Single-cell analyses reveal phosphate availability as critical factor for nutrition of *Salmonella enterica* within mammalian host cells

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**Abstract**

*Salmonella enterica* serovar Typhimurium (STM) is an invasive, facultative intracellular pathogen and acquisition of nutrients from host cells is essential for survival and proliferation of intracellular STM. The nutritional environment of intracellular STM is only partially understood. We deploy bacteria harbouring reporter plasmids to interrogate the environmental cues acting on intracellular STM, and flow cytometry allows analyses on level of single STM. Phosphorus is a macro-element for cellular life, and in STM inorganic phosphate (P\(_i\)) homeostasis is mediated by the two-component regulatory system PhoBR, resulting in expression of the high affinity phosphate transporter *pstSCAB-phoU*. Using fluorescent protein reporters, we investigated P\(_i\) availability for intracellular STM at single-cell level over time. We observed that P\(_i\) concentration in the *Salmonella*-containing vacuole (SCV) is limiting and activates the promoter of *pstSCAB-phoU* encoding a high affinity phosphate uptake system. Correlation between reporter activation by STM in defined media and in host cells indicates P\(_i\) concentration less 10 \(\mu\)M within the SCV. STM proliferating within the SCV experience increasing P\(_i\) limitations. Activity of the *Salmonella* pathogenicity island 2 (SPI2)-encoded type III secretion system (T3SS) is crucial for efficient intracellular proliferation, and SPI2-T3SS-mediated endosomal remodelling also relieves P\(_i\) limitation. STM that are released from SCV to enter the cytosol of epithelial cells did not indicate P\(_i\) limitations. Addition of P\(_i\) to culture media of infected cells partially relieved P\(_i\) limitations within the SCV, as did inhibition of intracellular proliferation. We conclude that availability of P\(_i\) is critical for intracellular lifestyle of STM, and P\(_i\) acquisition is maintained by multiple mechanisms. Our work demonstrates the use of bacterial pathogens as sensitive single-cell reporters for their environment in host cell or host organisms.

**Take Away**

- *Salmonella* strains were engineered to report their intracellular niche and the availability of inorganic phosphate (P\(_i\)) on level of single intracellular bacteria
• Within the *Salmonella*-containing vacuole (SCV), $P_i$ is limited and limitation increases with bacterial proliferation
• *Salmonella* located in host cell cytosol are not limited in $P_i$ availability
• Remodelling of the host cell endosomal system mediated by T3SS-2 reliefs $P_i$ limitation in the SCV

**KEYWORDS**
intracellular pathogen, invasion, pathogen-containing vacuole, reporter techniques, single-cell analyses, type III secretion system

1 | INTRODUCTION

Microbial pathogens have to adapt to the specific nutritional landscape provided by the host in order to survive and to proliferate. For intracellular bacteria, nutritional conditions within host cells may vary depending on the specific subcellular niche inhabited by the pathogen, the antimicrobial activity of the host cell and the pathogen burden. Given the current antibiotics crisis due to increasing resistance and lack of novel antimicrobial compounds, bacterial nutrition within the host may provide new targets for non-antibiotic strategies to combat infectious diseases. Understanding bacteria nutrition within the host and/or host cells is prerequisite for such approaches, and we set out to investigate the nutritional requirements of *Salmonella enterica*, a food-borne pathogen causing diseases ranging from gastroenteritis to systemic typhoid fever.

*Salmonella enterica* serovar Typhimurium (STM) is an invasive, facultative intracellular pathogen that resides in a specialised membrane-bound compartment termed *Salmonella*-containing vacuole (SCV) (Haraga, Ohlson, & Miller, 2008). Essential for survival and proliferation in the SCV is a type III secretion system (T3SS) encoded by *Salmonella* pathogenicity island 2 (SPI2) (Hensel et al., 1998). The SPI2-T3SS and the translocated effector proteins maintain the SCV integrity and mediate formation of specific tubular membrane compartments, called *Salmonella*-induced filaments (SIFs) (Brumell, Tang, Mills, & Finlay, 2001; Drecktrah, Knodler, Howe, & Steele-Mortimer, 2007; Figueira & Holden, 2012; Rajashekar, Liebl, Seitz, & Hensel, 2008). Formation of an extensive SIF network by STM was suggested to provide bacterial nutrition and enabling proliferation (Liss et al., 2017). Mutant strains defective in SPI2-T3SS are attenuated in systemic virulence and show reduced intracellular replication (Hensel et al., 1995; Hensel et al., 1998). An important SPI2-T3SS effector protein is SifA, as lack of this effector leads to defects in remodelling of the host cell endosomal system and maintenance of intact SCV (Beuzon et al., 2000; Stein, Leung, Zwick, Garcia-del Portillo, & Finlay, 1996). Deletion of sifA attenuates virulence, replication in macrophages (Beuzon et al., 2000; Brumell, Rosenberger, Gotto, Marcus, & Finlay, 2001) and results in hyper-replication of STM in the nutrient-rich cytosol of epithelial cells (Knodler, 2015).

Intracellular STM shows a remarkable heterogeneity regarding proliferation and response to environmental cues (Bumann & Cunrath, 2017; Helaine et al., 2014). Subpopulations, present in SCV or cytosol, are actively proliferating or dormant (Helaine et al., 2014; Knodler, 2015). For analyses of the intracellular environment of STM and responses to intracellular cues, this heterogeneity restricts the application of population-wide approaches such as transcriptomics or proteomics. Such analyses on the level of single intracellular bacteria are still pending. However, bacteria can be used as sensitive tools to interrogate the response to specific environmental cues, and fluorescent protein reporters in combination with flow cytometry (FC) allows efficient single-cell analyses of large and heterogeneous populations.

We set out to systematically analyse the availability of nutrients for intracellular STM in mammalian host cells on the single-cell level. Here, we focus on the role of phosphate for intracellular STM. Phosphorus is one of the most abundant elements in living organisms, found in membrane lipids, nucleic acids, proteins or carbohydrates, and involved in many enzymatic reactions. In bacteria, inorganic phosphate ($P_i$) homeostasis is mediated by the two-component regulatory system PhoBR. The membrane-bound sensor PhoR reacts to low $P_i$ and activates PhoB, a transcriptional regulator that promotes the expression of many genes involved in $P_i$ transport (Hsieh & Wanner, 2010). This includes transcription of the *pstSCAB-phoU* operon encoding a high affinity phosphate transporter. PstS is a periplasmic protein that binds $P_i$ with high affinity. PstC, PstA and PstB form an ABC transporter with PstC and PstA being inner membrane channel proteins and PstB an ATP-dependent permease component. PhoU is essential for the suppression of the Pho regulon under a high phosphate condition (Wanner, 1996). The exact mechanism of action of PhoU is not yet understood (Gardner, Johns, Tanner, & McCleary, 2014). Several studies link the $P_i$ homeostasis with virulence of bacteria as well as for STM (Lamarche, Wanner, Crepin, & Harel, 2008).

