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Intracellular *Salmonella enterica* Redirect Exocytic Transport Processes in a *Salmonella* Pathogenicity Island 2-Dependent Manner

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During intracellular life, *Salmonella enterica* proliferate within a specialized membrane compartment, the *Salmonella*-containing vacuole (SCV), and interfere with the microtubule cytoskeleton and cellular transport. To characterize the interaction of intracellular *Salmonella* with host cell transport processes, we utilized various model systems to follow microtubule-dependent transport. The vesicular stomatitis virus glycoprotein (VSVG) is a commonly used marker to follow protein transport from the Golgi to the plasma membrane. Using a VSVG-GFP fusion protein, we observed that virulent intracellular *Salmonella* alter exocytotic transport and recruit exocytotic transport vesicles to the SCV. This virulence function was dependent on the function of the type III secretion system encoded by *Salmonella* Pathogenicity Island 2 (SPI2) and more specifically on a subset of SPI2 effector proteins. Furthermore, the Golgi to plasma membrane traffic of the shingolipid C_5-ceramide was redirected to the SCV by virulent *Salmonella*. We propose that *Salmonella* modulates the biogenesis of the SCV by deviating this compartment from the default endocytic pathway to an organelle that interacts with the exocytic pathway. This observation might reveal a novel element of the intracellular survival and replication strategy of *Salmonella*.

Key words: bacterial virulence, cytoskeleton, intracellular pathogen, type III secretion

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Intracellular bacterial pathogens have evolved various virulence strategies to escape antimicrobial activities of infected eukaryotic cells and to proliferate within host cells. *S. typhimurium* is a facultative intracellular pathogen of man and animal that gains access to non-phagocytic cells via an invasion mechanism or survives phagocytosis by professional phagocytes such as macrophages or dendritic cells. Within a variety of infected host cells, *S. typhimurium* replicates within membrane-bound vesicles, commonly referred to as the *Salmonella*-containing vacuole (SCV) (1–3).

Previous studies indicated that virulent *S. typhimurium* interfere with the normal endocytic pathway of internalized material. The SCV has been characterized as a unique compartment that has acquired membrane markers characteristic of late endosomal/lysosomal vesicles and acidifies to a pH below 5.0. However, the SCV does not mature into a phagolysosome that ultimately would result in killing and degradation of the bacteria (2). Therefore, the SCV is commonly considered as a 'deviant phagosome'. Previous studies have also shown that intracellular *Salmonella* can manipulate host cell transport processes to avoid antimicrobial defense mechanisms (4,5).

A large number of virulence determinants contribute to the intracellular phenotypes of *S. typhimurium*. However, of central importance for the active manipulation of host cell processes is the function of a type III secretion system (T3SS) encoded by *Salmonella* Pathogenicity Island 2 (SPI2) (3). The SPI2-encoded T3SS (SPI2-T3SS) is specifically activated by intracellular *Salmonella* and translocates a group of effector proteins across the membrane of the SCV. Although the individual functions of the various effector proteins are largely unknown, the role of a subset of effectors in modification of vesicular transport has been observed.

In *Salmonella*-infected epithelial cells, so-called *Salmonella*-induced filaments (SIFs) are induced dependent on the function of the SPI2-T3SS and effector proteins SifA (6), SseF, and SseG (7,8). SIFs are tubular aggregates of lysosomal glycoprotein (lgp)-containing membranes that form along a scaffold of microtubules (9,10).

We have previously shown that a subset of SPI2 effector proteins, namely SseF and SseG, are targeted to microtubules (10). Furthermore, the infection with *S. typhimurium* can cause dramatic alterations in the host cell microtubule cytoskeleton such as condensation of microtubules around the SCV (11) and formation of massive bundles of microtubules (10). Interference with microtubule transport processes and the functions of motor proteins was observed by a number of research groups (11–13). It was shown that the intracellular proliferation required the function of kinesin and dynein (11), but more recent data suggest that

Salmonella prevents the excessive recruitment of kinesin to the SCV by action of SifA (14).

Previous work also suggested a possible interaction of intracellular *Salmonella* with the exocytic pathway, an assumption mainly based on the localization of the SCV in the vicinity of the Golgi apparatus in infected HeLa cells (15). It has been reported that expression in eukaryotic cells of the putative SPI2-T3SS effector SpiC causes a destabilization of the Golgi (16). Exocytic transport processes depend to a large extent on the movement of cargo vesicles along microtubules. For example, vesicles containing protein cargo bud off from the *trans*-Golgi network (TGN) and move along microtubules for anterograde transport.

In this study, we investigated the effect of intracellular *Salmonella* on microtubule-dependent transport processes. Our experiments revealed that in addition to modifications of the endocytic pathway, intracellular *Salmonella* can redirect exocytic transport to the SCV. These novel observations revise the understanding of the intracellular lifestyle of *S. typhimurium*.

Results

Intracellular Salmonella interfere with microtubule-dependent transport

We previously reported the effect of intracellular *S. typhimurium* on the structure of the microtubule network and defined a role of the SPI2-T3SS for these effects (10). We hypothesized that the interference of *Salmonella* with microtubule organization might affect microtubule-dependent transport processes in the host cell. Several model systems have been applied to study microtubule-dependent transport processes, and a commonly used marker is a temperature-sensitive (ts) derivative of the vesicular stomatitis virus glycoprotein (VSVG) (17). VSVG is located in the envelope of the vesicular stomatitis virus (VSV) and is essential for the binding and fusion of the virus envelope with the host cell membrane. After intracellular expression, VSVG traffics via the ER and Golgi to the cytoplasmic membrane of the host cell, where the assembly of the virus particle takes place (18,19).

