Proteomic analysis of *Salmonella*-modified membranes reveals adaptations to macrophage hosts

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Abbreviation

CLSM, confocal laser-scanning microscopy; ER, endoplasmic reticulum; LC, liquid chromatography; MTOC, microtubule-organizing center; MS/MS, tandem mass spectrometry; PCC, pathogen-containing compartment; SCV, *Salmonella*-containing vacuole; SIF, *Salmonella*-induced filaments; SMM, *Salmonella*-modified membranes; SPI, *Salmonella* pathogenicity island; T3SS, type III secretion system
Summary

Systemic infection and proliferation of intracellular pathogens require the biogenesis of a growth-stimulating compartment. The gastrointestinal pathogen *Salmonella enterica* commonly forms highly dynamic and extensive tubular membrane compartments built from *Salmonella*-modified membranes (SMMs) in diverse host cells. While the general mechanism involved in the formation of replication-permissive compartments of *S. enterica* is well researched, much less is known regarding specific adaptations to different host cell types. Using an affinity-based proteome approach, we explored the composition of SMMs in murine macrophages. The systematic characterization provides a broader landscape of host players to the maturation of *Salmonella*-containing compartments and reveals core host elements targeted by *Salmonella* in macrophages as well as epithelial cells. However, we also identified subtle host specific adaptations. Some of these observations, such as the differential involvement of the COPII system, Rab GTPases 2A, 8B, 11 and ER transport proteins Sec61 and Sec22B may explain cell line dependent variations in the pathophysiology of *Salmonella* infections. In summary, our system-wide approach demonstrates a hitherto underappreciated impact of the host cell type in the formation of intracellular compartments by *Salmonella*.

**Keywords:** immune cells/ immuno-proteomics/ intracellular trafficking/ *Salmonella*
Introduction

Macrophages are central players in innate and adaptive immune defense, directly neutralizing pathogens by phagocytosis and orchestrating responses of other immune cells. Beyond their role as guardians, macrophages themselves are also prime targets for several intracellular pathogens (1, 2). These pathogens evolved strategies to evade intracellular killing and are able to proliferate within macrophages.

*Salmonella enterica* strains are one of these pathogens, responsible for estimated 550 million incidences of salmonellosis with 155,000 annual deaths worldwide (3). Especially in immunocompromised and malnourished individuals, *S. enterica* infection can manifest in form of life-threatening systemic infections with mortality rates of 20-25% (4-6). After internalization by the host, *Salmonella enterica* subsp. enterica serovar Typhimurium (STM) modifies the phagosome in which it resides into the so-called *Salmonella*-containing vacuole (SCV) (7) to prevent its degradation by the host. Following an extensive interplay with the host endosomal system, the SCV matures into an extensive tubular network with certain properties of late endosomes (8, 13, 9, 10). The host remodelling processes are instigated in early stages by secreted virulence proteins, so-called effector proteins, of the *Salmonella* pathogenicity island 1 (SPI1) and subsequently substituted by SPI2 effectors (9, 11, 12). These effectors are responsible for the formation of an extensive network of *Salmonella*-induced tubules (SITs) arising from the SCV (13, 14). Within this tubular network, the *Salmonella*-induced filaments (SIFs) marked by lysosome-associated membrane protein 1 (Lamp1), are one of the most abundant tubular elements (9, 13). The fully developed compartment is here referred to as a pathogen-containing compartment (PCC) and this extensive maturation from a SCV to PCC is required for successful intracellular replication of STM (14, 15).

The origin and composition of host membranes forming the PCC is of special interest, as those components enable nourishment and protection, which ultimately stimulate STM proliferation. As a whole, we refer to the host membranes that have been acquired and modified by STM as *Salmonella*-modified membranes (SMM). While originally believed to mainly originate from the endosomal system, recent proteomic studies have identified a much more complex composition of the SCV as well as mature PCCs (16, 17). However,
these studies were conducted in HeLa cells and there is evidence that in macrophages the development of the PCC may be different (18-21). These differences likely contribute to phenotypic variations observed during the infection of macrophages by STM. For example, in HeLa cells STM frequently escape the SCV and replicate within the host cytosol (22-24), whereas in macrophages a stable population of cytosolic STM have not been detected (25-27). Replication rates of STM seem to vary with host cell types and studies suggest differences in effector protein expression and host target utilization (19, 20, 28-30). For example, STM target different small Rab GTPase when infecting macrophages compared to HeLa cells (31, 32). Together, these findings suggest that STM might hijack different host elements when infecting distinct cell types. As a consequence, it can be expected that the SMM composition will reflect these divergent host sources. However, only limited information is available regarding the SMM composition in infected macrophage cells.

Recently, we developed an affinity-based proteome approach for the systematic elucidation of the composition and sources of SMMs in HeLa cells (16). Here we applied a similar approach to investigate the SMM proteome in murine macrophages. A subsequent comparison of SMM proteomes from different cell lines provides insights into the variation of *Salmonella* PCCs and helps explain observed physiological variation in the intracellular life of *Salmonella*.
Experimental procedures

Chemicals. If not further indicated, chemicals were obtained from Sigma Aldrich.

