Title: *Salmonella* persisters undermine host immune defences during antibiotic treatment

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Abstract: Many bacterial infections are hard to treat and tend to relapse, possibly due to the presence of antibiotic-tolerant persisters that are considered dormant cells when bacteria are grown in laboratory medium. Non-growing persisters also form following uptake of *Salmonella* by macrophages but their nature is little understood. Here we show that *Salmonella* persisters arising during macrophage infection maintain an active state. Persisters reprogram macrophages by means of effectors secreted by the SPI-2 Type 3 Secretion System (T3SS), thereby dampening pro-inflammatory innate immune responses and inducing anti-inflammatory macrophage polarisation. Reprogramming allows non-growing *Salmonella* to survive for extended periods in their host. Persisters undermining the host immune defences might confer an advantage to the pathogen during relapse once the antibiotic pressure is relieved.

One sentence summary: Antibiotic-tolerant non-growing *Salmonella* persisters survive through modulating the macrophage immune response.

Main text:

During growth, genetically clonal bacterial populations always contain a small fraction of non-growing, non-dividing cells that arise from transient, reversible, phenotype switching.
These growth-arrested cells are usually tolerant to antibiotics and are called antibiotic-persisters (1). Previously, we showed that a large proportion of the intracellular pathogen Salmonella Typhimurium adopt a non-growing antibiotic-tolerant state within macrophages (2). We also showed that the first Salmonella persister cells that regrow upon release from their host cells are those that maintained metabolic activity during infection (2). Similar non-growing but metabolically active bacteria have also been observed in macrophages infected with Mycobacterium tuberculosis (3). By contrast when Salmonella and other bacterial species are grown in laboratory culture media, persisters are often observed to be inactive (i.e., dormant) (4–6).

To assess whether retention of transcriptional and translational activity might confer an additional physiological benefit to non-growing bacteria within a host cell, we infected mouse bone marrow-derived macrophages with wild-type Salmonella Typhimurium carrying a reporter plasmid allowing for tracking of bacterial proliferation and activity (7) (fig. S1-2). Spontaneous regrowth of non-growing Salmonella following infection and antibiotic treatment arose exclusively from the active rather than inactive bacteria, the latter of which failed to regrow even after days of incubation (Fig. 1A). In addition, we artificially generated a population of non-growing and translation incompetent Salmonella through exposure to bacteriostatic concentrations of chloramphenicol (fig. S3), and subsequently monitored survival of these dormant bacteria after exposure to the bactericidal antibiotic, cefotaxime. Although the inactive Salmonella withstood exposure to cefotaxime in laboratory medium, they did not survive within macrophages cultured with antibiotics (Fig. 1A).

Following entry into macrophages, Salmonella induces expression of the type 3 secretion system (T3SS), SPI-2, through which it translocates approximately 30 effectors that inhibit host cell processes that are detrimental to the pathogen (8, 9). We hypothesized that, as well as maintaining transcriptional and translational activity, persisters may also translocate SPI-2 effectors. We used the SPI-2 ssaG promoter fused to unstable eGFP (10) to test for SPI-2 gene expression in single cells. ssaG promoter expression was observed in non-growing bacteria that retained transcriptional and translational activity (Fig. 1B). Furthermore, Salmonella effector proteins were detected in the host-cell cytosol by Western blotting of macrophages containing pure populations of growing or persister cells (Fig. 1C and fig. S4).
To understand how persisters shape their host environment, we used dual RNA-seq (16) on infected macrophage sub-populations to analyse host and pathogen transcriptomes simultaneously (13) (fig. S5). The majority of dual RNA-seq reads from infected macrophages aligned to the host genome, with relative amounts of bacterial reads being proportional to the average number of bacteria present per cell (fig. S5d and Table S1).

Principal component (fig. S6a) and clustering analyses (Fig. 2A and Table S2) on the transcriptomic profiles of all macrophage populations confirmed the expected general difference between challenged and naïve macrophages (Fig. 2A, clusters I and II and fig. S6a). Among challenged macrophages, the greatest differences occurred between macrophages containing viable bacteria (growing or non-growing) and macrophages that have killed the bacteria they had engulfed (host-killed, HK) or bystander macrophages.

Clustering analysis pinpointed two large groups of genes (Fig. 2A, clusters III and IV) responsible for this separation (Fig. 2B). Of these, members of cluster III were enriched with genes involved in classical, pro-inflammatory macrophage activation (i.e., M1) and cluster IV was enriched with genes associated with alternative, anti-inflammatory (i.e., M2) macrophage activation. Inspection of the dual RNA-seq data for five representative M1 activation markers (Nfkb2, Cd40, Il1b, Nlrp3, Tnf; fig. S6b, left panel) confirmed that their upregulation during infection is dampened in macrophages containing viable bacteria. In contrast, five M2 activation markers (Il4ra, Arg1, Odc1, Ppard, Timp1; fig. S6b, right panel) were upregulated in macrophages containing viable bacteria. There was a significant overlap of these gene clusters with our previously defined M1 (p-value = 5.2 × 10^{-19}, hypergeometric test) or M2 (p-value = 1.2 × 10^{-38}, hypergeometric test) gene sets (Fig. 2C), deduced from single-cell RNA-seq data (11). As clusters III and IV contain considerably more genes, we consider them more complete M1 and M2 polarisation gene sets.

Principal component (fig. S6a) and clustering analyses of bacterial genes in the dual RNA-seq dataset (fig. S6c, Table S3) showed expected differences such as expression of flagellar genes in the inoculum by comparison with intracellular bacteria, which showed elevated expression of infection-associated genes (17). Remarkably, the transcriptomes of intracellularly growing and non-growing bacteria were similar (fig. S6a,c) and both expressed genes encoding the SPI-2 T3SS apparatus and its translocated effectors (fig. S6d).
We performed interspecies expression correlation analysis on the dual RNA-seq data to reveal the host consequences of bacterial SPI-2 T3SS expression. Of the 4,817 defined murine gene sets tested, SPI-2 T3SS gene expression showed the strongest positive correlation with M2 anti-inflammatory cluster IV genes, and the strongest negative correlation with M1 pro-inflammatory cluster III genes (Fig. 2D and E, Table S4). Of the Salmonella regulons, only PhoP/Q and SPI-2 T3SS showed this (anti-)correlation pattern with the M1/M2 host gene sets (Table S5, fig. S6f,g). Since PhoP/Q is required for SPI-2 T3SS activation (18), the data suggests SPI-2 T3SS effectors modulate M1/M2 polarization.