Here, we applied VSVG as a marker to follow microtubule-dependent transport processes. To elucidate the influence of *Salmonella* infection on VSVG trafficking, we transfected HeLa cells with the pVSVG3-EGFP plasmid, expressing an EGFP fusion to the temperature-sensitive VSVG-ts045 protein (20). The localization of VSVG-containing compartments in relation to the microtubule cytoskeleton was analyzed in *Salmonella*-infected cells as well as in non-infected control cells. As observed in previous studies using VSVG as a marker, an association of a minor fraction of the VSVG-containing compartments with microtubules was observed (Figure 1A). This association was not altered after infection with *Salmonella*.

To investigate the localization of SIF and VSVG-containing compartments, transfected HeLa cells were infected with *S. typhimurium* wild type, fixed at 16 h after infection, and stained with an anti-LAMP-2 antibody to visualize SIF. Although we observed the association of VSVG-positive compartments with SIF, especially in areas of filaments with increased diameter, the majority of SIF-positive cells did not show an association. Furthermore, VSVG-positive compartments were not always co-localized with LAMP-2-positive structures when present in the same filament (Figure 1B). The data show that VSVG transport occurs along microtubules despite the intracellular presence of *Salmonella* and that SIFs acquire VSVG-containing compartments to a low extent.

Intracellular Salmonella redirect transport of secretory vesicles

In the course of these experiments, we noted that in *Salmonella*-infected cells, VSVG was not randomly distributed or localized in the plasma membrane as observed in non-infected control cells. Surprisingly, the majority of VSVG-containing compartments were associated with microcolonies of intracellular *Salmonella* (Figure 1A,B). This unexpected observation prompted us to investigate the effect of intracellular *Salmonella* on exocytotic transport in more detail. At approximately 20 h after transfection, the cells were infected with *S. typhimurium* wild type expressing the red fluorescent protein (RFP). Differences in VSVG distribution between non-infected and infected cells were examined at different times after infection by immunofluorescence microscopy. In non-infected cells, VSVG-EGFP was predominantly found in vesicular compartments throughout the host cell cytoplasm with highest concentrations in the perinuclear region. By contrast, we observed the recruitment of VSVG-positive compartments in the vicinity of the SCV in infected cells. The VSVG-positive compartments were frequently located in the close vicinity of the SCV but did not always co-localize with the intracellular *Salmonella*. VSVG accumulation was not dependent on the perinuclear localization of the SCV but was also observed around SCV in the periphery of the cell (Figure 2A). Kinetic analyses indicated that recruitment of VSVG-containing compartments started around 4 h after infection and increased with the prolonged intracellular presence of *S. typhimurium* (Figure 2B).

To test whether the recruitment of VSVG-positive compartments requires biosynthetic activities of intracellular *Salmonella* or represents a response of the host cell to *Salmonella* infection, we treated the cells with chloramphenicol, an antibiotic that inhibits bacterial protein synthesis. VSVG recruitment was examined after 4 h of antibiotic treatment at 12 h post-infection (Figure 2C). We observed that VSVG association with the SCV prior to chloramphenicol addition at 8 h was higher (42.7 $\pm$ 2.5%)