Expression of SPI2-T3SS genes is activated within the SCV of host cells, and the proper spatiotemporal control ensures activity of virulence factors in the required phase of host–pathogen interaction. The intracellular habitat of STM is a complex environment with a multiplicity of stressors and nutritional limitations, thus specific stimuli are difficult to dissect. In contrast, systematic analyses of expression under defined in vitro conditions identified a slightly acidic pH, $Mg^{2+}$ limitation and $P_i$ starvation as stimuli inducing SPI2 gene expression (Deiwick & Hensel, 1999). The SPI2-T3SS-dependent endosomal...
remodelling and induction of a complex SCV/SIF continuum allows intracellular STM to relieve nutritional limitations (Liss et al., 2017). Accordingly, we anticipate a link between nutritional limitation in the intracellular environment, control of SPI2 gene expression, and SPI2-T3SS-mediated manipulation of the host cell.

Phosphate uptake and the regulation of $P_i$ homeostasis by pstSCAB-phoU were suggested to be essential for the pathogenicity of STM. Various studies indicated upregulation during infection (Garcia-del Portillo, Foster, Maguire, & Finlay, 1992; Hautefort et al., 2008). Thus, we investigated $P_i$ availability for STM at a single cell level over time within distinct intracellular habitats of different host cells. Therefore, we generated reporters and measured the presence and limitation of $P_i$ during infection of host cells by STM.

## RESULTS

### 2.1 The pstSCAB phoU-encoded phosphate uptake system is required for intracellular proliferation of STM

We investigated the relevance of $P_i$ uptake for the intracellular lifestyle of STM. Various genes involved in regulation of $P_i$ homeostasis were deleted, and intracellular proliferation of the resulting mutant strains in comparison to STM WT was determined using competitive index (CI) assays (Figure 1). The SPI2-T3SS-deficient strain ΔssaV served as negative control for intracellular replication (Hensel et al., 1998) and showed a low CI of 0.1, both in HeLa cells and

![Figure 1](image.png)  
**Figure 1** Role of phosphate transport systems for intracellular proliferation of *Salmonella enterica* serovar Typhimurium (STM). Competitive index (CI) assays for proliferation of STM strains in HeLa cells (a), or RAW264.7 macrophages (b). Cells were co-infected with STM WT in combination with mutant strains defective in ssaV, pstSCAB, phoB, phoR, phoU, or phoE at a total multiplicity of infection (MOI) of 1. Intracellular proliferation was determined as the ratio of CFU at 16 hr p.i. to CFU at 1 hr p.i. and CI of replication of WT versus mutant strain was calculated. A CI of 1.0 (dashed line) indicates identical intracellular proliferation of both strains, a CI lower than 1.0 indicates attenuated proliferation of the mutant strain. Shown are mean values and standard deviations (SDs) of three biological replicates, each consisting of three technical replicates. Statistical analyses were performed by one-way analysis of variance (ANOVA) in comparison to STM WT and are expressed as n.s., not significant; *, $p < .05$; **, $p < .01$; ***, $p < .001$. (c) Growth of STM WT and various mutant strains in LB medium. LB medium was inoculated with overnight cultures 1:100 and growth continued at 37°C with aeration by agitation at 180 rpm. Optical density at 600 nm (OD$_{600}$) was recorded at indicated time points.
RAW264.7 macrophages. The pstSCAB operon encodes for an ABC transporter, mediating the phosphate uptake (Lamarche et al., 2008). Deletion of this transporter resulted in a CI of 0.4 in both host cell types. This high-affinity phosphate transporter is regulated by the phoBR two-component system (Lamarche et al., 2008). Deletion of phoB or phoR attenuated intracellular replication. In HeLa cells, CI of 0.2 or 0.3 were determined for ΔphoB or ΔphoR strains, respectively, and within RAW264.7 macrophages as host cells, CI of 0.5 or 0.6 for ΔphoB or ΔphoR strains, respectively, were determined.

A particularly interesting phenotype was observed by deletion of phoU. While in RAW264.7 macrophages, phagocytosis was not affected, replication was impaired with a CI of 0.1. Growth kinetics of all mutant strains in LB broth were comparable to STM WT, and only slightly lower growth yield was observed for ΔphoU and ΔphoSCAB (line 145). STM ΔphoU formed smaller colonies on LB agar compared to STM WT, and ΔphoU and ΔphoSCAB failed to grow in PCN medium limited in P_i. PhoU plays a key role in phosphate homeostasis by repressing the pho regulon at high phosphate levels (Gardner et al., 2014). SPI1-T3SS-mediated invasion of HeLa cells was below the detection limit. This resulted in a highly reduced CI for ΔphoU versus WT in HeLa cells. In contrast, lack of PhoE, an outer membrane protein for uptake of inorganic phosphates (Spierings, Elders, van Lith, Hofstra, & Tommassen, 1992), did not affect intracellular replication as CI of 0.7 and 1 in HeLa cells and RAW264.7 macrophages, respectively, were determined. Therefore, we conclude that proper P_i homeostasis plays a central role for intracellular replication and virulence of STM.

2.2 Generation and validation of reporter strains for measuring inorganic phosphate levels

Phosphate uptake by the transporter PstSCAB, as well as regulation of this system by PhoBR, proved to be of critical importance for intracellular proliferation. This prompted us to investigate availability of P_i for intracellular STM in more detail. Intracellular STM diversity into various subpopulations, that is, bacteria being located vacuolar or cytosolic, proliferating slowly or rapidly, or forming non-replicating persisters. This heterogeneity demands analyses on the level of single cells of intracellular STM. In order to determine the intracellular P_i limitation more precisely, we generated a dual fluorescence reporter with constitutive expression of DsRed, and sfGFP under control of the promoter of pstS. PstS is a periplasmic protein that binds P_i with high affinity and is known to be upregulated intracellularly in P_i-poor environments (Hautefort et al., 2008).

STM was subcultured for 3.5 or 24 hr in media with various concentrations of P_i and expression of P_pstS::sfGFP was determined by FC. Induction of P_pstS::sfGFP was detected after growth in media containing P_i concentrations of 1 mM or lower, and sfGFP intensity increased as P_i concentration decreased (Figure 2a,b). Comparison of P_pstS::sfGFP intensities between cells of 3.5 and 24 hr subcultures revealed higher intensity in late cultures (Figure 2c). This would be in line with the consumption of the P_i pool during STM growth resulting in P_i limitation, and/or accumulation of the reporter.