Macrophages containing active non-growing Salmonella displayed an intermediate expression profile between the M1 and M2 phenotypes (Fig. 2B). Previous single-cell RNA-seq data led us to conclude that non-growing bacteria inhabit M1-like cells whereas growing Salmonella associated with M2-like macrophages (11). Re-analysis of our previous single-cell RNA-seq data (11) with the extended M1 and M2 gene sets actually shows bimodality of M1/M2 gene expression in macrophages containing non-growing bacteria (fig. S7a). By contrast, bystander macrophages show M1 bias and those containing growing bacteria show a clear M2 bias (fig. S7a). The levels of the macrophage surface M2 marker IL4RA were significantly increased in macrophages containing non-growing active Salmonella compared with macrophages containing non-growing inactive or host-killed bacteria (fig. s7b). Therefore, the intermediate population-level M1/M2 expression profile of the host cell probably reflects an underlying heterogeneity of transcriptional/translational activity and SPI-2 expression in non-growing intramacrophage Salmonella (Fig. 1 and (2)).

The correlation between SPI-2 effector expression and macrophage polarisation could be explained by the presence of subsets of macrophages prior to infection with depressed inflammatory responses and/or increased permissiveness. Alternatively, this could be a direct result of activity of SPI-2 effectors, some of which are known to downregulate pro-inflammatory responses such as NF-κB activation (9). Consistent with the latter, when we compared the levels of three mRNAs for established M1 markers (Cd40, Il1b and Nfkb2) between macrophages containing active non-growing wild-type or SPI-2 null (ssaV mutant) bacteria by RT-qPCR, wild-type Salmonella were associated with downregulated expression of the three markers during exposure to antibiotics, whereas cells infected with the SPI-2 null mutant bacteria were not (Fig. 3A). Therefore, active non-growing bacteria use SPI-2 T3SS
effectors to counteract macrophage M1 activation (9), and are able to do so despite sustained exposure to the pro-inflammatory IFNγ cytokine (fig. S8a-b).

In addition, analysis of the dual RNA-seq and our previous single-cell RNA-seq (11) data suggested that Salmonella actively promotes macrophage M2 polarisation. In a murine model of long-term infection, Salmonella resides in M2 macrophages (19) rather than in pro-inflammatory M1 macrophages (20–22), suggesting that the conditions within M2 macrophages enable prolonged bacterial survival. Consistently, in a mouse model of acute systemic infection (fig. S8c), the M2 marker IL4RA was highly expressed by splenic macrophages isolated three days after intra-peritoneal Salmonella injection, with the majority of growing bacteria inside macrophages with high IL4RA expression (Fig. 3B). There was greater heterogeneity in IL4RA expression within the macrophage population containing non-growing bacteria (Fig. 3B). Despite exposure to antibiotics, active non-growing wild-type Salmonella, but not SPI-2 null mutants, upregulated expression of the two M2 markers tested in macrophages (Fig. 3C).

Further investigation revealed that, during infection with a SPI-2 null strain, accumulation of IL4RA was abrogated in all macrophages infected with viable bacteria regardless of the bacterial growth state (Fig. 3D, fig. S8d) (15). The M2-like polarisation profile induced by Salmonella can be recapitulated best by exposure to both IL-4 and IL-10 (fig. S9). Screening a collection of all SPI-2 effector mutants revealed that M2-like polarisation is driven solely by the SteE (also known as SarA) effector (Fig. 3F, fig. S8f); a recent report shows that SteE promotes secretion of IL-10 from infected B cells (23). Although an steE mutant no longer triggered M2-like polarisation of host cells, the mutant still dampens the M1 response (fig. S8e-f, S10), suggesting that M1 suppression and M2 polarisation are independent of each other. Non-growing intracellular Salmonella can thus express and translocate sufficient quantities of effectors to cause major changes in the immune status of the infected host cell, even during exposure to antibiotics and IFNγ.

Finally, after 48 hours of antibiotic exposure within macrophages, non-growing mutant bacteria lacking a functional SPI-2 apparatus showed significantly reduced survival compared with their wild-type counterparts (Fig. 3G). However, in mixed strain infections, we found that wild-type persisters ‘rescued’ survival of SPI-2 null persisters in co-infected cells (Fig. 3H), illustrating that the decreased survival of SPI-2 null persisters was exclusively a
consequence of a failure to manipulate the intracellular host environment in which they reside.

Collectively, our data shows that following infection of macrophages, maintenance of transcriptional/translational activity enables non-growing *Salmonella* to translocate SPI-2 T3SS effectors into the host cell. Thus, unlike bacterial persisters in laboratory media, which apparently become dormant (2, 4–6), intracellular *Salmonella* maintain effector delivery but cease to grow. This strategy enables a subpopulation of intracellular bacteria to survive antibiotic exposure and reprogram their macrophage host cell to promote long-term bacterial survival (Fig. 4). Reprogramming of the host cell by *Salmonella* not only suppresses M1 bactericidal responses (9) but also increases permissiveness of the now M2-biased cells for the pathogen, potentially by modulating host-cell metabolism (20, 25). We showed that the capacity of *Salmonella* to direct macrophage M2 polarisation is driven by the SPI-2 T3SS effector SteE. Similarly, *M. tuberculosis* infections are reported to be accompanied by an expansion of a population of permissive macrophages (26–29)(30), raising the possibility that mycobacteria and other intracellular pathogens employ similar mechanisms for modulating host polarisation. Pathogen manipulation of host cells is accompanied by changes in the repertoire of secreted cytokines (23). Rather than being dormant, non-growing persister cells of intracellular bacterial pathogens are able to subvert host immune defences, even under antibiotic treatment, potentially making the host environment more permissive for recrudescence infection.

**References and notes:**


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Acknowledgements: We thank members from the Holden and Helaine labs for sharing constructs and protocols and scientific discussions; Izabela Glegola-Madejska for help with the animal experiments; and Prof. David Holden for careful reading of the manuscript.

Funding: This research was supported by an MRC Career Development Award (MR/M009629/1) from the Medical Research Council (UK) and Lister Institute Research Prize 2017 to SH, an EMBO long-term fellowship (ALTF 441-2015) to DACS, an Elite Advancement Ph.D. stipend from the Universität Bayern e.V., Germany to AJW. Author contributions: Outlined the study: SH, JV; Performed experiments: DACS, PWSH, AJW, RF, IB, SH; Bioinformatic analysis: PWSH; Analysed data: DACS, PWSH, AJW, RF, IB, ES, TT; Interpreted data: DACS, PWSH, AJW, SH; Wrote manuscript: DACS, PWSH and SH with contribution of other authors. Author order between DACS and PWSH determined randomly. Competing interests: Authors declare no competing interests. Data and materials availability: The dual RNA-seq data is summarised in Table S1; results of analyses are in Table S2-S5, S8, S9, and the data is archived on GEO (Accession: GSE104785).