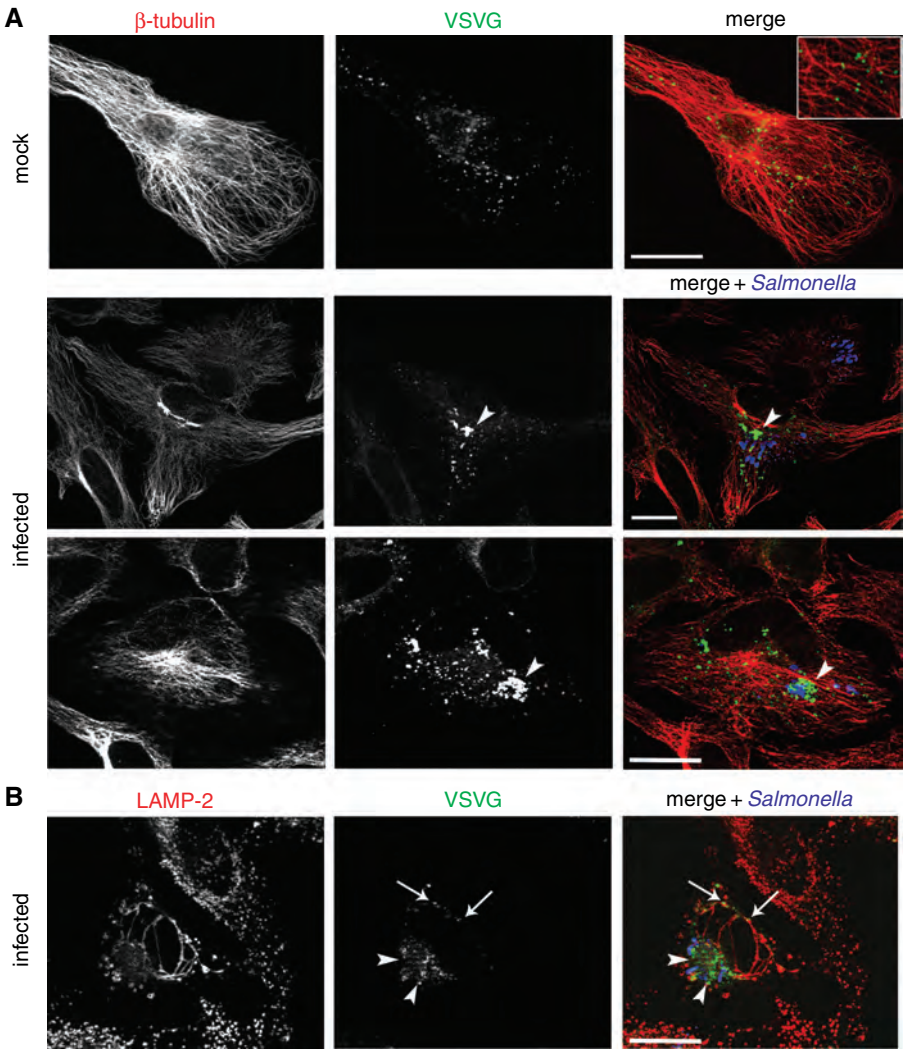

Figure 1: Association of VSVG-containing cargo vesicles with microtubules and *Salmonella*-induced filaments. HeLa cells were transfected with pVSVG3-EGFP for the expression of VSVG-EGFP (green). If indicated, cells were infected with wild-type (*S. typhimurium*) approximately 20 h after transfection. At 16 h after infection, cells were fixed and processed for immunostaining. (A) Cells were immunostained for β-tubulin (red), and *Salmonella* was detected by immunostaining with α LPS antisera (blue). Note the accumulation of large amounts of VSVG-containing compartments in the vicinity of the SCV (arrowheads). (B) *Salmonella*-infected HeLa cells were immunostained for *Salmonella* LPS (blue) and LAMP-2 (red) for the detection of SIF. Arrows indicate association of VSVG with SIF and arrowheads the position of the SCV. Scale bars represent 10 μM.

than at 12 h in the presence of the antibiotic (20.7 ± 1.9%). From these results, we conclude that proteins synthesized by intracellular *Salmonella* are required to redirect VSVG-positive compartments to the SCV and that continuous bacterial protein biosynthesis is necessary to maintain VSVG-containing vesicles in the vicinity of the SCV.

Post-Golgi vesicles accumulate in the vicinity of the SCV

The model system of VSVG protein traffic allowed a further dissection of the possible interference of *Salmonella* with exocytotic transport. VSVG is transported via the ER and Golgi to the plasma membrane, and previous studies have shown that transport of the temperature-sensitive ts045 variant of VSVG can be synchronized by incubation of transfected cells at different temperatures (20). The protein reversibly misfolds at 39.5 °C, leading to retention of the protein in the ER. At 19 °C, the protein refolds and is transported to the Golgi where it is retained in the *trans*-Golgi network (TGN). After shifting the cells to 32 °C, VSVG-positive compartments bud off from the TGN and traffic to the plasma membrane. To adjust the experimental setup to our infection conditions, we incubated HeLa cells at 37 °C, the default temperature for *Salmonella* intracellular growth. As incubation of HeLa cells at 39.5 °C led to increased cell death (data not shown) and also might have negative effects on intracellular *Salmonella*, cells were directly shifted from 37 to 19 °C. This modification achieved efficient targeting of VSVG-EGFP to the Golgi, even without preceding retention of the protein in the ER (Figure 3A). Comparison of Golgi to plasma membrane transport at 37 or 32 °C also showed no obvious difference. We also controlled the effect of the 37 to 19 °C shift on intracellular *Salmonella* and did not observe a reduction of intracellular proliferation (*Supplementary Figure S1A*) or reduced activity of a representative promoter of SPI2 virulence genes (*Supplementary Figure S1B*). To study the interference of *S. typhimurium* with the exocytic events, HeLa cells transfected with the pVSVG3-EGFP construct were infected with *S. typhimurium* wild type between 10 and 12 h