To address effects of accumulation of sfGFP on the reporter read-out, in vitro cultures and intracellular STM were analysed. STM was analysed during growth in PCN medium with maltose to achieve reduced growth rate (Schulte, Olschewski, & Hensel, 2021). For constitutive sfGFP expression, a rather constant sfGFP fluorescence was determined over 10 hr of culture (Figure S1a). In contrast, the P_pstS::sfGFP fusion after induction by AHT resulted in highly increased levels of sfGFP fluorescence. RAW264.7 macrophages were infected with STM WT, and the STM ΔssaV strain highly reduced in intracellular proliferation, both constitutively expressing sfGFP. Analyses of single STM recovered at various time points after infection indicated only minor fluctuations in levels of sfGFP fluorescence (Figure S1b). We conclude that accumulation, dilution by bacterial division, or degradation of sfGFP in intracellular STM have only minor effects on the read-out by FC analyses.

To precisely determine the P_i concentration critical for induction of phosphate reporter P_pstS::sfGFP, we performed phosphate shock experiments (Figure 2d,e). A logarithmic culture was used to inoculate media with various P_i concentrations, and incubation was continued for 1 hr. This regime minimised the change in P_i levels due to consumption by bacterial growth, and we detected induction of the phosphate reporter at concentrations below 100 μM. Decreasing P_i concentrations correlated with increasing sfGFP intensities, and sfGFP intensity of 10^9 relative fluorescence intensity (RFI) indicated a concentration of less than 10 μM P_i (Figure 2f). The effect of mutations in phosphate transporters or regulatory systems on the expression of the phosphate reporter was analysed (Figure S2). In PCN medium containing 25 mM P_i, here referred to as PCN (25), no induction of P_pstS::sfGFP was detected. However, a ΔphoU strain demonstrated strong induction of P_pstS::sfGFP in PCN (0.4), as well as in PCN (25) despite sufficient phosphate availability. Interestingly, only a subpopulation of STM ΔphoU showed induction of the reporter. Only moderate growth was observed for this mutant strain, indicating missing repression of pstS in the ΔphoU background. While P_pstS::sfGFP in STM WT showed increased intensity from PCN (25) to PCN (0.4), ΔpstSA::sfGFP, ΔphoB and ΔphoR strains did not respond to low phosphate concentrations. Therefore, no regulation of the high-affinity P_i uptake system was possible in these mutant strains.

One of the multiple environmental cues acting on intracellular STM is the acidic pH within the SCV. We tested response of the P_pstS::sfGFP reporter after shift of growing STM cultures to media with high, low, or limiting P_i concentrations at neutral or various acidic pHs (Figure 2g). Reporter induction was predominantly a function of P_i limitation, but we also observed limited induction after growth in media with 25 mM P_i and pH of 5.3 or lower. Acid growth media may indirectly lead to Pi starvation due to less efficient uptake by transporters, despite sufficiently high P_i levels in medium.

2.3 Heterogeneity of the intracellular bacterial populations

After validation of the phosphate reporter, we deployed the system in infection assays of HeLa cells and RAW264.7 macrophages. Due to
the different sfGFP intensities, we detected heterogeneous STM populations in HeLa cells and RAW264.7 macrophages. After infection of HeLa cells, three subpopulations were detected at 8 hr post infection (h.p.i.) (Figures 3a,c and S3). The classification of P$_i$ availability was based on the intensities determined by in vitro experiments. A very small population P1 was non-induced, induction of large population P2 (66%) with sfGFP intensities correlating to P$_i$ availability above 10 μM under in vitro conditions, and a smaller population P3 (27%) with sfGFP intensities correlating to P$_i$ limitation below 10 μM in vitro. At 16 hr p.i., P3 of STM WT (32%) and ΔssAV strains (21%) increased, whereas the ΔsifA strain remained predominantly in P2 (65%). Since a sifA mutant strain has access to cytosolic components due to compromised SCV integrity (Beuzon et al., 2000), we assumed a higher P$_i$ concentration in the host cell cytosol. Therefore, we deduced for HeLa cells P$_i$ limitation lower than 10 μM in the SCV, and P$_i$ availability higher than 10 μM in the cytosol. In RAW264.7 macrophages, conditions were slightly different (Figure 3b,d). At 8 hr p.i., we distinguished between induced (55% of STM WT and 52% of ΔssAV)
FIGURE 3 Determination of phosphate availability for intracellular *Salmonella enterica* serovar Typhimurium (STM) in HeLa cells or RAW264.7 macrophages. HeLa cells (a, c) or RAW264.7 macrophages (b, d) were infected at MOI 5 with STM WT (red), ΔssaV (orange) or ΔsifA (green) strains as indicated, each containing the phosphate reporter p5007 (P<sub>EM7</sub>::DsRed P<sub>pstS</sub>::sfGFP). (a, b) Host cells were lysed 8 hr or 16 hr p.i., released STM were fixed and subjected to FC to quantify sfGFP intensities of P<sub>pstS</sub>-positive STM. Three subpopulations of intracellular STM were distinguished based on P<sub>pstS</sub>::sfGFP intensity: P<sub>1</sub>, P<sub>pstS</sub>-negative; P<sub>2</sub>, P<sub>pstS</sub>-positive with sfGFP X-means equivalent to >10 μM P<sub>i</sub> in vitro; and P<sub>3</sub>, P<sub>pstS</sub>-positive with sfGFP X-means equivalent to <10 μM P<sub>i</sub> in vitro. Representative quantification of population size and X-means of sfGFP intensities of subpopulations P1, P2 and P3 for STM WT, ΔssaV and ΔsifA strains in HeLa cells (c), and STM WT and ΔssaV strains in RAW264.7 macrophages (d) at 8 hr and 16 hr p.i. P2 for STM in RAW264.7 was only determined at 16 hr p.i. Mean values and SDs of P<sub>pstS</sub>-positive bacterial subpopulations from triplicates of a representative experiment are shown. Statistical analyses were performed by one-way analysis of variance (ANOVA) for mutant strains compared to STM WT, or between time points, and are expressed as: n.s., not significant; *, p < .05; **, p < .01; ***, p < .001. HeLa cells (e) or RAW264.7 macrophages (f) were infected with STM WT [p5007] and pulse-chased with dextran-AlexaFluor 647 (blue) for labelling of the endosomal system. Live cell imaging was performed 16 hr p.i., and overview images show heterogeneous sfGFP (green) intensities of STM. Representative infected cells indicate red and green fluorescence signal for STM. Sections in the dashed box are shown magnified below. Scale bars, 10 μm.
and non-induced populations, but all induced STM reported Pi limitation corresponding to less than 10 μM Pi in vitro. While the ΔssaV strain showed 70% P_{pstS}-positive bacteria at 16 hr p.i., the STM WT showed 88% P_{pstS}-positive bacteria at 16 hr p.i., that can be distinguish in 73% of STM with high sfGFP intensities (P3), 15% of STM in P2 with lower DsRed and sfGFP intensity, indicative for concentrations of more than 10 μM Pi.