Cell lines, bacterial strains and their cultivation. RAW264.7 (ATCC no. TIB-71) and RAW264.7 LAMP1-GFP cells (10) were cultured in Dulbecco’s modified Eagle medium (DMEM) containing 4.5 g × L$^{-1}$ glucose and 4 mM stable glutamine (Biochrom, Berlin, Germany) supplemented with 6% inactivated fetal calf serum (iFCS). HeLa (ATCC No. CCL-2) and HeLa LAMP1-GFP were maintained in DMEM containing 4.5 g × L$^{-1}$ glucose, 4 mM L-glutamine, sodium pyruvate (Biochrom) and 10% iFCS. Cell lines were incubated at 37 °C in an atmosphere containing 5% CO$_2$ and 90% humidity. *Salmonella enterica* serovar Typhimurium strains NCTC12023 (wild type, WT) or ΔsseF (HH107) and ssaV (P2D6), both harboring p3711 for synthesis of SseF-2TEV-2M45, were used in this study (details see suppl. Table S1). Strain ssaV is defective in the *Salmonella* pathogenicity island 2 (SPI2)-encoded type III secretion system (T3SS) and unable to translocate SPI2-T3SS effector proteins. For live cell imaging, WT and ssaV harboring pFPV25.1 or pFPV-mCherry/2 were used for constitutive expression of GFP or mCherry, respectively (Suppl. Table S1). *Salmonella* strains were routinely cultured in Lysogeny Broth (LB) broth at 37 °C with aeration containing 50 μg × mL$^{-1}$ carbenicillin (Roth) or 12.5 μg × mL$^{-1}$ chloramphenicol if required for the selection of plasmids.

Transfection constructs. To generate plasmids expressing diverse candidate host proteins, host genes were amplified from the cDNA clone library (ASU, AZ, USA) and via Gibson assembly N- or C-terminally fused to eGFP encoded on pEGFP-N1 or pEGFP-C1 (Clontech). Transfection vectors used in this study are summarized in Table S1. Localization of fusion proteins were validated in non-infected cells.

Infection of RAW and HeLa cells. RAW or RAW LAMP1-GFP cells were infected with overnight cultures of *Salmonella* with a multiplicity of infection (MOI) of 25 or 50 for SMM enrichment or immunostaining. Bacteria were centrifuged onto the cells at 500 × g for 5 min, and infection was allowed to proceed for further 25 min at 37 °C in an atmosphere of 5% CO$_2$. Subsequently, cells were washed thrice...
with warm PBS and incubated with medium containing 100 μg × mL⁻¹ gentamicin (AppliChem) to kill non-invaded bacteria for 1 h. Finally, the medium was replaced by medium containing 10 μg × mL⁻¹ gentamicin for the rest of the experiment. HeLa cells were infected with 3.5 h subcultures as described before (16). For live cell imaging, a MOI of 50 was used for both cell lines.

**Transient transfection of RAW cells and HeLa cells.** Cell lines were cultured for one day in 8 well chamber slides (Ibidi) and transfected with FUGENE HD reagent (Promega) according to manufacturer’s instruction. In brief, 0.5-1 μg of plasmid DNA was solved in 25 μl cell culture medium without iFCS and mixed with 1-2 μl FUGENE reagents (ratio of 1:2 for DNA to FUGENE). After 10 min incubation at room temperature (RT) the transfection mix was added to the cells in DMEM with 10% iFCS for at least 18 h. Before infection the cells were provided with fresh medium without transfection mix. Transfection constructs are listed in suppl. Table S1.

**Immunostaining.** Immunostaining was performed as described before (33). Briefly, infected RAW LAMP1-GFP cells (MOI 50) were fixed with 3% PFA at 4, 8, 12, and 16 h p.i., washed with PBS and incubated for 30 min in blocking solution (2% goat serum, 2% BSA and 0.1% saponin in PBS) before incubated with anti-M45 (1:500) as primary antibody and anti-mouse-Cy5 as secondary antibody (Suppl. Table S1) for 1 h at RT.

**Confocal laser scanning microscopy.** Before live cell imaging, medium was replaced by Minimal Essential Medium (MEM) with Earle’s salts, without NaHCO₃, L-glutamine and phenol red (BioChrom) but supplemented with 30 mM HEPES, pH 7.4. Fluorescence imaging was mainly performed using the Leica SP5 confocal laser-scanning microscope (CLSM) with live cell periphery, equipped with an incubation chamber maintaining 37 °C and humidity. Used objectives were 10× (HC PL FL 10×, NA 0.3), 20× (HC PL APO CS 20×, NA 0.7), 40× (HCX PL APO CS 40×, NA 1.25–0.75) and 100× objective (HCX PL APO CS 100×, NA 1.4–0.7) and the polychromic mirror TD 488/543/633 for the three channels GFP/ RFP/ BF (Leica, Wetzlar, Germany). The LAS AF software (Leica, Wetzlar, Germany) was used for setting
adjustment, image acquisition and image processing. For time-resolved experiments, spinning disk microscope (Zeiss Cell Observer with Yokogawa Spinning Disc Unit CSU-X1a 5000, Evolve EMCCD camera, Photometrics, USA) with live cell periphery was used, equipped with an Alpha Plan-Apochromat 63× (NA 1.46) oil immersion objective (Zeiss). Images were acquired with the following filter combinations: mTurquoise2 with BP 485/30, GFP with BP 525/50, mCherry with LP 580 and processed by the ZEN2012 (Zeiss) software. Scale bars for all acquired images were added with Photoshop CS6 (Adobe). 3D movies were generated with the software Imaris (Bitplane, Zürich).

**Quantitation by flow cytometry analyses.** RAW cells were infected either with *Salmonella ΔsseF* or *ssaV*, harboring p3711 for synthesis of SseF-2TEV-2M45, as described before. At 4, 8, 12 and 16 h p.i. cells were fixed with 3% PFA in PBS, permeabilized with 0.1% saponin in 10% iFCS/PBS, stained primary with anti-M45 (1:1,000) and secondary with anti-mouse IgG Alexa Fluor 488 (1:1,000) (Suppl. Table S1) for subsequent flow cytometric analyses using FACSCalibur (BD Biosciences). Experiments were performed in triplicates at least three times. Data were analysed with FACS Express 4 (De Novo Software). Statistical analyses were performed using Student’s t-test with SigmaPlot 11 (Systat Software).