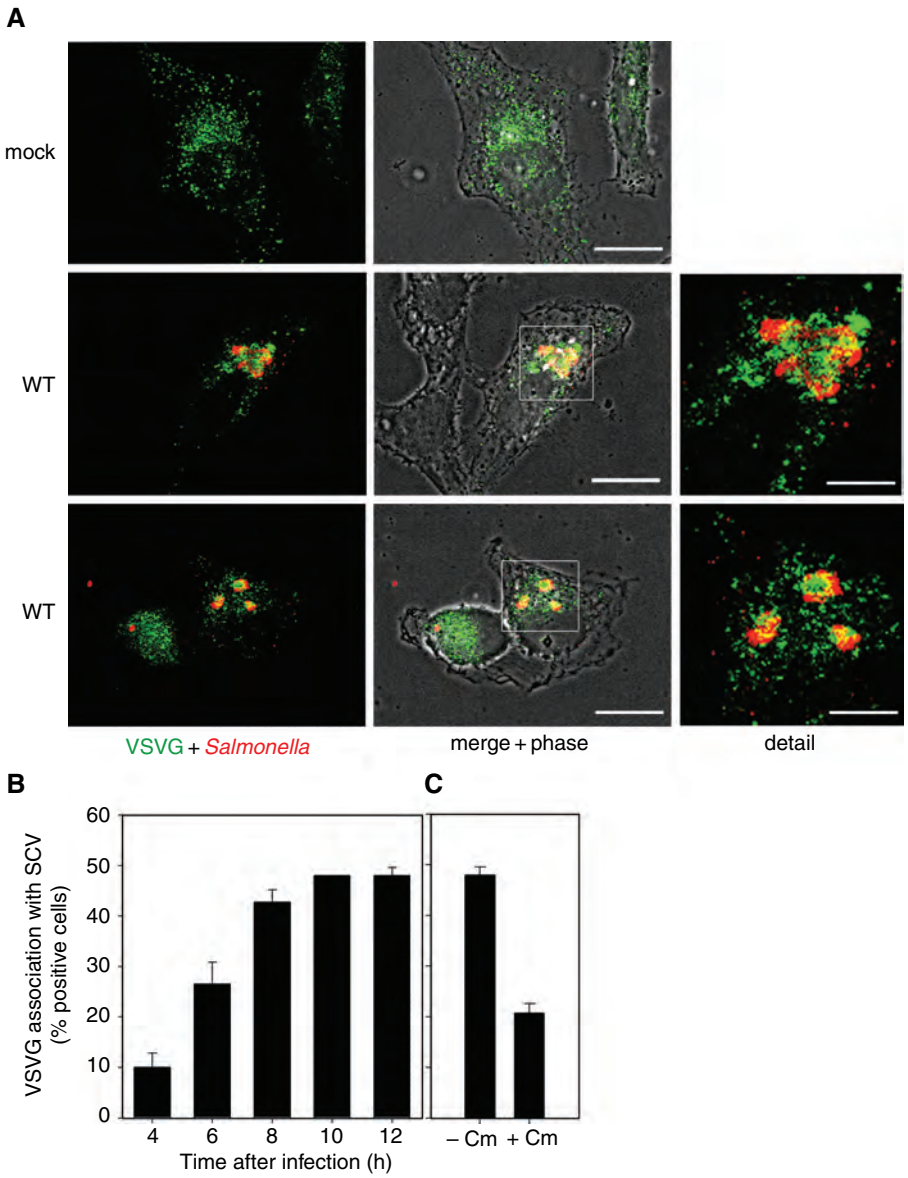

Figure 2: Accumulation of VSVG-positive compartments in the vicinity of the SCV. HeLa cells were transfected with pVSVG3-EGFP 20 h before infection for the expression of the VSVG-EGFP fusion protein (green). Subsequently, cells were infected with *S. typhimurium* wild type expressing RFP (red). At different times after infection, cells were fixed and immunofluorescence microscopy was used to quantify the distribution of VSVG-containing compartments with respect to the SCV. (A) Micrographs show representative non-infected (mock) and WT-infected (WT) cells at 12 h after infection. Detail micrographs show VSVG accumulation at *Salmonella* microcolonies. Scale bars represent 10 and 2 μm in overview and detail micrographs, respectively. (B) At various time-points after infection, cells were fixed and at least 50 transfected and infected cells were identified and analyzed for the distribution of VSVG. The percentage of transfected and infected cells showing VSVG association with the SCV similar to infected cells in (A) is given. (C) To investigate the role of bacterial protein biosynthesis, experiments were performed in the absence (– Cm) or presence (+ Cm) of chloramphenicol at a final concentration of 20 μg/mL added 8 h after infection. Cells were fixed 12 h after infection and scored for VSVG recruitment to the SCV as in (B). Representative data of three independent experiments are shown.

after transfection. Approximately 12 h after infection, cells were shifted to 19 °C for 2 h to arrest VSVG-EGFP in the TGN. Subsequently, cells were incubated at 37 °C for various periods of time. The accumulation of VSVG-positive compartments around the SCV was determined by immunofluorescence microscopy. Using this experimental setup, we also followed the trafficking of VSVG-containing compartments using time-lapse microscopy. A series of micrographs in Figure 3B shows the direction of VSVG-containing compartments to a microcolony of *S. typhimurium* wild type. A time-lapse movie showing the accumulation of VSVG-containing