We also observed variable fluorescence levels of constitutively expressed DsRed (Figure S3b,d). This heterogeneity is due to fluorescent protein dilution during STM replication and the rather long

![Figure 4](image)

**Figure 4** Kinetics of phosphate availability for *Salmonella enterica* serovar Typhimurium (STM) in HeLa cells and RAW264.7 macrophages. HeLa cells (a–d) or RAW264.7 macrophages (e–h) were infected at MOI 5 with STM WT (a, e) or ΔssaV (b, f) strains each harbouring p5007. Host cells were lysed at various time points p.i. as indicated, released bacteria were fixed and subjected to FC to quantify the population of P_{pstS}-positive STM (c, g), and X-means of sfGFP intensities (d, h) for STM WT (red lines) and ΔssaV (black lines) strains in HeLa cells or RAW264.7 macrophages. Mean values and SDs from triplicates of a representative experiment are shown. Statistical analyses are indicated as for Figure 3.
maturation time of DsRed (Schulte et al., 2021). Thus, DsRed fluorescence levels can be deployed as proxy for the level of proliferation of intracellular STM populations.

In overview images, STM WT indicated quite evenly distributed sfGFP intensities in HeLa cells (Figure 3e). However, in RAW264.7 macrophages, the heterogeneity of DsRed and sfGFP intensities was more visible (Figure 3f). The ΔssaV strain (Figure S4) also showed a relatively uniform distribution of sfGFP intensity in HeLa cells. In RAW264.7 macrophages, we distinguished between non-induced and induced bacteria, whereas the sfGFP intensity did not exhibit major differences.

We tested another phosphate reporter using the promoter of apeE, which codes for an outer membrane esterase. Expression of apeE was induced by phosphate limitation, and required the phoBR phosphate regulatory system (Conlin, Tan, Hu, & Segar, 2001). This reporter led to comparable results. An in vitro induction was measured at concentrations below 1 mM in a 3.5 hr subculture and the sfGFP intensity increased with decreasing Pi values (Figure S5a). The same subpopulations within HeLa cells (Figure S5b–d) and RAW264.7 macrophages (Figure S5e,f) were observed, but generally, the sfGFP intensity was lower.

Next, we analysed induction of the phosphate reporter over the time course of infection. By comparing the reporter induction of STM WT and ΔssaV strains in HeLa cells, we determined similar induction up to 10 hr p.i., as percentage of sfGFP-positive STM and intensity of single bacteria increased over time (Figure 4a,b). PpstS-positive bacteria increased from 5% at 2 hr p.i. to 85% for STM WT, and to 62% for the ΔssaV strain at 10 hr p.i. At later time points, the ΔssaV strain decreased to 34% PpstS-induced cells, while STM WT continued to increase to 89% (Figure 4c,d). This likely reflects the higher replication and metabolic activity of STM WT compared to STM ΔssaV (Liss et al., 2017). A steady increase of PpstS-positive cells was observed for STM isolated from RAW264.7 macrophages over time (Figure 4e,f). With an increase from 60% at 2 hr p.i. to 93% and 85% for STM WT and ΔssaV strains, respectively, at 24 hr p.i. The ΔssaV strain exhibited slight fluctuations, but over time between 60 and 85% of the bacteria were induced. In contrast, STM WT continued to increase to 89% (Figure 4c,d). This likely reflects the higher replication and metabolic activity of STM WT compared to STM ΔssaV (Liss et al., 2017).

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Due to the different populations observed for STM WT and ΔsifA strains in HeLa cells, we already assumed that access to cytosolic phosphate is limited for ΔsifA.
components led to increased phosphate availability. The use of a dual reporter with \( \text{P}_{\text{uhpT}}::\text{DsRed} \) and \( \text{P}_{\text{pstS}}::\text{sfGFP} \) provided information on phosphate concentration over time in distinct habitats in HeLa cells (Figures 5a and S6a). \( \text{P}_{\text{uhpT}} \) induction indicates cytosolic presence of the bacteria (Röder & Hensel, 2020). After invasion, most bacteria are still in a vacuole and therefore \( \text{P}_{\text{uhpT}}^{-}\)negative. However, the population of \( \text{P}_{\text{uhpT}}^{+} \)-positive bacteria increased in the early intracellular phase, that is, by replication in the cytosol. At late time points (\( \geq 10 \) hr p.i.) change in intracellular population occurred, with an increased vacuolar population, and declined of the cytosolic population. This is likely due to host cell death induced by the high burden of cytosolic STM. For STM WT, the \( \text{P}_{\text{uhpT}}^{-} \)-negative population increased from 54% at 10 hr p.i. to 81% at 24 hr p.i. The sfGFP intensity increased, indicating decreased availability of \( \text{Pi} \) in both habitats (Figure 5b,c). Similar results were obtained with a reporter using the correlation between \( \text{P}_{\text{ssAG}}::\text{DsRed} \) and \( \text{P}_{\text{pstS}}::\text{sfGFP} \) (Figures 5d and S6b). The ssAG gene is located in SPI2, encodes the needle subunit of the T3SS and was used to analyse expression of genes for the SPI2-T3SS (Lim, Kim, Choi, Lee, & Ryu, 2006). SPI2 induction is known for SCV-bound bacteria, but not for cytosolic bacteria (Knodler et al., 2010). Accordingly, vacuolar replication also started 10 hr p.i., as the \( \text{P}_{\text{ssAG}}^{-} \)-positive population increased from 39 to 75% at 24 hr p.i., but unlike the \( \text{P}_{\text{uhpT}}^{-} \)-positive population the intensity of the \( \text{P}_{\text{ssAG}}^{-} \)-negative population did not increase (Figure 5e,f). This may be attributed to the continuously rupture of the SCV by STM (Röder & Hensel, 2020), that is, STM escaped from a Pi-restricted SCV into the cytosol of host cells, and thus erroneously enhanced the overall sfGFP intensity. Equally, cytosolic bacteria (\( \text{P}_{\text{ssAG}}^{-} \)-negative) are unlikely to become vacuolar and exhibited continuously low sfGFP intensity. In RAW264.7 macrophages, however, all bacteria were \( \text{P}_{\text{ssAG}}^{-} \)-positive (vacuolar), and only few \( \text{P}_{\text{uhpT}}^{-} \)-positive bacteria (cytosolic) were detected (Figure S7). Therefore, STM mainly resides inside the SCV of RAW264.7 macrophages. The proportion of \( \text{P}_{\text{ssAG}}^{-} \)-sfGFP-induced STM increased over time, as well as fluorescence intensities (Figure S8). Induction of the reporter