**Immunoprecipitation, SDS-PAGE and Western blot.** SMM-enriched fraction and immunoprecipitation were prepared with some minor adjustments as described before (16). Briefly, about 1.6 × 10^8 RAW cells were used per immunoprecipitation (IP) and biological replicate. Before cell homogenization, infected host cells were rinsed thrice with PBS, scraped, resuspended in 10 ml osmostabilizing homogenization buffer (250 mM sucrose, 20 mM HEPES, 0.5 mM EGTA, pH 7.4), centrifuged at 1,000 × g for 10 min and resuspended in 1 ml of 4 °C pre-cooled homogenization buffer with 1 x protease inhibitor cocktail (Serva). Afterwards, host cells were mechanically disrupted with 0.5 mm glass beads (Scientific Industries) using the Vortex-2 Genie with Turbomix (Scientific Industries; 5 × 1 min strokes) with intermediate cooling. To remove non-lysed host cells, the lysate was centrifuged at 100 × g for 5 min at 4 °C. The immediate supernatant was centrifuged at 8,000 × g for 10 min at 4 °C to obtain the SMM-enriched fraction (pellet).
This pellet was then washed twice with pre-cooled homogenization buffer with protease inhibitor cocktail, before re-suspended in 500 μL homogenization buffer supplemented with 1.5 mM MgCl₂ and treated with DNaseI (50 μg × ml⁻¹) for 30 min at 37 °C. Protein concentration was afterwards determined via Bradford assay (BioRad). Per IP, 25 μl Protein G magnetic beads (GE) were coated with 40 μg purified anti-M45 antibody on a rotary shaker at 4 °C overnight. The beads were washed twice with PBS, cross-linked according to the manufacturer’s instruction and blocked for 30 min with 1% BSA in PBS at 4 °C. 500 μg of the SMM-enriched fraction were adjusted to a final volume of 200 μL in resuspension mix (1.5 mM MgCl₂, 10 mM KCl, 0.1% NP-40) and then incubated with 25 μl cross-linked anti-M45 antibody labelled Protein G magnetic beads on a rotary shaker at 4 °C overnight. To remove unbound proteins, the sample was five-times washed with 0.1% NP-40 in PBS. Finally, bound proteins were eluted in 25 μl 1 x SDS sample buffer (12.5% glycerol, 4% SDS, 2% mercaptoethanol, 50 mM Tris-HCl, pH 6.8). As before (16), proteins were separated on 12% SDS-PAGE for Western blotting and 4-12% gradient gels (NuPAGE Novex) for MS analysis using the NuPAGE MOPS buffer system.

Experimental design and statistical rationale. In total, six independent IP proteome experiments were performed; three biological replicates of RAW cells infected with STM ΔsseF [p3711] and three infected with the control strain STM ΔssaV [p3711], a mutant lacking (intracellular) replication and SIF network formation. Proteins were only considered as part of the SMM proteome if there were identified in at least in two biological replicates absent in the control (Suppl. Table S2). This comparison allows the identification of proteins specific to mature SMMs. Protein abundance was estimated using the exponentially modified protein abundance index (emPAI) and listed in suppl. Table S2. Statistical analyses of abundance differences were performed using Student’s t-test and adjusted for multiple hypothesis testing with the Benjamini-Hochberg procedure.

Protein digest, RP-LC separation, MS and data analysis. Digests, RP-LC and MS were conducted as described before (16). Briefly, eluted proteins were separated by SDS-PAGE, Coomassie Blue-stained,
sliced into 33 gel pieces and subjected individually to standard in-gel de-staining and trypsinolysis (34). Afterwards, digests were transferred into vials, resulting in a total of 198 digested samples. LC MS/MS analysis was performed using an UltiMate 3000 NCS-3500 nano-HPLC system (Dionex) controlled by Chromeleon chromatography software coupled to the AmaZon ETD speed ion trap MS with CaptiveSpray source (Bruker Daltonics). The UltiMate 3000 NCS-3500 nano-HPLC system (Dionex) was configured with a 2 cm PepMap 75-μm-i.d. C18 sample trapping pre-column (Thermo Fischer Scientific) and a 15-cm PepMap 75-μm-i.d. C18 microcapillary column (Thermo Fischer Scientific). 7 μL per samples were injected, trapped and separated by a 60-min linear gradient from 5 to 50% solvent B (80% ACN, 0.1% v/v formic acid) with 300 nL × min⁻¹ flow rate. For each MS scan, up to eight abundant multiply charged species in the m/z 400–1600 range were automatically selected for MS/MS but excluded for 30 seconds after having been selected twice. The HPLC system was controlled using Compass 1.5 (Bruker). Acquired MS/MS data were processed by the ProteinScape 3.1 software (Bruker) and searched against the UniProt Mus musculus database (12/2014, 16,696 entries) using the ProteinExtractor algorithm, a proprietary met-algorithm integrating Mascot Score (Bruker). Spectral data are available in PeptideAtlas (PASS01384, PASS01470, www.peptideatlas.org). Data analyses were conducted according the published guidelines (35). Mass tolerance values for MS and MS/MS were set at 0.8 Da and 4 Da. Fixed search parameters were tryptic digestion and miss cleavage up to 1. Variable search parameters used for the search were deamidation (NQ), carboxymethyl (C) and oxidation (M). Proteins were considered as identified with ProteinScape score >40 and two unique peptides with >95% confidence. Peptide Decoy (Mascot) and FDR was adjusted to 1% at protein and peptide level for all experiments.