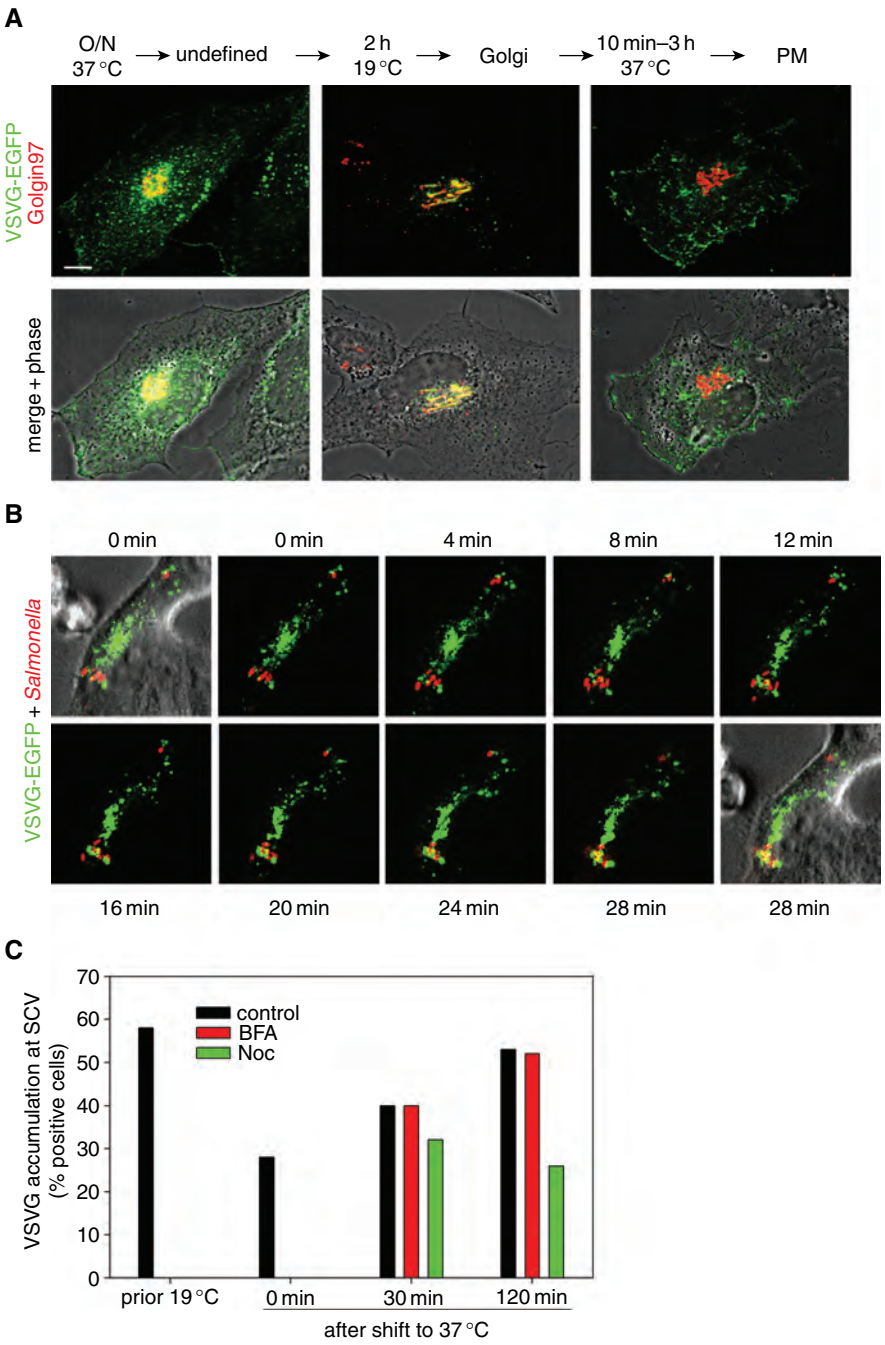

Figure 3: SCVs acquire post-Golgi compartments in a microtubule-dependent manner. (A) To follow VSVG transport in *Salmonella*-infected cells, a modified procedure was developed. HeLa cells were transfected with pVSVG3-EGFP (green) and incubated overnight at 37 °C resulting in an undefined distribution of VSVG. The position of the Golgi is visualized by immunostaining for the TGN marker Golgin97 (red). Subsequent incubation at 19 °C for 2 h resulted in targeting of VSVG to the TGN, as indicated by the co-localization with Golgin97. Increasing the temperature to 37 °C and further incubation for 10–180 min resulted in exit of VSVG from the TGN and transport to the plasma membrane. To study the effect of intracellular *Salmonella*, the procedure was further modified by infection with *S. typhimurium* wild type 12 h prior to the shift to 19 °C. (B) Kinetic analysis of the targeting of VSVG to SCV in living cells. VSVG (green) was targeted to the TGN as described above, and the localization of VSVG and the intracellular *Salmonella* (red) were imaged by time-lapse microscopy of a living cell at various time-points after shift to 37 °C. The first and the last images were merged with the phase contrast image. Note the re-distribution of VSVG to the SCV. (C) Effect of *S. typhimurium* on post-Golgi transport of VSVG. Transfected cells were infected with *S. typhimurium* WT, treated with 14 μg/mL of cycloheximide to inhibit the synthesis of new protein, incubated for 2 h at 19 °C, and shifted to 37 °C as described above. Subsequently, the assays were incubated without additional inhibitors (control, black) or treated with brefeldin A (BFA, red) or nocodazole (Noc, green) and incubated for 30–120 min at 37 °C. Cells were fixed with 3% PFA and VSVG accumulation in the vicinity of the SCV was determined by immunofluorescence microscopy. The percentage of transfected and infected cells showing VSVG accumulation at the SCV is indicated for various steps of the procedure. Representative data for one out of three experiments are shown.

compartments at an SCV is available as supplementary material (*Supplementary Movie 1*).