**FIGURE 6** Phosphate limitation for *Salmonella enterica* serovar Typhimurium (STM) in IFN-\( \gamma \)-activated RAW264.7 macrophages and monocyte-derived human macrophages. (a, b) RAW264.7 macrophages were cultured for 24 hr in medium without (blue) or with (red) 5 ng \( \times \) ml\(^{-1} \) IFN-\( \gamma \), and subsequently infected with STM WT [p5007] (\( \text{P}_{\text{EM7}}::\text{DsRed} \text{P}_{\text{pstS}}::\text{sfGFP} \)) at MOI 5. (c, d) Human peripheral blood macrophages (red, orange) or RAW264.7 macrophages (light green, dark green) were infected with STM WT [p5007] at MOI 25. Host cells were lysed as indicated at 8 hr p.i. or 16 hr p.i., released STM were fixed and subjected to FC to quantify \( \text{P}_{\text{pstS}}^{+} \)-positive STM and X-means of sfGFP intensities for \( \text{P}_{\text{pstS}}^{+} \)-positive STM (b, d). Data of representative experiments are shown, (a) for STM WT [p5007] in resting RAW264.7 (red) and activated RAW264.7 (blue), (c) for STM WT [p5007] in RAW264.7 (light green, dark green) or human macrophages (red, orange) Mean values and SDs from triplicates of a representative experiment are shown. Statistical analyses are indicated as for Figure 3.
occurred faster in RAW264.7 macrophages than in HeLa cells, indicating a more rapid exposure to harsh phagosomal conditions.

We also analysed the phosphate reporter in activated RAW264.7 macrophages and primary human macrophages, which provided a more restrictive environment for bacteria resulting in an attenuated replication (Lathrop et al., 2018; Rosenberger & Finlay, 2002). If RAW264.7 macrophages activated with interferon-γ (IFN-γ) were infected, we obtained mixed populations for STM WT or ΔssAV strains (Figure 6a). The induced bacterial population was smaller (70 and 95% in activated and non-activated RAW264.7, respectively) (Figure 6b).

In primary macrophages isolated from human peripheral blood, we determined an even smaller induced population (32 and 26% at 8 and 16 hr p.i., respectively), but it is known that proliferation for STM in these cells is highly restricted. Since only about 30% of the bacteria were induced, the rest of the population may be dead, or have entered persistent state (Figure 6c,d). Although the population was much more widely distributed in RAW264.7 macrophages, a similar average sfGFP intensity was measured at 16 hr p.i., whereas at 8 hr p.i. the sfGFP intensity was much lower in human macrophages. However, in activated RAW264.7 macrophages, as well as in primary human macrophages, induction and thus phosphate limitation was detected.

### 2.4 Reporters with destabilised sfGFP allow to measure rapid changes in phosphate availability

The sfGFP has a rather long half-life (Pedelacq, Cabantous, Tran, Terwilliger, & Waldo, 2006) that limits analyses of STM responses to changing environments. To modify the reporter system for analyses of rapid changes in phosphate concentration, the LVA tag was fused to sfGFP resulting in increased degradation (Andersen et al., 1998). Analysis of induction in vitro in a phosphate shock experiment showed that slightly delayed induction occurred and signals were measured at a concentration of 10 μM Pi, but not at 100 μM (Figure 7a). At a concentration of 10 μM Pi, an intensity of 10^3 RFI was determined for sfGFP-LVA, compared to 10^4 RFI for the parental reporter, probably as consequence of the continuous degradation of sfGFP (Figure 7b). Therefore, we compared the stability of sfGFP and sfGFP-LVA by growing subcultures in PCN (0.01) media for 2 hr. Then, protein synthesis was stopped by chloramphenicol, and incubation continued. The sfGFP intensities of the cultures were determined at various time points after block of synthesis (Figure 7c,d), and we observed that the LVA tag caused a continuous decline of sfGFP intensity and no sfGFP signals were detectable after 60 min. For normal sfGFP, a constant signal was still detectable after 210 min.

We used this reporter in STM infection of HeLa cells and observed hardly any difference between P_pstScsfGFP and P_pstScsfGFP-LVA (Figure 7e,f). The percentages of induced bacteria were almost identical, while sfGFP intensity was lower if LVA was present. Nevertheless, the intensity of the destabilised sfGFP constantly increased, thus confirming a decrease in Pi concentration over time of intracellular proliferation. In RAW264.7 macrophages, sfGFP intensity dropped after 10 hr p.i. both for sfGFP without and with LVA tag (Figure 7g,h). With LVA labelling, however, not only the change in the intensity was detected, but also the proportion of P_pstSc-positive bacteria highly decreased. The overview images showed that especially in heavily loaded HeLa cells and RAW264.7 macrophages, no further sfGFP signals were visible for LVA-tagged version (Figure 7i,j).

### 2.5 Intracellular proliferation limits phosphate availability for STM

We found that STM WT generally exhibited stronger P_pstSc::sfGFP induction in HeLa cells than in RAW264.7 macrophages. Furthermore, the percentage of P_pstSc-induced bacteria was lower in activated RAW264.7 macrophages and in human macrophages compared to resting host cells, or in STM ΔssAV strain compared to STM WT. This might be explained by different composition of compartments in distinct cell types, or by different levels of STM proliferation affecting intracellular Pi levels due to Pi consumption. To investigate the influence of replication on Pi availability, we infected HeLa cells or RAW264.7 macrophages and added Cotrimoxazole to inhibit intracellular replication. In HeLa cells, we observed no changes at 8 hr p.i., but significantly lower sfGFP intensity at 16 hr p.i. indicating higher Pi availability (Figure 8a–c). Quantification of CFU of intracellular STM indicated lower bacterial counts in Cotrimoxazole-treated cells at 8 and 16 hr p.i. (Figure 8d). No significant increase in CFU was observed, showing that STM remained viable but restricted in proliferation. In RAW264.7 macrophages, both the percentage and the intensity were lower at 16 hr p.i. due to inhibition of bacterial replication (Figure 8e–g). Cotrimoxazole treatment also reduced CFU of STM in macrophages, but proliferation between 8 and 16 hr p.i. was not fully ablated (Figure 8h). However, Cotrimoxazole-inhibited STM in macrophages showed a lower frequency of sfGFP-positive cells at 16 hr p.i., with sfGFP intensities as low as at 8 hr p.i. We conclude that inhibition of intracellular proliferation relieves the limitation of Pi due to decreased consumption of the Pi pool available for intracellular STM.