Supplementary materials:

Materials and Methods

Figures S1-S10

Tables S1-S9
Figure legends

**Fig. 1** *Salmonella* antibiotic persisters during macrophage infection are metabolically active, transcribing, translating and translocating SPI-2 T3SS effectors.

(A) (Left) Regrowth on laboratory medium of sorted inactive (iNG), active (aNG) non-growing or growing (G) bacteria after 24 hours of infection under exposure to cefotaxime (cefo) or gentamicin (genta) respectively and as indicated. (Right) Survival of translationally-blocked non-growing *Salmonella* subsequently exposed to bactericidal concentrations of cefotaxime in laboratory medium or macrophages (Mφ) for 24 hours. (Unpaired (top: aNG vs. G) and paired t-test (rest) on log-transformed data; *p*-values are indicated; error bars depict mean and SD.)

(B) Expression of unstable eGFP controlled by a SPI-2 promoter (PssaG) by intracellular bacteria at 24 hours post-uptake depending on their growth and activity status. Representative histogram is shown on the left, quantification of six independent repeats on the right (paired t-test; *p*-value is indicated; error bars depict mean and SD).

(C) Translocation of HA-tagged SPI-2 T3SS effectors detected in bacterial pellet or host cell cytosol after lysis of bystander Mφ or Mφ containing growing (G), non-growing (NG), or a secretion deficient (ssaV) mutant 20 hours after uptake. * indicate detected effector.

**Fig. 2** Dual RNA-seq implicates SPI-2 in dampening M1 and promoting M2 macrophage polarisation

(A) Clustering analysis of host genes differentially expressed between any two subpopulations, with selected enriched terms. Analysed sub-populations of Mφ were naïve, bystander, or containing host-killed (HK), non-growing (NG) or growing (G) bacteria.

(B) Principal component analysis of Mφ transcriptomes based on M1 (cluster III, Fig. 2B) and M2 (cluster IV, Fig 2B) polarisation genes. The subsets of Mφ are colour-coded and the three biological repeats indicated with different symbols.

(C) Venn diagram showing the relation between M1 (top) and M2 (bottom) polarisation genes identified in (11) and extended M1 and M2 genes identified here (clusters III and IV, Fig. 2B). Number of genes within each gene set are indicated.
(D) Interspecies correlation analysis between SPI-2 apparatus and effector gene expression patterns in *Salmonella* and gene expression patterns of defined host gene sets in infected dual RNA-seq samples. Plot of the GSEA enrichment score (x-axis) and -log10 FWER-adjusted *p*-value (y-axis) based on correlations between Z-score-normalised host gene expression and average Z-score-normalised SPI-2 apparatus and effector expression for all 4,817 tested murine gene sets.

(E) Boxplots depicting the distribution of correlations of interesting gene sets in (E).

Box: median with upper and lower quartiles. Whiskers: lowest and highest values; FWER-adjusted *p*-values from (E) are indicated.

**Fig. 3 Growing and non-growing *Salmonella* use SPI-2 to dampen M1 and drive M2 macrophage polarisation**

(A) mRNA levels of M1 genes upon infection by WT or *ssaV* mutant active non-growing *Salmonella* 18 hours after uptake (cefotaxime-treated). Relative expressions are measured by qRT-PCR and calculated by the delta-delta-CT method relative to control DsRed RNA and bystander Mφ (paired ANOVA with multiple testing amongst shown groups; adj. *p*-values are indicated; error bars depict mean and SD).

(B) Representative histograms of IL4RA expressed by splenic Mφ from one mouse (left) and the proportion of IL4RA positive Mφ in multiple mice. Subpopulations of Mφ from the same mouse are connected with a dotted line. (Paired ANOVA, multiple testing against bystander Mφ; adj. *p*-values are indicated) (right).

(C) Expression of M2 polarisation genes as in (A).

(D) Proportion of IL4RA positive infected Mφ 24 hours after uptake of WT, *ssaV* mutant or *ssaV* complemented bacteria. Either Mφ containing active non-growing (aNG) bacteria (cefotaxime-treated, left), or Mφ containing similar amount of growing (G) bacteria for each strain (right) were analysed (paired ANOVA with multiple testing against corresponding bystander Mφ; adj. *p*-values are indicated; error bars depict mean and SD).

(E) Screen of SPI-2 effector mutants for accumulation of IL4RA in Mφ infected with growing bacteria 24 hours after uptake (unpaired ANOVA with multiple testing (Dunnett’s test) against corresponding Mφ infected with WT *Salmonella*; adj. *p*-values are indicated; error bars depict SD).

(F) IL4RA expression in bystander Mφ and Mφ with host-killed (HK), non-growing (NG)
or growing (G) *Salmonella* continuously exposed to IFNγ 24 hours after uptake. Populations of Mφ containing WT or *ssaV* mutant bacteria were gated to contain similar bacterial loads. (Paired ANOVA, repeated testing against bystander Mφ; adj. p-values are indicated; error bars depict mean and SD).

(G) Intramacrophage long-term survival of NG *Salmonella* after 48 hours cefotaxime treatment, corrected for cytotoxicity (paired ANOVA with multiple testing against WT; adj. p-values are indicated; error bars depict mean and SD).

(H) Intramacrophage survival of NG *ssaV* mutant *Salmonella* after 24 hours cefotaxime treatment, following sorting of Mφ singly infected with mCherry-expressing *ssaV* mutant, or Mφ infected with a mixed population of mCherry-expressing *ssaV* mutant and gfp-expressing WT bacteria (unpaired *t*-test against single infection; p-values are indicated; error bars depict mean and SD).