All identified proteins were searched against the UniProt-GOA database (36). Only proteins identified in two biological replicates were considered as a candidate of the SMM proteome (Suppl. Table S2A) and grouped with PANTHER Classification System (www.pantherdb.org) (37) according their Gene Ontology (GO). To compare the composition of pathogen modulated host compartments from different pathogens
and hosts, the MGI vertebrate homology database to extract human-mouse homologues (www.informatics.jax.org/homology.shtml) was used (38).

**Functional analyses of SMM candidates.** We performed site-directed mutagenesis in transfection vectors containing genes encoding Rab GTPases Rab8B, Rab18 and Arl8B in order to create dominant negative (DN) alleles. The mutations were confirmed by DNA sequencing and the expression of the fusion proteins was confirmed by transient transfection of RAW264.7 macrophages. For transfection, plasmid DNA was isolated using Endofree plasmid preparation kits (Qiagen). RAW264.7 macrophages were cultured for one day in 12-well cell culture plates (Techno Plastics Products) and transfected with FUGENE HD reagent (Promega) according to manufacturer’s instruction. In brief, 21.5 μg of plasmid DNA was solved in 50 μl cell culture medium without iFCS and mixed with 43 μl FUGENE reagent (ratio of 1:2 for DNA to FUGENE). After 10 min incubation at room temperature (RT) the transfection mix was added to the cells in DMEM with 10% iFCS for at least 18 h. Before infection, the cells were provided with fresh medium without transfection mix. RAW264.7 cells were infected with STM WT or ΔssaV strains as described before. The bacterial strains harboured pFPV-mCherry for constitutive expression of mCherry. At 1 and 16 h p.i, macrophage cells were washed, detached from the culture plates, and analyzed by flow cytometry using an Attune NxT cytometer (Life Technologies, Thermo Fischer). At least 10,000 GFP-positive RAW264.7 cells were analysed, mCherry fluorescence intensity quantified and mean intensities for mCherry fluorescence were calculated.
Results

*SIF network formation and translocation of effector SseF in RAW macrophages*

First we characterized the SIF development in the murine macrophage cell line RAW264.7 (RAW). We infected the stably transfected RAW LAMP1-GFP cell line with wild type *Salmonella enterica* sv. Typhimurium (STM WT) constitutively expressing mCherry and visualized the infection process by live cell microscopy from 4-16 h post infection (p.i.). The lysosomal membrane-integral glycoprotein LAMP1 was used as marker to continuously monitor SIF development and SMM formation. As controls we used non-infected cells (mock), as well as cells infected with STM* ssaV*, a strain that is unable to translocate effector proteins, and thereby is deficient in inducing SIF network formation. As expected, we observed no indication of SIF network formation in the controls (mock and *ssaV* mutant) at any time point, whereas STM WT-infected macrophages showed small SIF networks at 8 h p.i. (Fig. 1). During the following hours, the network size in STM WT-infected macrophages increased until it reached its maximum at 12 h p.i. 3D-representation of maximum intensity projections revealed that at this time point SIFs were extensively branched and the network was completely enclosing the nucleus (Fig. 2). Furthermore, pulse-chase labeling with the endocytic tracer Dextran Alexa647 showed that most endocytic vesicles co-localized with LAMP1-GFP at the SIF networks and the SCV.

As we use the prominent membrane-integral translocated SPI2-T3SS effector protein SseF as bait for SMM enrichment (16), we next analyzed its presence in macrophages SIFs. We used flow cytometry for quantitation of the M45-tagged SseF and immunostaining to visualize its translocation in macrophages (Fig. 3). At 4 h p.i., we observed 2% effector-positive M45-stained macrophages (Fig. 3A). The rate increased to roughly 20% at 8 h p.i. and reached its maximum of around 30% at 12 h p.i. Afterwards the rate declined to roughly 20%. Measuring the relative fluorescence intensity (RFI) of the M45 signal enabled us to determine the relative amount of effector in proportion to M45-positive cells or all cells. The highest values for the M45-positive cells were obtained at 12 h and 16 h p.i. (Fig. S1A). In addition, RFI measurement of the M45 signal in the complete cell population displayed the highest RFI values at 12 h p.i. (Fig. S1B).
Immunostaining confirmed co-localisation of the bait protein SseF with LAMP1 and a maximum of SseF abundance in SIFs at 12 h p.i. (Fig. 3B).

In summary, in macrophages the maximal SIF network extension is reached at 12 h p.i., coinciding with the highest number of SseF-positive cells and amount of detectable effector per cell. Thus, we used this infection condition for SMM profiling in murine macrophages.

**SMM composition in RAW macrophages**

To determine the SMM composition in murine macrophages, we used the previously established three-step approach (16), in which cell purification/lysis is followed by intracellular compartment enrichment and affinity immuno-precipitation (IP) (Fig. 4A). Briefly, we infected ~1.6 × 10⁸ RAW cells with STM ΔsseF [p3711], or STM ssaV [p3711] as negative control and harvested cells at 12 h p.i. After homogenization, the SMM fraction was enriched by differential centrifugation. We confirmed the presence of the tagged effector SseF-2TEV-2M45 in the SMM-enriched fraction via Western blotting (Suppl. Fig. S2A), before the samples were used for IP followed by LC-MS/MS analyses. We profiled six IP preparations, three biological replicates of RAW cells infected with STM ΔsseF [p3711] and three infected with the control strain STM ssaV [p3711], a mutant lacking replication and SIF network formation. Protein samples were pre-separated by SDS gel electrophoresis and bands were excised and digested for LC-MS/MS analyses. In total we identified 728 host proteins (Fig. 4B). Of these, 212 proteins were also detected in the negative control proteome of cells infected with STM ssaV. Statistical analysis of their protein abundance revealed no significant difference between the control proteins and the STM ΔsseF [p3711] of infected RAW proteins. Accordingly, the shared proteins do not appear to be specific to the infection process and were not further considered. We note that without adjustment for multiple hypothesis testing we found an increase predominantly of ribosomal and ER-associated proteins.