The kinetics of VSVG accumulation at SCVs were determined by co-localization studies of cells fixed before and at various time-points after shift from 19 to 37 °C (Figure 3C). VSVG accumulation at *Salmonella* microcolonies clearly decreased during incubation at 19 °C, indicating the transient nature of the recruitment of VSVG-containing compartments. Temperature shift to 37 °C rapidly led to re-accumulation of VSVG-positive compartments around the SCV (approximately 1 h) reaching levels comparable to those seen before the 19 °C incubation. We also analyzed the role of the microtubule cytoskeleton and Golgi integrity in VSVG association with the SCV. Prior to the shift from 19 to 37 °C, the transfected and infected cells were treated with nocodazole or brefeldin A at concentration sufficient to disrupt microtubules or to dissociate the Golgi apparatus, respectively (controls shown in *Supplementary Figure S2*). Although the procedure of transfection, *Salmonella* infection, and subsequent addition of inhibitors resulted in severe loss of viability of host cells, we observed that addition of nocodazole prevented the transport of new VSVG-containing compartments to the SCV, as the percentage of SCV associated with VSVG did not increase within 2 h after the temperature shift. By contrast, the addition of brefeldin A had no effect on the transport of VSVG-containing compartments, as the percentage of SCV associated with VSVG increased within 2 h similar to the control without inhibitor. From these observations, we conclude that under the experimental conditions applied here, VSVG-containing compartments were already available for recruitment to the SCV despite the brefeldin A-induced collapse of the TGN. However, this transport and especially the association with the SCV were dependent on the integrity of the microtubule cytoskeleton.

A previous study reported that targeting of wild-type *Salmonella* to the Golgi is a requirement for intracellular replication of the pathogen (15). We questioned whether the observed association of VSVG-containing compartments with the SCV is a consequence of the association of both compartments with the TGN and investigated the relative localization of the TGN, intracellular *Salmonella*, and VSVG (Figure 4). Using various TGN markers, such as Golgin97, Giantin, and GM-130 (Figure 4) or TGN46 (data not shown), we observed that microcolonies of wild-type *Salmonella* are located in a juxtanuclear position near to the Golgi. In 3D reconstructions of epi-fluorescence images, however, the TGN and intracellular *Salmonella* appear as distinct subcellular entities (*Supplementary Figure S3, Supplementary Movies 2 and 3*). At 12 h after infection, only low amounts of VSVG-EGFP were present in the TGN. The majority of VSVG-EGFP, however, was associated in vesicles recruited to *Salmonella* microcolonies but not within the membrane of the SCV as indicated by 3D reconstructions (*Supplementary Movies 2 and 3*). The VSVG-EGFP association of microcolonies was quantified, and 51.7 ± 3.5% (mean of three experiments ± SD) of microcolonies formed by wild-type *Salmonella* showed VSVG-EFGP accumulation that was distinct from the TGN.

VSVG recruitment is dependent on the SPI2 effector molecules SifA, SseF, and SseG

Intracellular phenotypes of *Salmonella* such as replication, acquisition of late endosomal compartments, and rearrangement of the microtubule cytoskeleton are dependent on the function of the SPI2-T3SS. Furthermore, SPI2-dependent phenotypes initiate with a delay of several hours after infection. This kinetic is similar to the redirection of VSVG-positive compartments shown in Figure 2. We speculated that the SPI2 virulence system might also play a role in the observed recruitment of VSVG-positive compartments to the SCV. To test this hypothesis, we infected pVSVG3-EGFP-transfected cells with *S. typhimurium* wild type or an *ssaV* mutant strain, lacking a functional SPI2-T3SS. At 12 h after infection, cells were fixed and VSVG recruitment was examined by immunofluorescence microscopy. While VSVG recruitment was observed for about 50% of the SCV harboring wild-type *Salmonella*, the marker was almost absent in the vicinity of SCV harboring the *ssaV* strain (Figure 5). As this result suggested a role of the SPI2-T3SS, we next set out to identify the effector protein(s) of the SPI2-T3SS responsible for the observed phenotype. Various mutant strains deficient in known SPI2 effector proteins were used for infection, and recruitment of VSVG to the SCV was quantified (Figure 5). Reduced VSVG recruitment was observed in HeLa cells infected with a *sifA* strain as well as strains deficient in *sseF* and *sseG*. A slight reduction of VSVG acquisition was also observed for a *sopD2* mutant. A more pronounced VSVG recruitment in comparison to the wild-type strain was observed for SCV harboring the *sseI* mutant strain. The SPI2 effector proteins SseI and SspH 1 have been shown to interact with the actin cytoskeleton (21). Mutant strains deficient in other SPI2 effector proteins, i.e. SspH1, SspH2, PipB2, SseJ, SlrP, or SifB, showed an extent of VSVG recruitment similar to that of the wild-type strain. The reduced association of VSVG with SCV containing SifA-, SseF-, or SseG-deficient strains was restored in strains harboring plasmids for complementation. These results indicate that the same subset of effector proteins previously shown to be involved in modifications of the microtubule cytoskeleton and the recruitment of lgp-positive vesicles are also involved in the redirection of exocytic transport.

Intracellular Salmonella cause redirection of TGN to plasma membrane transport of shingolipids

To investigate whether the interference of intracellular *Salmonella* with VSVG transport represents a phenomenon of general significance, we investigated further markers of the exocytic pathway. We initially tried to quantify activity of secreted alkaline phosphatase (SEAP), but this marker was not useful due to the high background