**Fig. 4** Persisters undermine host innate immune response and enable long-term survival

Model of macrophage manipulation by *Salmonella* persisters. Growing *Salmonella* translocate SPI-2 effectors that manipulate host cell polarisation and create a less hostile environment. Proliferation makes the bacteria susceptible to antibiotic killing (left). Inactive non-growing *Salmonella* cannot translocate SPI-2 effectors and are killed in the strongly antimicrobial environment (middle). Active persisters manipulate host cell polarisation through translocation of SPI-2 effectors, in turn retaining their activity and maintain the ability to survive in the host while being antibiotic-tolerant (left).
MATERIALS AND METHODS

Bacterial strains and plasmids
Salmonella enterica serovar Typhimurium strain SL1344 was used for the dual RNA-seq, and strain 12023, and isogenic mutants, for all other assays. Mutants were made via lambda-red recombination. Where required, strains carried the fluorescence dilution plasmid pFCcGi or pDiGi. Activity of the ssaG promoter was detected with pDiGssaG. This vector was constructed by PCR linearisation of the pDiGc plasmid and replacement of rpsM promoter and gfp by an insert containing the 450 bp upstream of the ssaG start site and an unstable GFP between EagI/SphI restriction sites. See Table S6 for details about all strains, plasmids and primers.

Cell culture
Bone marrow was extracted from tibia and femur of female C57BL/6 mice (Charles River), in accordance with a UK Home Office Project Licence in a Home-Office designated facility. Red blood cells were lysed in 0.83% NH₄Cl for 3 minutes and the remaining progenitor cells were cultured in Dulbecco’s modified eagle medium with high glucose (DMEM; Sigma), containing 20% (vol/vol) L929 culture supernatant (ATCC #CCL-1) (LCM), 10% (vol/vol) fetal calf serum (FCS; Gibco, Life Technologies), 10 mM HEPES (Sigma), 1 mM sodium pyruvate (Sigma), 0.05 mM beta-mercaptoethanol (Sigma), 100 U/ml penicillin (Sigma), and 100 µg/ml streptomycin (Sigma). Fresh medium was supplemented at day 3 and the differentiated bone-marrow derived macrophages (bmm) were harvested at day 7. Bmm were seeded in DMEM without LCM or antibiotics in 24-well plates (Cellstar, Greiner Bio-one, 2E5 bmm/well), 6-well plates (Thermo Scientific; 1E6 bmm per well) or in tissue-culture treated petri dishes (Sarstedt; 7E6 bmm per petri dish) to be infected the next day. When indicated, 50 ng/ml IFNγ was added 23 hours prior to infection and/or during infection.

Bacterial infection of macrophages
Bacteria were grown in minimal medium (MgMES: 170 mM 2-(N-morpholino)ethanesulfonic acid (MES) at pH 5.0, 5 mM KCl, 7.5 mM (NH₄)₂SO₄, 0.5 mM K₂SO₄, 1 mM KH₂PO₄, 8 mM MgCl₂, 38 mM glycerol, and 0.1% casamino acids) (with 0.2% w/vol L-arabinose if induction of GFP from pFCcGi or DsRed from pDiGi or 0.05M IPTG for induction of GFP from pDiGi was required in the inoculum) for 18 hours. Bacteria were opsonized with 8% mouse serum (Sigma) for 20 minutes, and added to the bmm in an MOI of 5 (to obtain a phagocytosis rate
of one bacterium per macrophage) unless stated otherwise. Infection was synchronized by 5 minutes centrifugation at 110 g. Phagocytosis was allowed to occur for another 25 minutes at 37°C with 5% CO₂. To analyse all intracellular (NG and G) bacteria, bmm were washed 3x with PBS and incubated in medium with 30 µg/ml gentamicin (Sigma) for 1 hour. Then, cells were washed 3x with PBS again and incubated in 10 µg/ml gentamicin-containing infection medium for the remainder of the infection. Higher concentrations of gentamicin interfere with the ability of intracellular bacteria to express inducible fluorescent reporters and are therefore suspected to alter bacterial activity in general. To specifically analyse intracellular NG bacteria, bmm were treated with 200 µg/ml cefotaxime after the first wash. To discriminate aNG from iNG, the expression of the inducible fluorescent reporters from FD plasmids were not induced in the inoculum, but instead arabinose was added during the last 4 hours of the infection. At indicated time points, bmm were washed with PBS once and detached from the surface with cold PBS and scraping. To directly harvest the bacteria, bmm were washed 3x with PBS and lysed with 0.1% (vol/vol) triton X-100 (Sigma) in PBS. Analysed populations per experiment are summarised in Table S7.

Fluorescence-activated cell sorting (FACS)

FACS was used to discriminate different subsets of bmm or bacteria as described in the main text, and in Table S7, and in (11). In all experiments apoptotic macrophages and doublets were excluded by gating. In addition, gates were set to only include macrophages in which a single phagocytosis event had taken place, based on the GFP intensity. Unfixed samples were sorted under continuous cooling to 4°C by a BD FACS Aria III. Subsequently, samples were prepared for dual RNA-seq, effector translocation, RT-qPCR or regrowth. For bacterial regrowth, samples were sorted at room temperature (RT).

Bacterial regrowth after FACS

From sorted bacteria (Fig. 1B): 200-1,000 cefotaxime-treated aNG and iNG bacteria and 200-500 gentamicin-treated growing bacteria were sorted into tubes with 1 ml LB and directly plated onto LB-agar plates (LB broth base (Invitrogen) and agar (Oxoid Ltd)) containing 30 µg/ml chloramphenicol or 50 µg/ml carbenicillin to minimise the chance of contamination. Plates were incubated for at least 2 days to count all appearing colonies.

From sorted macrophages (fig. S5c): bmm were sorted at RT into tubes with 1 ml PBS. From gentamicin-protection assays bystander bmm (2E4), bmm+HK (1E4), bmm+G (500), bmm+NG (500); and from cefotaxime-treated assays bystander bmm (2E4), bmm+iNG (1E4),
bmm+aNG (500) events. Bmm were lysed by adding 50 µl 2% (vol/vol) triton for 5 minutes at RT, and plated onto LB-agar plates containing 30 µg/ml chloramphenicol. Plates were incubated for at least 2 days to count all appearing colonies.

From sorted macrophages for transcomplementation assay (Fig. 3H): Bmm were infected with ssaV mutant Salmonella expressing mCherry constitutively from pFCcGi (15) with or without additional WT Salmonella expressing GFP constitutively from pFPV25.1 with a combined MOI of 5, for 48 hours in presence of cefotaxime. Bmm were harvested, and 2,000 bmm were sorted containing just ssaV mutant Salmonella that had not been incubated with WT, and 1,000 bmm (that had been incubated with both strains) were sorted containing both WT and ssaV mutant Salmonella. Bmm were sorted at RT into tubes with 1 ml PBS. Bmm were lysed by adding 50 µl 2% (vol/vol) triton for 5 minutes at RT, and plated onto LB-agar plates containing 50 µg/ml kanamycin. Plates were incubated for at least 2 days to count all appearing colonies.