Further 232 proteins were only observed in one biological replicate, resulting in a final reproducible set of 262 host proteins as the SMM proteome isolated from macrophages (Suppl. Table S2).
We then classified the identified macrophage SMM proteins according to their predicted or experimentally determined subcellular location (GO cellular component) and their involvement in biological functions (GO biological process) using the PANTHER classification system (37). These analyses revealed that most of the identified SMM proteins are part of vesicles (58%). In addition, a significant number of SMM proteins was found to originate from diverse host organelles, including mitochondria (32%), nucleus (19%), ER (13%), ribosomes (13%), endosomes/lysosomes (10%), Golgi (8%) and the cytoskeleton (21%) (Fig. 4C). Comparing the distribution using quantitative values yielded similar results with a slight increase of proteins associated with nucleus (24%) and ribosomes (21%) (Suppl. Fig. 2B). Many of the identified SMM proteins are involved in transport processes (40%, Fig. 4D). A closer analysis of these proteins revealed that among the transport proteins a plurality is involved in vesicle-mediated and protein transport. A smaller subgroup is specifically involved in the directed transport from Golgi to ER, intra-Golgi or ER to Golgi, endosomal trafficking, endocytosis, recycling, and exocytosis processes (Fig. 4E).

As we already generated an SMM proteome data set for HeLa cells (16), we proceeded to compare both SMM proteomes. In both studies we identified a similar number of SMM proteins (RAW: 262 and HeLa: 247) and observed a similar distribution according to PANTHER GO cellular compartment and biological processes (Suppl. Fig. S3A, B). Minor differences were found in nuclear and vesicle-related proteins as well as number of proteins involved in translation and transport. However, when focusing on transport-related processes, overall fewer proteins involved in vesicle-mediated transport were identified in infected macrophages (11%) compared to HeLa cells (22%) (Fig. 4E). Likewise, exocytic pathways (2% in RAW and 11% in HeLa) as well as transport from ER to Golgi (2% in RAW and 6% in HeLa) were also less prominent at PCCs in macrophage. A comparison of the identified proteins on the individual level only showed a 19% overlap between the identified SMM proteins (Suppl. Fig. S3C). A closer analysis revealed that part of the difference can be attributed to the identification of different subunits from multimeric protein complexes in the SMM proteomes. When we group different subunits of a given protein complex together, we found that the overlap between the two data sets increased to 59%. As expected, among the proteins
identified in both SMM proteomes a high proportion were components of diverse host trafficking routes, such as elements of the endocytic and secretory vesicle transport including small GTPases Rab5c, Rab14A, Rap1A, RalA and G3bp2, components of the COPI-mediated transport (e.g. CopA, CopG1) or clathrin-mediated endocytosis as the adaptor protein complex (e.g. Ap2B1). Likewise, cytoskeletal components known to mobilize vesicles and stabilize PCC such as myosin-9, dynein, α-actinin-1, Arp2/3 and the actin capping complex, were prominent in both cell lines. In contrast, we did not find any evidence for an interference of STM in vesicle trafficking mediated by the COPII system in infected RAW cells, which is in contrast to our previous proteomic data in HeLa cells. In addition, we identified organelle-associated proteins such as the ER proteins Rpn2, VapA and Tmed10 or the mitochondrial protein Trap1 in both studies.

Together, the data suggest that STM targets a similar core set of host membrane sources in both cell lines, but also seems to fine-tune them on the individual protein level to the respective host cell type. In addition, the proteomic investigation also suggests a significant impact of non-canonical host elements such as the ER and mitochondria.

**Live cell imaging of SMM proteins**

To elaborate on the proteome data, we conducted live cell imaging with selected SMM candidates identified by our affinity-based proteome approach as chemical fixation rapidly fragment the mature PCC. We designed transfection vectors for expression of GFP-fusions of 18 SMM candidates from different known trafficking routes and host organelles. We then transiently transfected RAW cells with those GFP-fused SMM candidates and monitored expression and cellular localization of the GFP-fusions in non-infected host cells to establish their regular expression pattern (Suppl. Fig. S4). Subsequently, we analyzed the localization of these SMM candidates during STM infection. We infected transiently transfected RAW cells with STM WT constitutively expressing mCherry for visualization of the pathogen, and pulse-chased host
cells with endocytic fluid tracer Dextran Alexa647 to follow the endocytic traffic. The first host proteins we analyzed were part of various vesicle trafficking systems previously found to be involved in PCC formation and maturation in *Salmonella*-infected HeLa cells (16, 39). These include small Rab, Arf and Ras-like GTPases (Rab5c, Rab14, Arf1, Arf3, Rap1), components of the COPI-mediated transport (CopG) and lysosomal traffic (Arl8B and Ifitm3), parts of the retromer complex moderating the transport from endosomes to the trans-Golgi network (Vps35) and the adapter protein complex 2 for endocytic secretory pathways (Ap2B1). In all cases, we observed recruitment of these proteins to the *Salmonella* PCC in infected macrophages between 10-12 h p.i. (Fig. 5A, Suppl. Fig. S5). Thus, the live cell imaging study confirmed the proteomic results and demonstrates vesicle recruitment from various trafficking routes during the PCC maturation in macrophages directed by STM.