Finally, we manipulated phosphate availability in host cells and determined the effects on intracellular STM. For this, (i) additional phosphate was added to the cell culture medium to increase Pi uptake (Candeal, Caldas, Guillen, Levi, & Sorribas, 2014), (ii) acidification of the SCV was inhibited by bafilomycin (Rathman, Sjaastad, & Falkow, 1996), or (iii) the proton gradient was uncoupled by protonophore CCCP (Candeal et al., 2014). We added Pi to the host cells 24 hr before infection, so that a higher Pi level was already present in the host cells. When we infected HeLa cells with STM WT, fewer bacteria showed induction of P_pstSc::sfGFP (Figure 9a–c). In infected RAW264.7 macrophages, additional Pi had no effect on P_pstSc::sfGFP expression by intracellular STM (Figure 9d–f). In contrast, the addition of bafilomycin and thus blocking the acidification of the SCV increased sfGFP intensities of STM in RAW264.7 macrophages.
but not for STM in HeLa cells. Uncoupling by CCCP resulted in slightly higher sfGFP intensity of STM in HeLa cells, but no changes were detected in RAW264.7 macrophages. In conclusion, the higher external $P_i$ levels affect $P_i$ availability of STM in HeLa cell, while neutralisation of the pH of SCV in RAW264.7 macrophages increased $P_i$ limitation, which may be a consequence of reduced transport of $P_i$ into the SCV lumen, and/or higher $P_i$ consumption by increased STM proliferation in a more permissive compartment.
DISCUSSION

3.1 Main phosphate transporter PstSCAB-PhoU is required for intracellular replication of STM

This study investigated nutrient availability for intracellular STM with focus on phosphate. Pi is essential for many important functions like DNA and RNA synthesis and energy metabolism. We developed and applied a sensitive quantitative approach to investigate, at a single cell level, the availability of Pi within the intracellular environments of STM. Our study demonstrates the importance of intracellular Pi homeostasis for virulence of STM. Deletions of \textit{phoB}, \textit{phoR}, \textit{phoU} or \textit{pstSCAB} encoding the ABC transporter led to a reduced replication of STM in macrophages and epithelial cells. Several previous studies on intracellular STM reported upregulation of genes such as \textit{pstSCAB} or \textit{phoBR} (Eriksson, Lucchini, Thompson, Rhen, & Hinton, 2003; Garcia-del Portillo et al., 1992; Hautefort et al., 2008). Deletion of \textit{pstSCAB} was recently reported to attenuate systemic virulence of STM in the murine infection model (Zhang et al., 2019), and our work extends these findings to further components of the phosphate metabolism. The reduced replication of a \textit{ΔphoB} strain in RAW264.7 macrophages and a reduced systemic virulence in mice was recently reported. Defect of PhoU leads to constitutive expression of the Pst transporter and non-physiological accumulation of Pi in the cytosol of STM, indicating that the proper phosphate homeostasis is of critical importance (Jiang et al., 2020). Defects in phosphate metabolism also impaired virulence of other pathogens such as \textit{Shigella flexneri}, \textit{Yersinia} spp., or \textit{Escherichia coli}, and several studies have shown that the Pho regulon is part of a complex network important for bacterial virulence and stress response (Lamarche et al., 2008).

3.2 Reporter strains reveal phosphate availability on level of single intracellular salmonella

Using a dual fluorescence reporter, we measured Pi availability and concentrations in distinct intracellular habitats of STM. PstS is a periplasmic protein that binds Pi with high affinity and the promoter of \textit{pstS} was repressed in culture by Pi concentration above 1 mM, as described for \textit{E. coli} (Rosenberg, Gerdes, & Chegwidden, 1977), while a phosphate down-shock experiment revealed Pi concentration above 100 \textmu M as repressing for PstS. Increasing expression of \textit{pstS}::GFP with decreasing phosphate concentrations was reported for \textit{S. flexneri} and in addition a very strong induction measured by concentration of 10 \textmu M Pi.
Using STM as reporter, we determined Pi concentration of less than 10 μM within the SCV of HeLa cells and RAW264.7 macrophages based on the PpstS::sfGFP intensity. However, in the cytosol of HeLa cells, concentrations above 10 μM were determined (Figure 10a). The released bacteria indicated a heterogeneous distribution and a pronounced change over time. Remarkable was the shift at 10 hr p.i. in HeLa cells, where the cytosolic population became smaller in relation to the vacuolar population. At this time, the ΔssaV strain differed from STM WT probably due reduced replication in the SCV (Hensel et al., 1998). In contrast, in RAW264.7 macrophages, strong Pi deficiency was measured shortly after invasion. After 10 hr p.i., a decreased percentage of PpstS-positive STM and induction of sfGFP intensity was obvious. We therefore assumed that non-induced STM consisted of metabolically inactive, temporarily cytosolic, as well as dead and dormant bacteria (Helaine et al., 2014; Röder & Hensel, 2020). In activated RAW264.7 macrophages and in primary human macrophages, both exhibiting higher antimicrobial activity (Rosenberger & Finlay, 2002; Schwan, Huang, Hu, & Kopecko, 2000), the induced subpopulation was smaller compared to STM WT in resting RAW264.7 macrophages, and indicated availability of less than 10 μM Pᵢ.

Serovars of S. enterica cause systemic infections in humans, and macrophages are important for immunity to infection. However, what renders macrophages permissive or restrictive for survival and replication of S. enterica serovars is only partially understood. Human M1 macrophages (classically activated) were used here that restrict proliferation of STM (Lathrop et al., 2018). Based on analyses of fluorescence reporter strains in human macrophages, together with the results obtained by treatment with Cotrimoxazole to inhibit the intracellular replication, we conclude that consumption of and demand for Pi was lower due to the lack of replication in these habitats, similar to an ΔssaV strain. All these results clearly showed that Pi homeostasis allows STM to survive and replicate intracellularly. Therefore, pstSCAB-phoU seems to be the main transporter of Pi and essential for replication and intracellular survival. Inhibition of such important transporter or interference with proper sensing and regulation may be considered as new non-antibiotic strategy.

### 3.3 Multiple effects of phosphate in regulation of virulence factors

It is known that low levels of Pi in the SCV trigger the activation of SPI2 genes (Löber, Jäckel, Kaiser, & Hensel, 2006). The translocation of effectors via the SPI2-T3SS leads to the maturation of the SCV and the formation of the SIFs (Rajashekar et al., 2008). In RAW264.7 macrophages and HeLa cells, we ascertained a...
correlation between SPI2 activity and \( P_{\text{pstS}} \) induction. Nearly all bacteria with \( P_{\text{ssaG}} \) induction were also positive for \( P_{\text{pstS}} \) induction (Figures S6b and S7). In HeLa cells, a further \( P_{\text{pstS}} \)-positive population without SPI2 activity was present, mainly the cytosolic subpopulation. Additionally, the PhoBR two-component system represses \( \text{hilA} \) expression under low extracellular phosphate conditions (Lucas et al., 2000). Therefore, low phosphate levels are important to control \( \text{hilA} \) expression in the intestinal environment (Baxter & Jones, 2015). The transcriptional activator \( \text{hilA} \) controls expression of SPI1-T3SS, as well as some effector proteins for invasion. This may explain reduced invasion of the \( \Delta \text{phoU} \) strain. Deletion of \( \text{phoU} \) resulted in a missing activation of the SPI1-T3SS due to the permanent repression of \( \text{hilA} \) by \( \text{phoBR} \).