**Chloramphenicol pre-treatment persister survival assays**

Salmonella was grown in LB or MgMES pH 5.0 for 18 hours. A dormant non-growing population was generated by incubation with 100 µg/ml chloramphenicol (Cm) for 45 minutes, as previously reported (5). This population was challenged with bactericidal antibiotics (200 µg/ml cefotaxime) in liquid medium (LB, for 5 hours) or macrophages (24 hours) with (for pre-treated population) or without (for non-pretreated population) 100 µg/ml chloramphenicol. Both antibiotics were washed away, macrophages were lysed (when applicable) and viability was assessed by plating on LB agar.

**ssaG-promoter activity**

Bacteria containing pDiGssaG were harvested from lysed bmm and fixed for 15 minutes in 3% (w/vol) paraformaldehyde (PFA, Sigma). To facilitate discrimination from debris, bacteria were stained with CSA-1 in 10% (vol/vol) horse serum (HS, Sigma) /PBS for 30 minutes at RT, washed once, and stained with an Alexa-647 conjugated secondary antibody in HS/PBS for 30 minutes at RT. Samples were measured on a BD Aria III flow cytometer. Bacteria were discriminated from debris by gating on SSC/A647 before analysing the DsRed and GFP signal.

**Effector translocation**

Bmm were infected with WT (MOI 5) or ssaV mutant (MOI 25) bacteria carrying pFCCeGi, induced for expression of GFP (Fig. 1D) or not (fig. S3a). In order to sort bmm+aNG, expression of GFP was induced during the last 4 hours of infection (fig. S4a). After 20 hours
gentamicin-protected infection, 5E4 events per gate were sorted and intracellular bacteria were released by incubation in cold 0.1% (vol/vol) triton/PBS for 10 minutes. Released bacteria were pelleted for 10 minutes at 16,000 g. Both supernatant and pellet were analysed by 12% (vol/vol) SDS-PAGE and subsequent immunoblot on PVDF membrane (Merck Millipore). Equal amounts of sample were loaded, except for the bmm+G (1/4 of the total sample), to slightly correct for the higher number of bacteria per bmm. Membranes were blocked in 5% (w/vol) milk powder (Marvel milk) in 0.1% (vol/vol) Tween20 (Sigma)/PBS (PBST). Membranes were probed with antibodies (Table S6) for 18 hours at 4°C and subsequently with peroxidase-conjugated secondary antibodies for 2 hours at RT. Bands were visualised with ECL plus (Pierce, Thermo Scientific) on a Biorad ChemiDoc Touch.

**Dual RNA-seq sample preparation**

For all mentioned subpopulations 1E5 events were sorted and total RNA was isolated using the mirVana kit (Thermo Fisher Scientific) following the manufacturer’s recommendations for total RNA extraction and DNase I-treated (NEB) for 45 minutes at 37°C. Generation of cDNA libraries and sequencing was as previously described (16). Briefly, bacterial and murine rRNA was depleted using the RiboZero Gold (epidemiology) kit (Illumina) according to the manufacturer’s guidelines. Strand-specific cDNA libraries were prepared by Vertis Biotech. AG (Freising, Germany) and sequenced on the NextSeq500 platform (Illumina) to >10 million reads per library. To compensate for the differential bacterial genome coverages between samples with non-growing and growing Salmonella (see fig. S5d), libraries derived from macrophages with non-growing bacteria were sequenced to 3-5-fold greater depth. For each condition, biological triplicates were analysed.

**Dual RNA-seq alignment and downstream analysis**

Fastq reads were first trimmed using Trimmomatic (version 0.35) with options ILLUMINACLIP:${adapt}:2:30:10, where ${adapt} refers to the TruSeq SE adaptors. Alignment was always done using a two pass approach.

For final alignment against the mouse genome, trimmed reads were first aligned to the Salmonella SL1344 assembly (ASM21085v2, including the Chromosome and pSLT_SL1344, pRSF1010_SL1344, and pCol1B9_SL1344 plasmids) using TopHat (version 2.0.2, with options -N 2 --library-type=fr-unstranded --b2-very-sensitive --b2-L 25). The gtf annotation for SL1344 (ASM21085v2) was downloaded from EnsemblBacteria and manually extended.
with small RNA annotations (34) as a gene model with TopHat. Unaligned reads were then used for alignment against the mouse genome (GRCm38, Ensembl) using TopHat. The gtf annotation for mouse (GRCm38.p5, Ensembl 87) was downloaded and used as a gene model with TopHat. Read counts per annotated mouse gene were then computed using HTSeq (version 0.6.1) and expression levels of each gene were quantified by computing the number of fragments detected per kilobase per million of reads (FPKM) using a custom R script (Table S8).

For final alignment against the Salmonella SL1344 genome, trimmed reads were first aligned to the mouse genome (GRCm38, Ensembl) using TopHat. Unaligned reads were then used for alignment against the Salmonella SL1344 assembly (ASM21085v2, including the Chromosome and pSLT_SL1344, pRSF1010_SL1344, and pCol1B9_SL1344 plasmids) using TopHat. As above, the gtf annotation for SL1344 including small RNA annotations was used as a gene model with TopHat. Read counts per annotated Salmonella gene were then computed using HTSeq and FPKM were calculated as above (Table S8).

Differential expression analysis was performed using DESeq2 (version 1.14.1), and genes with an adjusted \( p \)-value < 0.01 were considered differentially expressed. Principal component analysis was carried out on Z-score-normalised rlog values from DESeq2 using all genes differentially expressed between at least two conditions. Clustering analysis was performed on Z-score-normalised rlog values from DESeq2 using densityClust for R (version 0.2.1). To robustly identify genes contributing to separation along principal component 1 (dim 1) and 2 (dim 2) in macrophages (fig. S6a), clustering was first carried out on genes differentially expressed between at least two groups using only challenged macrophages (Table S9), and then on genes differentially expressed between any two groups using all macrophages (with genes contributing to separation along dim 2 removed first). Clusters were then visualised together. Gene ontology (GO) and KEGG pathway analysis was carried out in DAVID for both macrophage and Salmonella gene clusters.

For determining genes correlated with SPI-2 expression (Fig. 2D,E), the average Z-score-normalised rlog values of SPI-2 genes (ZSPI2) from DESeq2 were calculated for all infected macrophage samples (i.e. bmm+G, bmm+NG, bmm+HK samples), accounting for the number of bacteria within each sample by combining the mouse and Salmonella read count tables prior to generating rlog values in DESeq2. The Pearson correlation value between the Z-score-
normalised rlog values of each mouse gene and ZSPI2 was computed in R. This was transformed into a ranked gene list (ranging from -1 to +1) and used for GSEA analysis against all GO terms, canonical pathways from KEGG and Biocarta, and GSEA hallmark gene sets, and manually added gene sets.