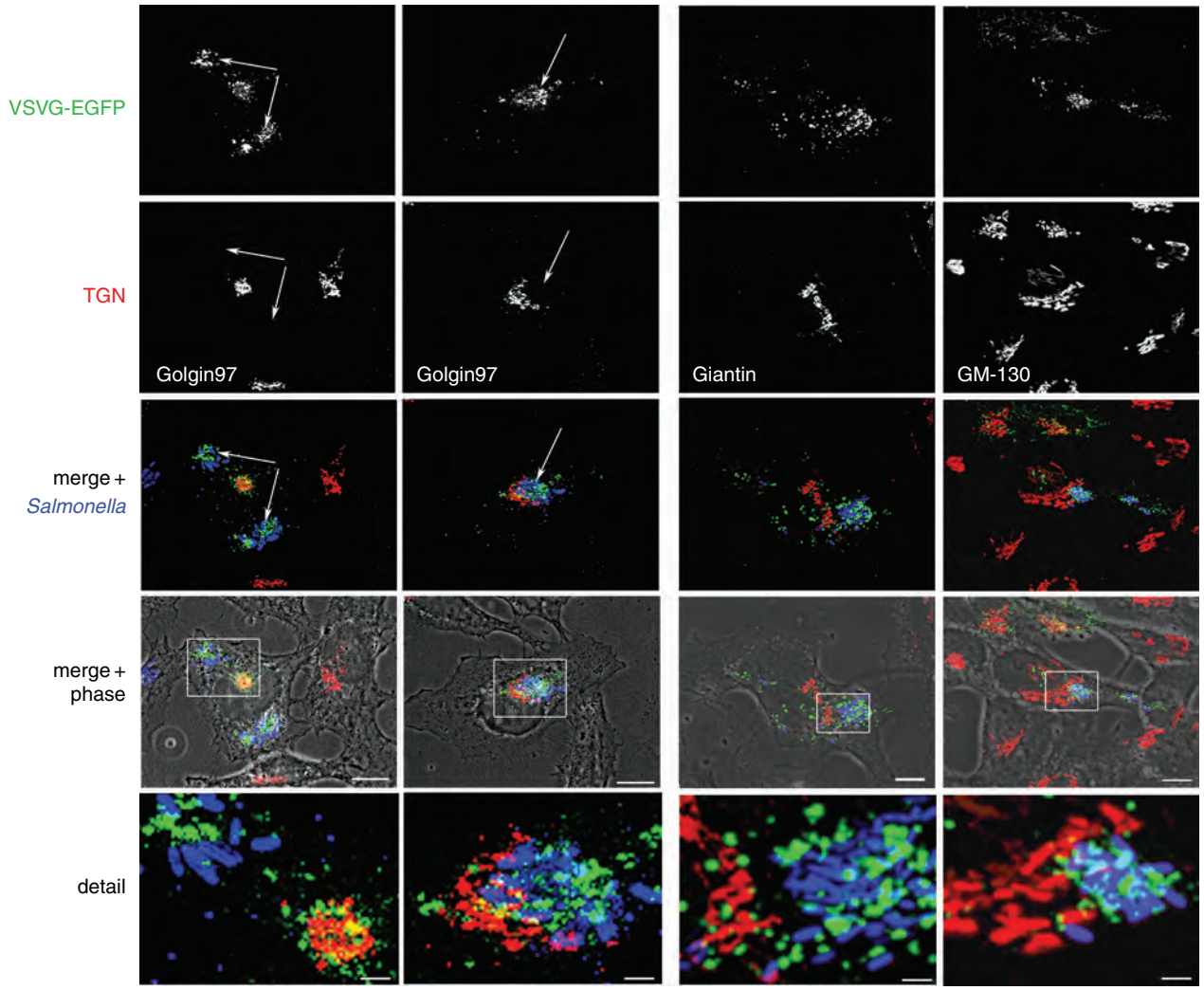

Figure 4: Accumulation of VSVG at the SCV is distinct from association with the Golgi apparatus. HeLa cells were transfected with pVSVG3-EGFP (green) and infected with *S. typhimurium* WT. Cells were fixed 12 h after infection and stained for *Salmonella* (blue) and TGN markers Golgin97, Giantin or GM130 (red). Representative transfected and infected cells are shown and the position of *Salmonella* microcolonies is indicated by arrows. Note the appearance of two microcolonies in a cell in the left panel. Scale bars represent 10 and 2 μm in the overview and detail micrographs, respectively.

of SEAP activity released from cells that died over the course of the experiments. Shingolipids such as C_5-ceramides labeled with fluorochromes have been used to follow exocytotic processes (22). These molecules are internalized, accumulate in the Golgi, and are subsequently transported to the plasma membrane. Here, the transport of Bodipy-TR C_5-ceramide was investigated. Approximately 30 min after uptake, the tracer accumulated in the Golgi (Figure 6A) and later showed a dispersed localization in non-infected cells. By contrast, C_5-ceramide-containing compartments were concentrated in the vicinity of intracellular microcolonies in *Salmonella* wild type-infected HeLa cells (Figure 6A). This accumulation of C_5-ceramide-containing compartments to the SCV initiated about 1 h after the pulse and a massive accumulation of the tracer was obvious after 2 h. Microcolonies of the *ssaV* strain were not associated with C_5-ceramide-containing compartments at 2 h after the pulse, indicating that the SPI2-T3SS influences the cellular transport and spatial distribution of the C_5-ceramide-containing compartments similar to the transport of VSVG-containing compartments.

