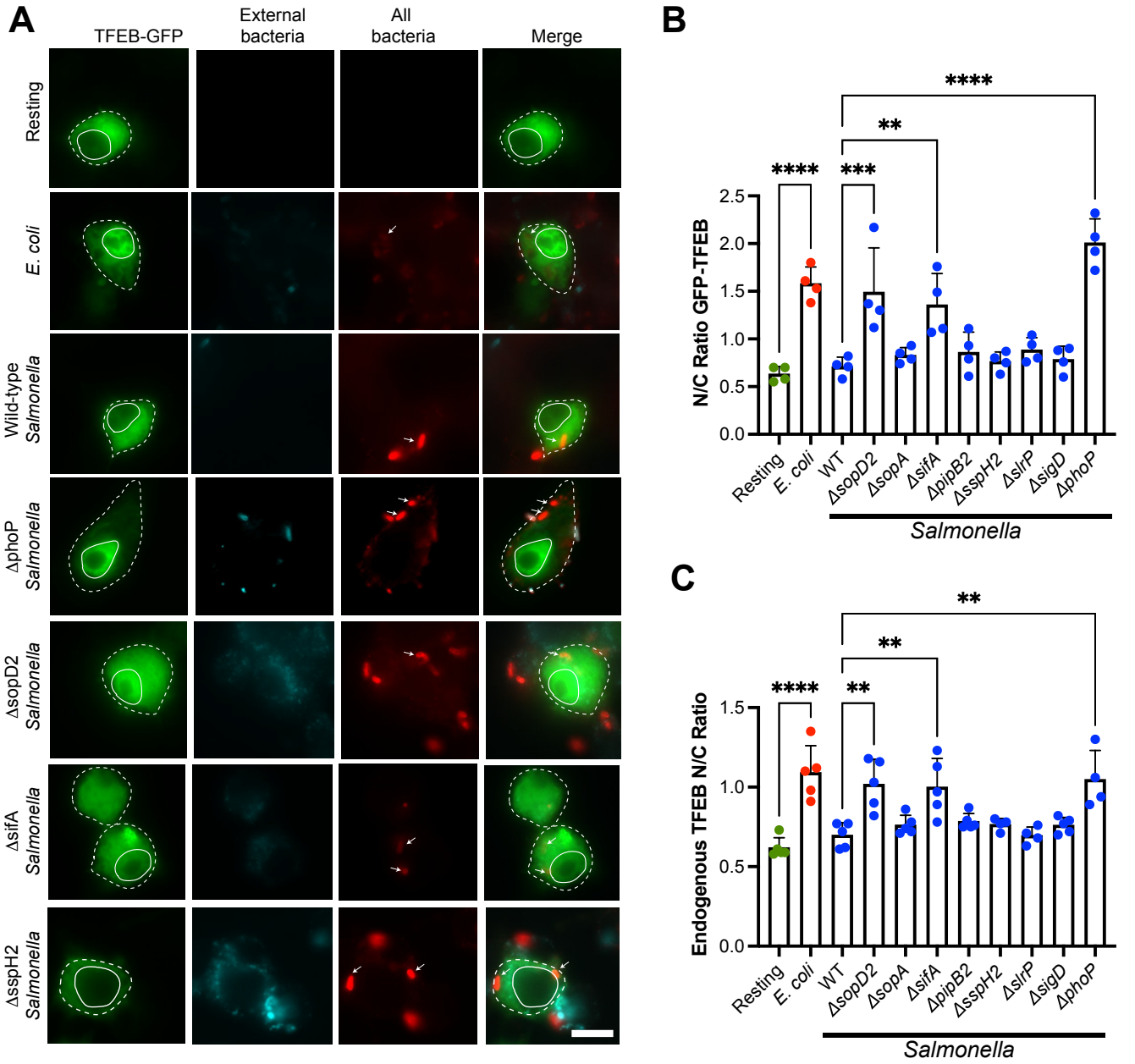

A
TFEB-GFP
External bacteria
All bacteria
Merge
Resting
E. coli
Wild-type Salmonella
ΔphoP Salmonella
ΔsopD2 Salmonella
ΔsifA Salmonella
ΔsspH2 Salmonella
B
N/C Ratio GFP-TFEB
2.5
2.0
1.5
1.0
0.5
0.0
****
***
**
****
Resting
E. coli
WT
ΔsopD2
ΔsopA
ΔsifA
ΔpipB2
ΔsspH2
ΔslrP
ΔsigD
ΔphoP
Salmonella
C
Endogenous TFEB N/C Ratio
1.5
1.0
0.5
0.0
****
**
**
**
Resting
E. coli
WT
ΔsopD2
ΔsopA
ΔsifA
ΔpipB2
ΔsspH2
ΔslrP
ΔsigD
ΔphoP
Salmonella