For determining genes correlated with M1 or M2 genes (fig. S6f,g), the average Z-score-normalised rlog values from DESeq2 of either M1 (cluster III, Fig. 2A; ZM1) or M2 (cluster IV, Fig. 2A, ZM2) was calculated for all infected macrophage samples (i.e. bmm+G, bmm+NG, bmm+NV samples). The Pearson correlation value between the Z-score-normalised rlog values of each *Salmonella* gene and either ZM1 or ZM2 was computed in R. This was transformed into a ranked gene list (ranging from -1 to +1) and used for GSEA analysis of *Salmonella* regulons.

For re-analysis of scRNA-seq datasets (11) (fig. S7a), gene read count data was downloaded from GSE79363. Principal component analysis was carried out on Z-score-normalised rlog values from DESeq2 using genes within macrophage cluster III and cluster IV (i.e. expanded M1/M2 gene sets, Table S3) only.

**RT-qPCR**

For each population, 1E5 bmm (Fig. 3A,C) or 3E4 bmm (fig. S8) were sorted. Sorted bmm were pelleted (300 g, 5 minutes) and snap-frozen to store o/n at -80°C before generating cDNA. To generate cDNA, pellets were thawed, 4 ng RNA was spiked in to facilitate normalisation between samples, and total RNA was isolated using the RNeasy kit (Qiagen). cDNA was generated using the QuantiTect Reverse Transcription kit (Qiagen). After validating the specificity of the RT-qPCR primers by analysing the melt curves of the amplicons and a dilution series over a 1E4 dilution range, the RT-qPCR was performed with 200 nM primers and SYBR Green master mix (Thermo Scientific) and measured on a StepOne Plus thermocycler (Applied Biosystems). Gene expression relative to the spiked-in DsRed (Fig. 3A,C) or the geometric mean of three stable control genes (*beta-2-macroglobulin* (*b2m*), *hypoxanthine phosphoribosyltransferase 1* (*hprt1*), *hydroxymethylbilane synthase* (*hmbs*)) (35) (fig. S8) and bystander bmm exposed to WT *Salmonella* (Fig. 3A,C; fig. S6e,f) or unpolarised bystander bmm (fig. S6b,d) was calculated via the delta-delta-Ct method. (Spike-in RNA was generated from stationary phase *Salmonella* expressing DsRed from the pDsRed and RNA was...
isolated via the RNeasy bacterial RNA isolation protocol (Qiagen)).

**Mouse infections**

All animal experiments were carried out under and in accordance with a UK Home Office Project Licence in a Home-Office designated facility. Female C57Bl/6 mice of 8-10 weeks old were infected intra peritoneally with 5E4 CFU/mouse of S. Typhimurium 12023 with arabinose-induced pFCCgi-Cb. Animals were kept for 3 days. They were sacrificed by cervical dislocation and their spleens were harvested. Splenic macrophages were isolated by homogenising the spleens in cold Hank’s buffered salt solution (HBSS, Gibco, Life Technologies) with 2% (vol/vol) FCS and 10 mM HEPES, enriching for CD11b+ cells over a MACS column (Miltenyi Biotec) per manufacturer’s instruction. Isolated splenic cells were fixed for 15 minutes in 3% PFA) and stained for IL4RA and F4/80 in 10% HS / 0.1% (w/vol) saponin (Sigma)/PBS (Table S6) and analysed on a BD Fortessa flow cytometer. During analysis doublets were excluded by gating.

**Polarisation marker analysis**

At the indicated time points, infected macrophages were harvested and fixed for 15 minutes in 3% PFA. Macrophages were blocked and permeabilised by 30 minutes incubation with 10% HS/0.1% (w/vol) saponin/PBS. The presence of the polarisation markers was visualised with directly conjugated antibodies (Table S6) in 10% (vol/vol) HS/0.1% (w/vol) saponin/PBS. IL4RA staining in absence of saponin showed the same results. Samples were measured on a BD Fortessa flow cytometer. During analysis, apoptotic macrophages and doublets were excluded by gating. In addition, gates were set to only include macrophages in which a single phagocytosis event had taken place, based on the GFP intensity. When multicolour panels were used, spill over was automatically compensated via the BD FACS DIVA software.

**Intramacrophage survival**

At 2 hours, 24 hours, or 48 hours post uptake, bmm were lysed with 0.1% (vol/vol) triton-X100 and bacteria enumerated by serial dilution and CFU count. In parallel, bmm from one well were harvested by scraping and counted in a hemocytometer. Survival ratio was calculated as (CFU per bmm at final time point) / (CFU per bmm at 2 hours), and plotted relative to WT.

**Immunofluorescence microscopy**
Bmm were seeded onto 13 mm coverslips in 24-wells plates. Bmm were infected with WT (MOI 25) or ssaV (MOI 50) bacteria in a gentamicin protection assay. After 18 hours, 10 µg/ml MG132 (Sigma) was added to inhibit protein degradation by the proteasome. After 2 hours, bmm were washed with PBS and fixed using 4% (w/vol) PFA for 15 minutes at room temperature (RT) and quenched in 100 mM ammonium chloride for 1 hour at RT. Coverslips were washed twice in 0.1% (w/vol) saponin/PBS, incubated with the primary anti-HA antibody at RT in the dark for 2 hours, washed twice in 0.1% (w/vol) saponin/PBS, washed once in PBS, and incubated with the secondary antibody and 50 ng/ml DAPI (Invitrogen) for 45 minutes at RT in the dark. Then, coverslips were washed twice in 0.1% (w/vol) saponin/PBS, once in PBS and once in ddH₂O before placing into Aqua Poly/Mount (Poly sciences Ink) on a glass slide. Slides were imaged using a Zeiss LSM 710 confocal laser scanning microscope. The four colours were scanned sequentially.

Statistics

Statistical tests were performed with GraphPad Prism 7.0 as indicated in the figure legends, unless stated otherwise.
SUPPLEMENTARY TABLES

Table S1: Summary of dual RNA-seq datasets. Read counts aligning to mouse and \textit{Salmonella} genomes.

Table S2: Clustering analysis of differentially expressed mouse genes.

Table S3: Clustering analysis of differentially expressed \textit{Salmonella} genes.