Interestingly, the proteomic investigation of the SMM in macrophages, as well as in HeLa cells in our previous study (16), suggests that membranes from organelles such as the ER and mitochondria may also be redirected to the PCC by STM. To investigate this finding in more detail we analyzed the recruitment of three integral ER proteins, the dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2 (Rpn2), the vesicle-membrane-protein-associated protein A (VapA), and the transmembrane emp24 domain-containing protein 10 (Tmed10), as well as the mitochondrial protein Trap1 in infected HeLa as well as RAW cells via life-cell imaging. For this investigation, we transiently transfected HeLa or RAW cells for synthesis of fusion proteins of GFP and the respective SMM proteins, and monitored the infection process as previously described. In HeLa cells we detected these organelle marker proteins closely located to the PCC 8 h p.i. (Suppl. Fig. S6), whereas in RAW cells the same proteins were detected between 10 and 12 h p.i. (Fig. 5B). Thus, the data indicate that in both cell lines mitochondria and ER contribute to the maturation of STM PCCs. However, the timing appears to be different, with the maturation of the PCC in macrophages being delayed by approximately 4 h compared to epithelial cells.

Next, we focused on differences between the SMMs formed in the two cell lines. Based on the proteome data, the SMM in macrophages seems to lack components of the retrograde vesicle transport, like for
instance the small GTPases Rab2A, Rab11 and Sar1A. Using live cell imaging we could clearly detect these proteins at the PCC in HeLa cells (Fig. 6A). However, the PCC in macrophages was devoid of these proteins, indicating that they are indeed only manipulated by STM in epithelial cells.

We further investigated proteins that were only found in SMMs of macrophages. Here we focused on the Rab GTPase Rab8B, the mitochondrial protein Aifm1 and ER transport proteins Sec61 and Sec22b. Live cell imaging validated the localization of these proteins at the PCC in RAW cells but they were absent in HeLa cells (Fig. 6B). Thus, the data suggest that while *Salmonella* accesses similar structures in its host, there are subtle differences in the specific routes that are manipulated. We then set out to investigate the potential impact of three Rab GTPases for the intracellular life-style of *Salmonella*. Here, we focused on Rab8B and Rab18, which were exclusively identified in SMM proteome of macrophages and ARL8B, which was identified in SMM proteomes of both RAW and HeLa cells. To perturb GTPase function, we generated dominant-negative (DN) versions, and expressed WT as well as mutant alleles of those GTPases as GFP fusions in transiently transfected cells.

Since efficiency of transfection of macrophages generally is rather low, and transfection often reduces the efficacy of bacterial uptake, we developed a flow cytometry-based approach that allowed selective analyses of the transfected and STM-infected subpopulation of RAW cells (Suppl. Fig. S7). STM infection was performed with strains constitutively expressing mCherry, and the intensity of mCherry fluorescence served as proxy for phagocytosed STM and intracellular proliferation. We gated on GFP-positive RAW264.7 cells to identify transfected cells and quantified the mCherry fluorescence. We observed that perturbation of the GTPase function of Rab8B, Rab18 and Arl8B led to a mildly increased intracellular proliferation of STM in infected cells.

In sum, our systematic approach revealed communalities as well as compelling differences in the infection process both host cell types, which we summarized in Fig. 7.
Discussion

*Salmonella* is able to infect diverse hosts and cell types and commonly forms characteristic, highly dynamic and extensive tubular membranous PCC which are required for intracellular replication (13, 14). While it is well established that STM builds a replication-permissive niche in diverse host cells by deploying a complex set of effector proteins, the PCC composition and their cellular origin are only partially explored. Until recently, identification of STM PCC components were mainly executed by fluorescence imaging (immunostaining, GFP-fusions) (18, 31, 40-44) limiting the analyses per study onto a few host proteins. Recently established proteomic approaches enabled a more systematic viewpoint on PCCs (16, 17, 45). In addition, an increasing number of studies revealed that STM modifies the expression of its effector repertoire depending on the infected host cell type (19, 20, 46, 47), suggesting a careful modulation of the PCC biogenesis in different host cells. To elaborate this hypothesis, we applied our recently established affinity-based proteome approach to profile the SMM composition of STM-infected murine macrophages and compared it with the SMM from the epithelial cell line HeLa (16).

Our data highlight communalities between the compartment in epithelial and phagocytic cells, but also provide evidence for cell-specific adaptations in STM PCCs. A common strategy for biogenesis of PCCs is the manipulation of host traffic routes (48). It was therefore not surprising that the main components of SMM proteomes in HeLa and RAW cells are transport-associated components indicative for diverse intracellular host membrane trafficking routes (49). Conserved elements found in the PCC of HeLa as well as RAW cells were for instance the small Rab GTPases Rab5c and Rab14. Rab14 is known to modulate traffic between the Golgi and endosomes (50-52) and Rab5 plays a key role in the early endosome and phagosome maturation (50). Both have previously been shown to be essential for intracellular replication of STM in epithelial cells and phagocytic cells (53, 54), which is further supported by our proteomic surveys.
In our studies, we also found conserved trafficking routes whose functions were previously only analyzed in epithelial cells. Examples include proteins of the retromer-mediated protein sorting system (Vps35), which controls the endosome to Golgi retrieval pathway, the COPI-mediated transport (α/β2/δ/ε/γ1-COP), which is responsible for vesicle transport between cis-Golgi back to the ER, the endosomal-lysosomal trafficking via Arf GTPase Arl8B and the clathrin-mediated endocytosis facilitated via adapter protein complex 2 (Ap2B1).

The manipulation of these routes seems to confer diverse benefits to the STM infection process, though their impact is only partially understood. Manipulation of the retromer or endolysosomal system for example, affects PCC dynamics by enhancing PCC growth as well as allow its migration to the cell periphery and promoting cell-to-cell spread of the bacterium (39, 40, 55). However, at least in epithelial cells it is still disputed whether these mechanisms are essential for intracellular survival (39, 56). Likewise, by influencing COPI transport and clathrin- and adapter-mediated endocytosis, STM is able to enhance effector protein translocation and reduce cellular antigen presentation, respectively (57, 58). The relevance of these systems has, to the best of our knowledge, not been studied in macrophages. Since in our study we found these marker proteins to be present in STM-infected HeLa as well as RAW cells, it is likely that they play similar roles in both host cell types.