Recently, PagR was identified as \( S. \text{enterica} \)-specific integrator of low \( P_i \) and low \( \text{Mg}^{2+} \) levels leading to activation of expression of SPI2-T3SS genes (Huang et al., 2020). The study proposed that PhoB is activated by low \( P_i \), and PhoQ in response to low \( \text{Mg}^{2+} \), both activated PagR. PagR activates SlyA, and SlyA induces expression of ssrAB. Increased levels of the two-component system SsrAB then result in increased expression of genes encoding the SPI2-T3SS and cognate effector proteins. This cascade explains an amplification loop resulting in additional copies of SsrAB. However, the sensor SsrA also responds directly to physicochemical signals in the SCV in order to activate expression of SPI2-T3SS functions, and phosphate limitation and mildly acidic pH were identified as signals Deiwick (Deiwick, Nikolaus, Erdogan, & Hensel, 1999; Löber et al., 2006). Therefore, \( \text{phoBR} \) and \( \text{pstSCAB-phoU} \) have two critical roles for virulence, (i) to switch off SPI1-T3SS after invasion due to the low \( P_i \) values in the intracellular space and (ii) to activate SPI2-T3SS, thus enabling the replication of STM during intracellular lifestyle.

**FIGURE 10** Model of factors that influence \( P_{\text{pstS}} \) induction of *Salmonella enterica* serovar Typhimurium (STM) in host cells. (a) The \( P_{\text{pstS}} \) induction depends on the vacuolar or cytosolic presence of STM, and the level of intracellular replication. (b) Replication-arrested STM demand less phosphate. An enlargement of the *Salmonella*-containing vacuole (SCV)-*Salmonella*-induced filament (SIF) continuum leads to an increased phosphate import, but also to increased replication and phosphate consumption. (c) External addition of phosphate led to increased intracellular phosphate concentration in host cell cytosol, but not within SCV. (d) Addition of CCCP led to decreased intracellular phosphate concentration in host cells cytosol, but not within the SCV. (e) Bafilomycin changed the pH within the SCV and led to lower phosphate availability.
Another study observed that MgtC interacts with PhoR leading to activation of the Pho regulon via PhoB and increased Pi uptake (Choi et al., 2019). Interestingly, and in contrast to our findings, inactivation of phoB, or interference with MgtC-PhoR interaction resulted in decreased Pi transport but increased proliferation in macrophages, and higher virulence in a murine model (Choi et al., 2019). These phenomena were explained by dual roles of MgtC in controlling F0F1-ATPase and intracellular pH (Lee, Pontes, & Groisman, 2013) and the new interaction with PhoR.

Work by Pontes and Groisman (2018) suggested a further regulatory cascade. Starvation of STM in the SCV for Mg2+ results in ribosome instability decreased protein biosynthesis and decreased ATP hydrolysis leading to lower levels of Pi in the cytosol of STM. This situation induces the Pst transport system, despite sufficient Pi availability in the extracellular space. This model also supports the function of PhoR as sensor for Pi in the bacterial cytosol, in line with the absence of the periplasmic domain in PhoR for sensing Pi in periplasm and thus extracellular milieu (Gardner & McCleary, 2019).

Future work has to clarify the interconnected effects of extracellular and cytosolic Pi concentration, transport, the Pi consumption by anabolism during STM proliferation, and the effect of ATP hydrolysis. For this, STM reporter strains are useful tools, yet need to be complemented by single cell sensors such as Pi-sensitive fluorescent proteins that directly sense phosphate levels.

### 3.4 Increase of the SCV-SIF continuum enables further transport of Pi

In vertebrates, Pi homeostasis is mediated by sodium-dependent Pi transporters that use the inwardly directed electrochemical gradient of Na+ ions. In HeLa cells, the major Na+-Pi cotransporter is PIT1 (Bon et al., 2018). Gradients are established by the Na+-K+-ATPase to control Pi influx (Virikki, Biber, Murer, & Forster, 2007). It is not known if specific Pi transporters exist in endosomal membranes that control luminal Pi. Previous work by Vorwerk, Krieger, Deiwick, Hensel, and Hansmeier (2015) investigated the proteome of host cell endomembranes modified by intracellular STM and identified a mitochondrial phosphate carrier protein (MCPC) (Vorwerk et al., 2015) that catalyses the transport of Pi by H+ cotransport into the mitochondrial matrix. Therefore, we assume that Pi influx in the SCV/SIF continuum also requires a H+- or Na+- gradient. Enlargement of membrane surface and lumen of the SCV-SIF continuum may lead to reduction of H+- or Na+- luminal concentration, thus increase the gradient resulting in increased H+ and Pi cotransport. Furthermore, an improved fusion with vesicles through the enlarged membrane is also possible to increase Pi transport (Liss et al., 2017). Therefore, we conclude that STM WT increases Pi transport due to the enlargement of SCV-SIF continuum resulting in bacteria replication. An ΔssaV strain, without the SCV-SIF continuum, cannot replicate due to the lack of access to Pi (Figure 10b).

In conclusion, we used STM as reporter to monitor, at single-cell level, phosphate availability in distinct intracellular niches and host cell types. Proper Pi homeostasis is critical for the intracellular lifestyle of STM and the non-redundant nature of the high affinity Pi transporter may indicate a new target for therapeutic interference with systemic S. enterica infections.

### 4 MATERIALS AND METHODS

#### 4.1 Bacterial strains and growth conditions

STM strains NCTC 12023 (identical to ATCC 14028) and isogenic mutant strains are summarised in Table 1. STM strains were routinely cultured in Luria-Bertani (LB) broth containing 50 μg x ml⁻¹ carbenicillin (Roth) if required for selection of plasmids. Bacterial cultures were routinely grown in glass test tubes at 37°C with aeration in a roller drum at ~60 rpm. For invasion of HeLa cells, fresh LB medium was inoculated 1:3 with o/n cultures of STM and incubated for 3.5 hr with agitation in a roller drum. To test induction of reporters by inorganic phosphate (Pi), PCN media were supplemented with various amounts of Pi, ranging from 100 nM to 25 mM.

#### 4.2 Construction of plasmids

Plasmids used in this work are listed in Table 2. Oligonucleotides for generation of recombinant DNA molecules were obtained from IDT and are specified in Table S1.

The promoter of pstS or apeE of STM was cloned as 300 bp fragment upstream of the translational start site of pstS or apeE using 1f-PstS and 1r-PstS or 1f-apeE-p4889 and 1r-apeE-p4889 for amplification of the insert. Primers Vf-p4889 and Vr-p4889 were used for

| Designation | Relevant characteristics | References |
|-------------|-------------------------|------------|
| NCTC 12023  | wild type               | Lab collection |
| MvP503 ΔpstS::FRT | (Chakravortty, Hansen-Wester, & Hensel, 2002) |
| MvP1886 ΔssaV::aph | (Noster et al., 2019) |
| MvP1890 ΔssaV::FRT | (Noster et al., 2019) |
| MvP2893 ΔphoU::aph | This study |
| MvP2894 ΔphoU::FRT | This study |
| MvP2897 ΔphoE::aph | This study |
| MvP2899 ΔphoE::FRT | This study |
| MvP2900 ΔphoB::aph | This study |
| MvP2901 ΔphoB::FRT | This study |
| MvP2911 ΔphoR::aph | This study |
| MvP2898 ΔpstSCAB::aph | This study |
| MvP2898 ΔpstSCAB::FRT | This study |

Abbreviation: STM, Salmonella enterica serovar Typhimurium.