Table S4: Interspecies correlation of SPI-2 (apparatus/effector) genes with pre-defined mouse gene sets. Gene Set Enrichment Analysis for the correlation between genes contained within defined macrophage gene sets (GO terms, KEGG pathways, Biocarta, GSEA Hallmark) and genes associated with \textit{Salmonella} SPI2 transolocon and effectors.

Table S5: Interspecies correlation of cluster III and IV with \textit{Salmonella} regulons. Gene Set Enrichment Analysis for the correlation between genes contained within \textit{Salmonella} regulons and genes contained within macrophage clusters III and IV. List of genes contained within each \textit{Salmonella} regulon (identified from RegPrecise and (16)) also included.

Table S6: Used strains, plasmids, primers, and antibodies.

Table S7: List of populations analysed or sorted by flow cytometry.

Table S8: Dual RNA-Seq FPKM values for all mouse and \textit{Salmonella} genes.

Table S9: Dual RNA-Seq pairwise differential expression analysis between any two challenged macrophage subpopulations.

SUPPLEMENTARY FIGURE LEGENDS

fig. S1 Detectable subpopulations

(a) The different bacterial sub-populations analysed throughout this study. The inoculum was a stationary phase population of \textit{Salmonella} containing a majority of growing bacteria and a minority of inactive non-growing bacteria (1:10$^{6}$). The intracellular populations of bacteria are either host-killed (HK), growing (G), or non-growing (NG). The NG population is further divided in inactive (iNG) and active (aNG) sub-populations.

(b) Macrophage subpopulations: bacteria carry a vector to express two different fluorescent proteins that are under the control of different promoters and differ in their stability upon killing of the bacteria. Also see Table S7.

\textit{(genta)} Gentamicin kills extracellular bacteria and leaves all intracellular bacterial populations intact. Bacteria express one constitutive (mCherry) and one inducible
fluorescent protein (GFP). Accumulation of the constitutive marker is observed in macrophages containing growing bacteria. Fluorescence of the pre-induced fluorescent reporter allows to ascertain only one uptake event occurred in each macrophage: one unit of fluorescence originating from a single non-growing bacterium or several growing bacteria diluting the signal but all adding up to one unit which is contained within one macrophage. Host-killed bacteria lose unstable GFP fluorescence faster than highly stable mCherry fluorescence.

(cefo) Cefotaxime kills extracellular and intracellular growing bacteria, thus enriches for non-growing populations in macrophages. Bacteria carry pFCcGi so that the constitutive marker (mCherry) allows detection of infected macrophages and induction of GFP expression in this case allows to reveal macrophages containing active non-growing bacteria. Macrophages without induction of GFP contain either inactive or non-viable bacteria.

(c) Bacterial subpopulations: bacteria carry a vector to express two different fluorescent proteins that are under the control of different promoters. Also see Table S7.

(pDiGi) Fluorescence Dilution of the pre-induced fluorescent reporter (GFP) allows discrimination between growing and non-growing bacteria. Induction of the second inducible fluorescent protein (DsRed) during infection discriminates inactive from active bacteria.

(pDiGssaG, genta with immunolabeling) Fluorescence Dilution of the pre-induced fluorescent reporter (DsRed) allows discrimination between growing and non-growing bacteria. Induction of GFP reports ssaG promoter activity during infection. Immunolabeling of bacteria is required to recognise bacteria from macrophage debris.

(pDiGssaG, cefo with immunolabeling) Induction of the inducible fluorescent protein (DsRed) during infection discriminates inactive from active bacteria. Induction of GFP reports ssaG promoter activity during infection. Immunolabeling of bacteria is required to discriminate bacteria from macrophage debris.

**fig. S2** Experimental setup to test the importance of activity for survival of non-growing *Salmonella* in macrophages.

Activity is tested through production of an inducible fluorescent protein for survival of non-growing *Salmonella* after their release from bmm. Regrowth on laboratory medium of sorted inactive (iNG), active (aNG) non-growing or growing (G) bacteria after 24 hours of infection.
fig. S3 Generating non-growing dormant *Salmonella*

(a) IPTG-induced GFP expressing stationary phase *Salmonella* (carrying pDiGi) culture was pre-treated with chloramphenicol, before being sub-cultured in fresh media containing arabinose with continued treatment with chloramphenicol to monitor translational activity. As a control, non-pretreated IPTG-induced GFP expressing stationary phase *Salmonella* (carrying pDiGi) culture was sub-cultured in fresh media containing arabinose for 90 minutes.

(b) Experimental set-up to test survival of translationally-blocked non-growing *Salmonella* generated through exposure to bacteriostatic concentrations of chloramphenicol (Cm) and subsequently exposed to bactericidal concentrations of cefotaxime in laboratory medium or bmm.

fig. S4 Intramacrophage non-growing *Salmonella* translocate SPI-2 effectors

(a) Translocation of HA-tagged SPI-2 effectors detected in cytosol or bacterial pellet after lysing the bmm. Infection was carried out for 20 hours in presence of gentamicin and arabinose was added during the last 4 hours to facilitate analysis of aNG. MΦ populations were sorted, lysed and fractions separated by SDS-PAGE and subsequent Western blot. * indicate detected effector.

(b) Translocation of an HA-tagged SPI-2 effector (SseL) detected by immunofluorescence confocal microscopy, 20 hours after uptake. The fluorescence dilution (FD) plasmid was used to discriminate non-growing (bottom) from growing bacteria (top). In parallel, bmm were infected with *ssaV* mutant bacteria (middle), which cannot translocate effectors.

fig. S5 Sorting strategy and dual RNA-seq

(a) (Left) Experimental design for infection, sorting, and dual RNA-seq. (Right) Gates used for sorting bystander bmm, bmm containing host-killed (HK), non-growing (NG), and growing (G) *Salmonella*.

(b) Bmm from the sorted populations visualised by confocal microscopy. GFP of non-growing *Salmonella* was as bright as that of the inoculum; GFP of growing *Salmonella* had been diluted between daughter cells; bmm containing host-killed bacteria showed mCherry fluorescence either dispersed throughout the cell (left, below detection levels
by microscopy), or still in the shape of a bacterium (right), whereas GFP fluorescence had been lost.

(c) Regrowth of *Salmonella* from sorted bmm. At 20 hours after uptake, bmm were sorted as in (a), lysed, and plated on LB-agar to enumerate surviving CFU. Error bars depict mean and SD. Colours of the symbols are the same as in the rest of the manuscript for clarity.