Figure 6

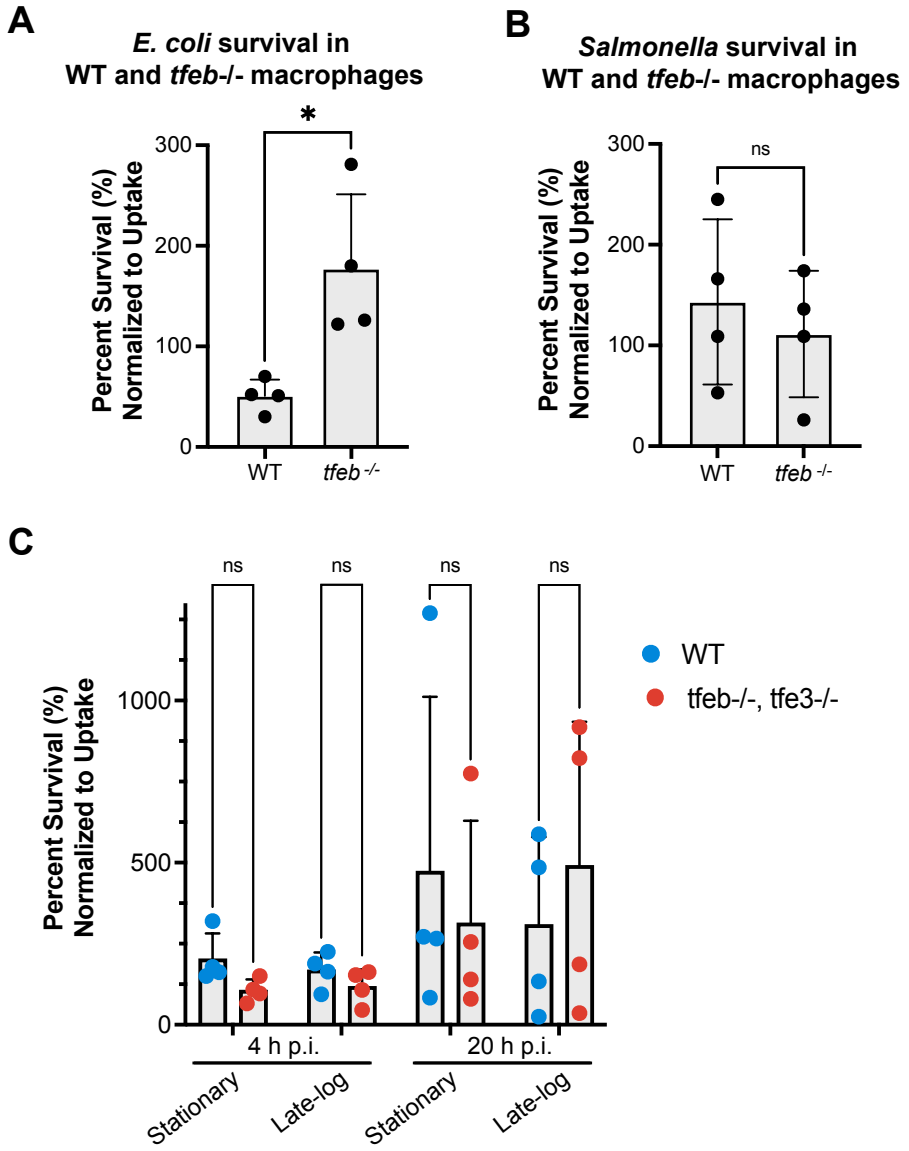
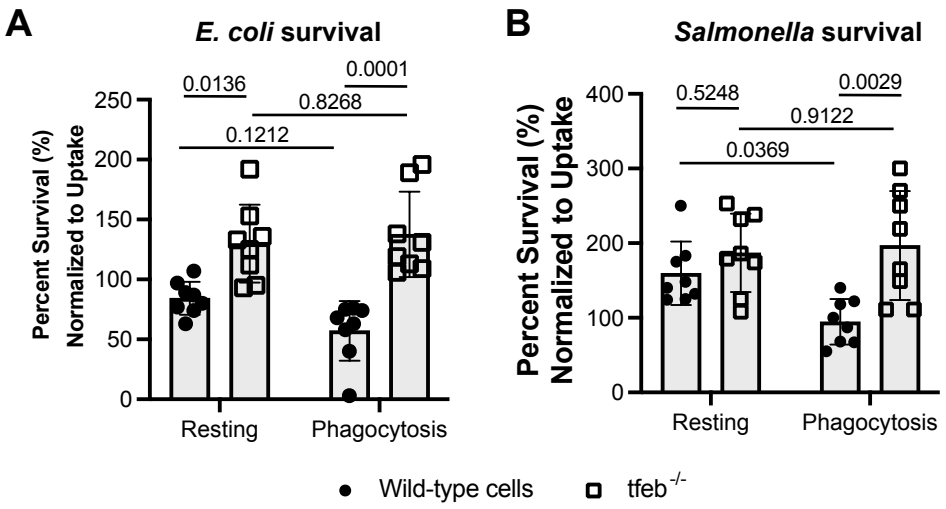