*All mutant strains are isogenic to STM NCTC 12023.*
amplification of vector p4889, and fragments were fused by Gibson assembly (GA) to generate p5007 or p5081. Dual fluorescence reporter p5007 and p5081 harbour P\textsubscript{EM7}::DsRed for constitutive expression of DsRed, and P\textsubscript{pstS}::sfGFP or P\textsubscript{apeE}::sfGFP for sfGFP expression.

Plasmid pM937 was used to generate p3775 by insertion of transcriptional terminators and P\textsubscript{EM7}::TagRFP-T amplified from pWGRG338 using primers pEM7-RFP For and RFP-T2 Rev and cloning as NotI/SacI fragment. The eGFP gene in p3775 was replaced by sfGFP obtained as Xhol/BamHI fragment from pWGRG167 to generate p3776.

The promoter of ssaG was cloned as 320 bp fragment upstream of the translational start site of ssaG. EM7 was replaced by P\textsubscript{ssaG} using GA to create p5457 with primers Vf-p4889-exEM7 and Vr-p4507 for amplification of vector p5007, and 1f-PssaG-p4889 and 1r-PssaG-p4889 for amplification of the insert from p3776. The promoter of \textit{uhpT} was cloned as 251 bp fragment upstream of the translational start site of \textit{uhpT}. The module P\textsubscript{EM7}::DsRed was replaced by module P\textsubscript{uhpT}::DsRed using GA to create p5192 with primers Vf-p4889 ex EM7 and Vr-p4889 ex EM7 for amplification of vector p5007, and 1f p5407 PuhpT and 1r-p4507 Dsred for amplification of the insert from p5407.

For destabilisation of sfGFP, the LVA tag according to BioBricks (AGGCTGCTGCAAACGACGAAAATACGCCTGTTAGCTG) was fused to the C-terminus to sfGFP via SDM with primers p4889-LVA For and sfGFP-LVA Rev to create p5440.

### 4.4 Preparation of primary human macrophages

Primary human macrophages were prepared from buffy coat from pooled blood samples of anonymous donors (obtained from the Deutsches Rotes Kreuz) as described in Bonfaccino, Dasso, Harford, Lippincott-Schwartz, and Yamada (2004) and in Röder, Felgner, and Hensel (2021). Preparation of lymphocytes by Ficoll–Hypaque gradient was performed as described, alternatively to whole blood, buffy coat was mixed 1+1 with PBS. For differentiation into monocytes/macrophages, the isolated lymphocytes were thawed, seeded and maintained in RPMI-1640 (Biochrom), supplemented with 20% FCS and 2.5 mg x ml\(^{-1}\) GM-CSF (Peprotech). After 5–7 days, the purity of the monocyte/macrophage population was checked by staining with FITC anti-human CD14 antibody (BioLegend) and FC, and subsequently used for infection.

### 4.5 Infection of host cells

Host cell infections were performed as previously described (Rajashekar et al., 2008). Briefly, RAW264.7 macrophages and human macrophages were infected with o/n cultures, and HeLa cells were infected with 3.5 hr subcultures of STM at multiplicity of infection (MOI) of 5 or 25. Infected cells were centrifuged at 500g for 5 min to synchronise infection, incubated for 25 min at 37°C in an atmosphere of 5% CO\(_2\), before extracellular bacteria were removed by washing thrice with PBS. Subsequently, host cells were maintained in growth media containing 100 μg x ml\(^{-1}\) gentamicin for 1 hr, followed by media containing 10 μg x ml\(^{-1}\) for remaining time of incubation.

### 4.6 CI assay

CI assay was performed as previously described (Segura, Casadesus, & Ramos-Mora, 2004). Briefly, HeLa cells or RAW264.7 macrophages were seeded in surface-treated 24-well plates (Fast) and grown to 80% confluency at the day of infection. WT without antibiotic resistance and mutant strains harbouring the \textit{aph} cassette were separately grown in LB+1 and STM recovered 1 and 16 hr with PBS. For differentiation into monocytes/macrophages, the isolated lymphocytes were thawed, seeded and maintained in RPMI-1640 (Biochrom), supplemented with 20% FCS and 2.5 mg x ml\(^{-1}\) GM-CSF (Peprotech). After 5–7 days, the purity of the monocyte/macrophage population was checked by staining with FITC anti-human CD14 antibody (BioLegend) and FC, and subsequently used for infection.

### 4.7 Pulse-chase with fluid phase markers

For tracing the endocytic pathway, fluid phase markers were used. HeLa cells and RAW264.7 macrophages were incubated with 100 μg x ml\(^{-1}\)...
AlexaFluor 647-conjugated dextran, (molecular weight 10,000, Molecular Probes) o/n, washed, and incubated with dextran-free media. Cells were infected for 25 min, incubated 1 hr with 100 μg × ml⁻¹ gentamicin, cultivated 15 hr in growth media with a decreased gentamicin concentration of 10 μg × ml⁻¹ and processed for imaging.

4.8 | Live cell imaging

HeLa cells and RAW267.4 macrophages cultured in 8-well chamber slides were infected at MOI of 5. The infection was performed as described above and 16 hr p.i., the medium was replaced by Imaging Medium supplemented with 10 μg × ml⁻¹ gentamicin. Live cell imaging was carried out by CLSM on a Leica SP5 with an environmental incubation chamber maintaining a humidified atmosphere of 5% CO₂.

4.9 | Quantification by flow cytometry analyses

HeLa cells and RAW264.7 macrophages were infected at MOI of 5 for 25 min. At various time points ranging from 2 to 24 hr p.i., host cells were lysed by addition of 0.1% Triton X-100 in PBS and fixed with 3% PFA for subsequent FC analyses using the Attune Nxt Cytometer (Thermo Fischer). Further details on gating strategies and quantification of populations size and expression levels can be found in prior reports (Röder, Felgner, & Hensel, 2021; Röder & Hensel, 2020; Schulte et al., 2021). Experiments were performed in triplicates at least three times. Data were analysed with Attune Nxt 2.5. Statistical analyses were performed using one-way analysis of variance Bonferroni using SigmaPlot 13 (Systat Software).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Jennifer Röder and Michael Hensel: Conceived the study. Jennifer Röder and Pascal Felgner: Performed experimental work. Jennifer Röder, Pascal Felgner and Michael Hensel: Analysed the data. Jennifer Röder and Michael Hensel: Wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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