We also identified differences in the endosomal contribution to PCC formation in dependence on the infected cell line. Based on our proteome and microscopy data, some SMM elements that were considered to be canonical in STM appear to be specific to endothelial cells and seem to be less relevant in macrophages. For instance, the PCC of HeLa cells showed accumulation of small GTPases Rab2A and Rab11 (16, 31), whereas they were absent in the SMM proteome as well as during live cell imaging of infected RAW cells. Both GTPases are known key players of protein recycling. Rab2A associates with the vesicular tubular clusters and sorts anterograde-directed cargo from recycling proteins to the ER (59). Rab11 regulates early trafficking into recycling endosomes (60). Targeting these Rab GTPases is essential for intracellular replication for other intracellular bacteria such as *Brucella* (61) or *Chlamydia* (62),
respectively. However, limited studies in STM indicate this may not be the case for STM (43). Even if they are relevant for STM in HeLa cells, our data suggest that they do not appear to play a role within the mature PCC in RAW cells.

More information is available on the role of the GTPase Sar1A, which was also found to be a target for STM in HeLa, but not in RAW cells. Sar1A is a key regulator of the COPII transport (63) and it was shown earlier that COPII complexes accumulate on the early SCV membrane in HeLa cells (17). These COPII complexes destabilized the SCV membrane through an unknown mechanism, permitting STM to escape the SCV and hyper-replicate within the cytosol of epithelial cells. The absence of Sar1A at the PCCs of infected macrophages could stabilize the PCC and thereby explain the lack of cytosolic persistence of STM in this cell line. Consequently, COPII-mediated interactions may be a factor determining whether STM persist stably in the PCC or establishes a population in the host cell cytosol.

We also observed a number of trafficking markers that were only present at the PCC of macrophages. For instance, Rab8 and Rab18 were detected at the PCC of macrophages in this study, but were absent at the PCC in HeLa cells (16). Similar observations were made by Smith et al. (31) who found both Rab GTPases recruited to the early SCV (60 min p.i.) in macrophages, but not in HeLa cells. Our study therefore indicates that this difference persists late into the mature PCC. Both GTPases are suggested to allow the STM PCC to maintain early endosomal characteristics and thereby enable persistence in the SCV (28, 47, 64). We furthermore could show that disruption of both GTPases led to slightly increased intracellular proliferation of STM indicating that these GTPase might be part of host cell mechanisms that control proliferation of intracellular STM. Often these systems are involved in phagosomal maturation (such as e.g. Rab32). This restriction seems to be relieved by inhibition of the function of Rab8 and Rab18 as molecular switch. It is not certain why this mechanism is not required in HeLa cells, but there are regulatory differences of both Rab GTPases between epithelial and phagocytic cells (65) which may be a contributing factor.
Another interesting observation involves the origin of the redirected host components. In both SMM proteomes, we detected endosomal, lysosomal, Golgi, ER, mitochondrial and nuclear membrane proteins, indicating an intriguing complexity of pathogenic interactions with these host organelles. In STM the role of mitochondrial proteins and ER components at the PCC is underexplored, which is in contrast to several other intracellular pathogens such as Legionella, Mycobacteria, Simkania and Chlamydia (45, 66-68). For instance, the vacuole of Legionella pneumophila is known to fuse with ER-derived secretory vesicles, associate with mitochondria, and later interact with ER membranes (69, 70). Chlamydia recruits exocytic vesicles, ER-derived lipid droplets and forms tight connections with mitochondria (71, 72). Moreover, Chlamydia caviae uses mitochondrial transporters to translocate its effector proteins directly inside mitochondria to stimulate its vacuole biogenesis and generate infectious bacterial progeny (73). Other pathogens like the protozoan parasite Encephalitozoon cuniculi cluster their replicative compartments over mitochondrial porins to scavenge ATP directly from the host (74). Considering that a direct interaction with these host organelles and compartments is crucial in a diverse group of intracellular pathogens, it is intriguing to speculate about a similar but unrecognized role for STM. The proteomic comparison of HeLa and RAW SMM proteomes provides some tentative support for this notion. In both studies, we observed that the mature PCC maintains close contact with mitochondria. We further observed the presence of the mitochondrial protein Trap1 in the SMM proteome of both host cell lines. Trap1 is a member of the HSP90 family which controls a variety of physiological functions, including cell proliferation, differentiation, and cellular survival. It is best known for its anti-apoptotic role (75, 76), but it has not been investigated further in Salmonella infections yet. We further observed several ER proteins as part of the SMM proteome, such as the dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit 2 (Rpn2), which is part of the proteasome complex at the ER. Rpn2 interacts with Trap1 (75) and assists in refolding of damaged proteins to prevent apoptosis (77). Other ER proteins found to be conserved in the SMM of RAW and HeLa cells include the vesicle-membrane-protein-associated protein A (VapA) and the transmembrane emp24 domain-containing protein 10 (Tmed10). Tmed10 assists in Golgi-ER trafficking and was observed to be exploited...
by cytomegalovirus to delay immune recognition (78). The integral ER protein VapA participates in establishing ER contacts with multiple membranes by interacting with different tethers. Furthermore it was recently shown that depletion of VapA impairs autophagosome biogenesis (79). Together the data might suggest that STM interacts with ER and mitochondrial components in order to modulate and delay host functions such as apoptotic or immune responses. However, further studies are required to validate these functions.