A
E. coli survival in
WT and tfeb-/- macrophages
*
Percent Survival (%)
Normalized to Uptake
300
200
100
0
WT
tfeb -/-
B
Salmonella survival in
WT and tfeb-/- macrophages
ns
Percent Survival (%)
Normalized to Uptake
300
200
100
0
WT
tfeb -/-
C
ns
ns
ns
ns
Percent Survival (%)
Normalized to Uptake
1000
500
0
WT
tfeb-/-, tfe3-/-
4 h p.i.
20 h p.i.
Stationary
Late-log
Stationary
Late-log

Figure 7


A
E. coli survival
Percent Survival (%)
Normalized to Uptake
250
200
150
100
50
0
0.0136
0.0001
0.8268
0.1212
Resting
Phagocytosis
B
Salmonella survival
Percent Survival (%)
Normalized to Uptake
400
300
200
100
0
0.5248
0.0029
0.9122
0.0369
Resting
Phagocytosis
Wild-type cells
tfeb -/-

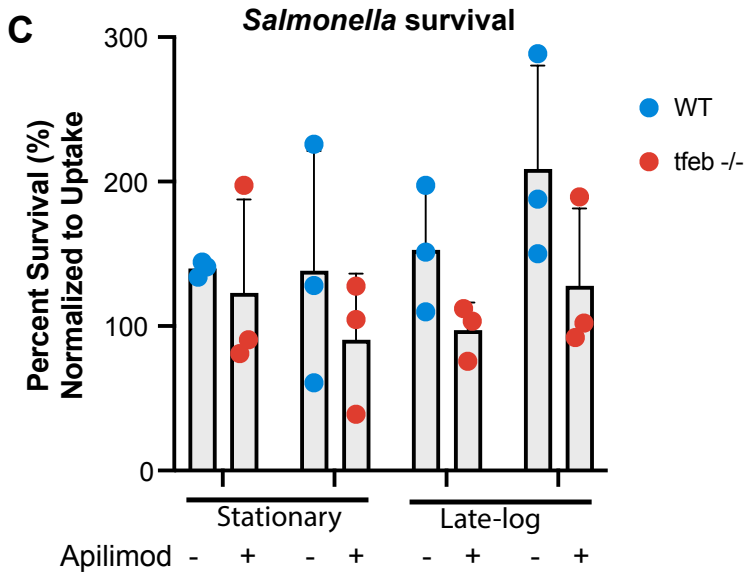

C
Salmonella survival
Percent Survival (%)
Normalized to Uptake
300
200
100
0
WT
tfeb -/-
Stationary
Late-log
Apilimod - + - + - + - +

Figure 8