(d) Mapped reads as aligned to the individual genomes. Numbers depict the proportion of reads aligned to the *Salmonella* or mouse genome (N=3).

(e) RNA species recovered from each dual RNA-seq sample. Split to alignment to either host (mouse, top) or pathogen (*Salmonella*, bottom) genome.

**fig. S6 Dual RNA-seq analysis**

(a) Principal component analyses of macrophage (left) or *Salmonella* transcriptomes (right) using genes differentially expressed between any two subpopulations. The sorted subpopulations are colour coded and the three biological repeats indicated with different symbols.

(b) Z-score-normalised expression of representative M1 (left) and M2 (right) polarisation genes over all dual RNA-seq samples. Dots indicate mean of biological replicates and error bars represent standard deviation (SD). Statistical significance was tested by a paired ANOVA with multiple testing amongst all groups; adj. *p*-values are indicated.

(c) Clustering analysis of *Salmonella* genes differentially expressed between any two subpopulations. Selected GO or KEGG terms and representative genes per cluster are mentioned on the right.

(d) Expression of SPI-2-apparatus (left) and SPI-2-translocated effectors (right) genes in dual RNA-seq experiment. Depicted is a boxplot (box: median with upper and lower quartiles; whiskers: lowest and highest values). Statistical significance was tested by a paired ANOVA with multiple testing amongst each group; adj. *p*-values are indicated.

(e) Interspecies correlation analysis between M1 (left) and M2 (right) gene sets with 42 pre-defined *Salmonella* regulons in infected dual RNA-seq samples. Plot of the GSEA enrichment score (x-axis) and -log10 FWER-adjusted *p*-value (y-axis) based on correlations between Z-score-normalised *Salmonella* gene expression and average Z-score-normalised M1 or M2 expression (cluster III and IV, Fig. 2A).

(f) Boxplots depicting the distribution of correlations of interesting gene sets in (e). Box: median with upper and lower quartiles. Whiskers: lowest and highest values; FWER-
adjusted $p$-values from (e) are indicated.

**fig. S7 Intermediate M2 expression profile of the host cell reflects heterogeneity in non-growing *Salmonella***

(a) Principal component analysis of macrophage single-cell transcriptomes from (II) using genes from the extended macrophage polarisation gene sets (cluster III and IV, Fig. 2A).

(b) The distribution of IL4RA expression on bmm with inactive and non-viable (iNG/HK), active (aNG) non-growing *Salmonella* (representative sample, left) and the proportions of IL4RA positive bmm (multiple samples, right) after 24 hours of infection. Populations of bmm contained similar bacterial loads (Paired ANOVA, repeated testing amongst shown groups; adj. $p$-values are indicated; error bars depict mean and SD).

**fig. S8 M1 dampening and M2 promoting are independent**

(a) Expression of IFN-$\gamma$-induced markers CD86 and MHC-II on unpolarised and IFN-$\gamma$-polarised bmm. Expression of IL4RA is included as reference. (Error bars depict mean and SD; paired t-test; $p$-values are indicated.)

(b) mRNA expression of M1 genes upon infection of unpolarised or IFN-$\gamma$-polarised bmm at 24 hours after uptake. Relative expressions are measured by qRT-PCR and calculated by the delta-delta-CT method relative to expression of control genes ($b2m/hprt1/hmbs$) and unpolarised bystander bmm (paired ANOVA with multiple testing amongst shown groups; adj. $p$-values are indicated; error bars depict mean and SD)

(c) Experimental setup to track macrophage polarisation during acute systemic infection.

(d) mRNA expression of M2 genes as in (b)

(e) mRNA expression of M1 genes upon infection by WT or *steE* mutant *Salmonella* 24 hours after uptake. Relative expressions are measured by qRT-PCR and calculated by the delta-delta-CT method relative to expression of control genes ($b2m/hprt1/hmbs$) and bystander bmm exposed to WT *Salmonella* (paired ANOVA with multiple testing amongst shown groups; adj. $p$-values are indicated; error bars depict mean and SD)

(f) mRNA expression of M2 genes as in (e)

**fig. S9 *Salmonella*-induced polarisation shows mixed hallmarks of exogenous
polarisation with IL-4 and IL-10

(a) Expression of polarisation genes by bystander bmm and bmm containing growing (G) bacteria as measured in the dual RNA-seq experiment. Bars depict mean of the three biological repeats; error bars depict “logfoldStandard Error” as calculated in DESeq2.

(b) Expression of polarisation markers upon exogenous polarisation with classical M1 (LPS, IFNγ) and M2 (IL-4, IL-10) stimuli as measured by flow cytometry. Bars depict mean of at least three independent experiments; error bars depict SD; individual measurements are indicated.

fig. S10 SteE mutant Salmonella survives equally well in bmm as WT Salmonella

Survival (the result of intracellular killing and replication) of WT, steE mutant, and steE mutant Salmonella complemented with plasmid-encoded SteE after 24 hour-infection. For reference, two repeats of ssaV mutant Salmonella and ssaV mutant Salmonella complemented with plasmid-encoded SsaV are included. (paired ANOVA with multiple testing against WT on indicated data sets; adj. p-values are indicated; error bars depict mean and SD).
Figure 1: Activity is essential for antibiotic persistence of *Salmonella* during macrophage infection and involves SPI-2 T3SS activity.
Figure 2: SPI-2 predicted to dampen M1 and promote M2
Figure 3: Non-growing and growing Salmonella dampen M1 and promote M2 polarisation through SPI-2 T3SS effectors
Figure 4: Metabolically active persisters undermine host innate immune response and enable long-term survival.
figure S1: Detectable subpopulations

figure S2: Experimental set-up to test the importance of activity for survival of non-growing *Salmonella*
figure S3: Generation of dormant non-growing bacteria
figure S4: Translocation of SPI-2 effectors by NG *Salmonella*
figure S5: Sorting strategy and dual RNA-seq
a) Macrophages and Salmonella

b) M1 and M2 genes

c) Inoculum, NG, G

d) SPI-2 apparatus and effectors

e) Salmonella gene sets

f) Correlation with M1 and M2 genes

Figure S6: Dual RNA-seq analysis
figure S7: Intermediate M2 expression profile reflects heterogeneity in non-growing Salmonella
figure S8: M1 dampening and M2 promoting are independent
figure S9: *Salmonella*-induced polarisation shows mixed hallmarks of exogenous polarisation with IL-4 and IL-10
figure S10: steE mutant does not show a large replication / survival defect in Mφ (at 24h genta)