Yet, despite these overarching similarities in the utilization of host organelles, it appears that STM also targets different sub-elements of these organelles, depending on the host cell type. For instance, in our data set we detected mitochondrial and ER proteins that appear to be specific to colonization of macrophages. Examples include the mitochondrial protein Aifm1, which is involved in regulation of apoptosis, as well as the ER proteins Sec22b and Sec61β. Intriguingly, Sec22b and Sec61β were also found to be important for other intracellular pathogens during infection of macrophages. In Legionella Sec22b partakes in the biogenesis of the Legionella-containing vacuole (80, 81). Additionally, manipulation of Sec22b was also essential for the formation of the parasitophorous vacuoles of the intracellular parasites Leishmania pifanoi and L. donovani (82, 83), indicating that the manipulation of antero- and retrograde transport between ER and Golgi may be a common target for the infection of macrophages by pathogens. Sec61β controls protein transport into the ER and was identified as part of Legionella- as well as the Brucella-containing vacuole in macrophages (68, 84). For these bacteria, interaction with Sec61β was associated with the avoidance of macrophagosomal killing of the pathogen. Overall, there is increasing evidence that intracellular pathogens, including Salmonella, target conserved as well as macrophage-specific pathways that are not utilized to the same degree in other cell lines.

In summary, this study provides compelling insights into the maturation and maintenance of the PCC of STM inside murine macrophages and highlights cell type-specific adaptation of its niche host cells. The data revealed not only common host traffic routes usurped by the pathogen, but also the selective retention of specific Rab proteins and divergent acquisition strategies of ER proteins by re-routing of ER trafficking
in HeLa cells and ER mediated phagocytosis in macrophages. Furthermore, the study opens up numerous targets for future investigations to elucidate cell type-specific adaptations.

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**Data availability**

Spectral data are available at the PeptideAtlas deposit (PASS01384 and PASS01470; [www.peptideatlas.org](http://www.peptideatlas.org)).

**Author’s contributions**

SV, NH and MH designed the experiments; SV, TR and VL performed experiments; SV, TR, VL and NH, provided the figures; SV and NH analyzed data and NH, SV, TC and MH wrote the manuscript.

**Conflict of Interest**

The authors declare that they have no conflict of interest.
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Figure legends

Fig. 1. SIF network in STM infected macrophages is maximal extended at 12 h p.i.
RAW cells expressing LAMP1-GFP (green) were infected with STM WT or ssaV expressing mCherry (red). Regular LAMP1-expression was monitored in uninfected cells (mock). Live cell imaging was performed every hour from four to 16 h p.i. Representative images of a STM-infected or non-infected cells are shown. Scale bar: 5 μm.

Fig. 2. Salmonella forms a complex SIF network in macrophages.
A, RAW cells expressing LAMP1-GFP (green) were pulse-chased with fluid tracer Dextran Alexa 647 (red) and infected with STM WT expressing mCherry (STM, blue). Live cell imaging was performed 12 h p.i. Endocytic fluid tracer Dextran Alexa647 co-localizes with LAMP1-GFP positive SIF structures. Merge is shown as maximum intensity projection (MIP); magnifications are presented as individual layer.
B, 3D visualization of SIF network as MIP. Scale bar: 10 μm.

Fig. 3. SseF effector accumulation and SseF-positive macrophages are maximal at 12 h p.i.
A, percentage of infected RAW cells positive for M45 were determined at indicated time points by flow cytometry. Shown are the mean values (+ standard deviation) of three independent experiments. B, stable transfected RAW cells expressing LAMP1-GFP (green) were infected with STM ΔsseF synthesizing SseF-2TEV-2M45 (SseF, blue) and mCherry (STM, red), fixed and immuno-stained with anti-M45 and anti-IgG-Cy5 at 4, 8, 12, and 16 h p.i. Representative micrographs are shown as maximum intensity projection. Scale bar: 5 μm.

Fig. 4. Global analyses of the macrophages derived SMM proteome.
SMM proteome in macrophages

A, scheme for SMM profiling. B, Venn-diagram of identified proteins. In total 728 proteins were identified, of which 262 were reproducibly identified as part of SMM. C, classification of the 262 SMM proteins according to PANTHER Gene Ontology (GO) subcellular component. D and E, categorization of identified SMM proteins according to PANTHER GO biological processes as well as transport processes. Proteins in C - E can have multiple assignments.

Fig. 5. SMM proteins are localized at SCV or SIFs in infected macrophages.

RAW cells were transfected with plasmids encoding GFP-fusions of diverse host proteins (green) involved in A. different trafficking processes such as Rab5c, Rab14A, Arf1, Arf3, Rap1, CopG, Arl8B, Ifitm3, Vps35 and Ap2B1 or B. organelle marker proteins including Rpn2, VapA, Tmed10 and Trap1. Before visualization by CLSM, cells were pulse-chased with Dextran Alexa647 (red) and infected with Salmonella WT continuously expressing mCherry (STM, blue) for 10-12 h p.i. Dextran Alexa647 served as a marker for PCC. Representative overview images (large) are shown as maximum intensity projections. White squares indicate a magnified area. In magnifications, an individual z-plane for each channel is shown. Scale bars: 10 μm (overview); 2 μm (magnification).

Fig. 6. Differences between SMMs formed in phagocytic and epithelial host cells.

A, CLSM of transfected HeLa and RAW cells show recruitment of small GTPases Rab2A, Rab11 and Sar1A to the PCC in HeLa cells, absent in macrophages. B, re-location of SMM proteins Rab8B, mitochondrial protein Aifm1 and ER transport proteins Sec61 and Sec22B to the PCC of RAW cells. CLSM were performed as described before. Shown are presentative overview images (large) and magnifications of marked areas displayed as individual channel. Scale bars: 10 μm (overview); 2 μm (magnification).
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The model indicates communalities (grey) and differences (blue, only observed in RAW; violet, only observed in HeLa) in trafficking proteins relocated to the SMM during the infection of HeLa and RAW cells.
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