Previous studies indicated that the maturation of the SCV is, to a certain extent, influenced by the SPI1-mediated invasion of host cells (23,24). We investigated the effect of intracellular *Salmonella* on C_5-ceramide distribution in RAW 264.7 macrophage-like cells. In this infection model, RAW 264.7 cells phagocytoze non-invasive *Salmonella*. We observed that after uptake of the wild-type strain, condensations of C_5-ceramide were present at *Salmonella* microcolonies similar to the observations in

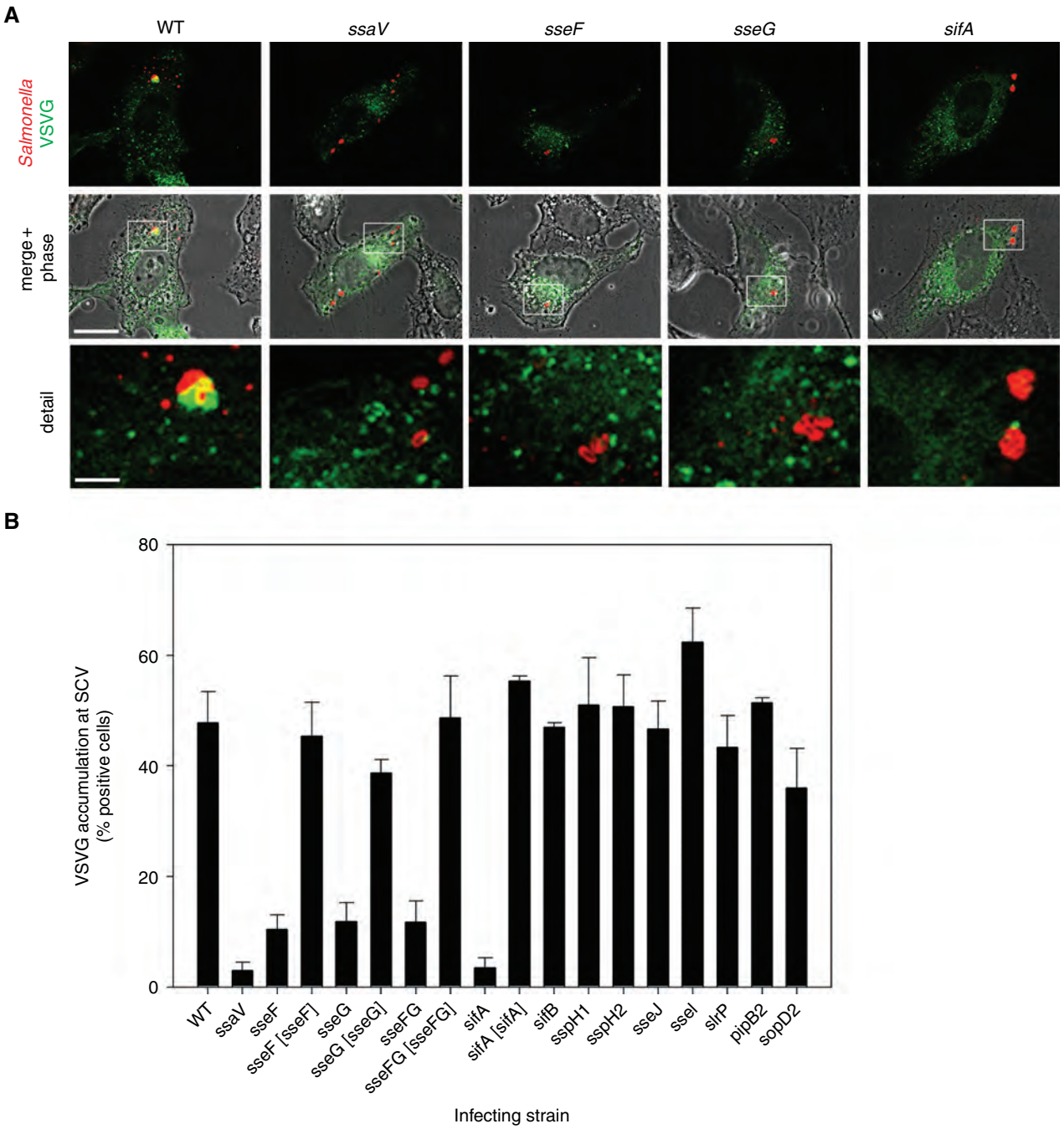

Figure 5: Role of the SPI2-T3SS and SPI2 effector proteins in redirecting secretory vesicles to the SCV. HeLa cells were transfected with pVSVG3-EGFP and infected with *S. typhimurium* wild type (WT) or various mutant strains deficient in the SPI2-T3SS system (*ssaV*), in genes for individual effector proteins (*sseF*, *sseG*, *sifA*, *sifB*, *sspH1*, *sspH2*, *sseJ*, *sseI*, *slrP*, *pipB2*, or *sopD2*) or a subset of effector proteins (*sseFG*). Furthermore, mutant strains harboring plasmids for complementation of the deleted genes were used. In detail, the *sseF* (HH107), *sseG* (HH108), *sscB sseFG* (MvP373), or *sifA* (P3H6) strains were complemented with plasmids p2095, p2788, p2096, or p2104, respectively. VSVG accumulation around the SCV was determined by immunofluorescence microscopy. (A) The spatial distribution of VSVG (green) and *Salmonella* (red) is shown for transfected cells infected with *S. typhimurium* wild type or mutant strains deficient in *ssaV*, *sseF*, *sseG*, or *sifA*. Representative cells with similar numbers of intracellular bacteria were selected. Scale bars represent 10 and 2 μm for the overview and detail micrographs, respectively. (B) For each infecting strain, 50–100 transfected and infected host cells were examined for the distribution of VSVG. The figure shows mean values and SD of at least three independent experiments of the various strains.

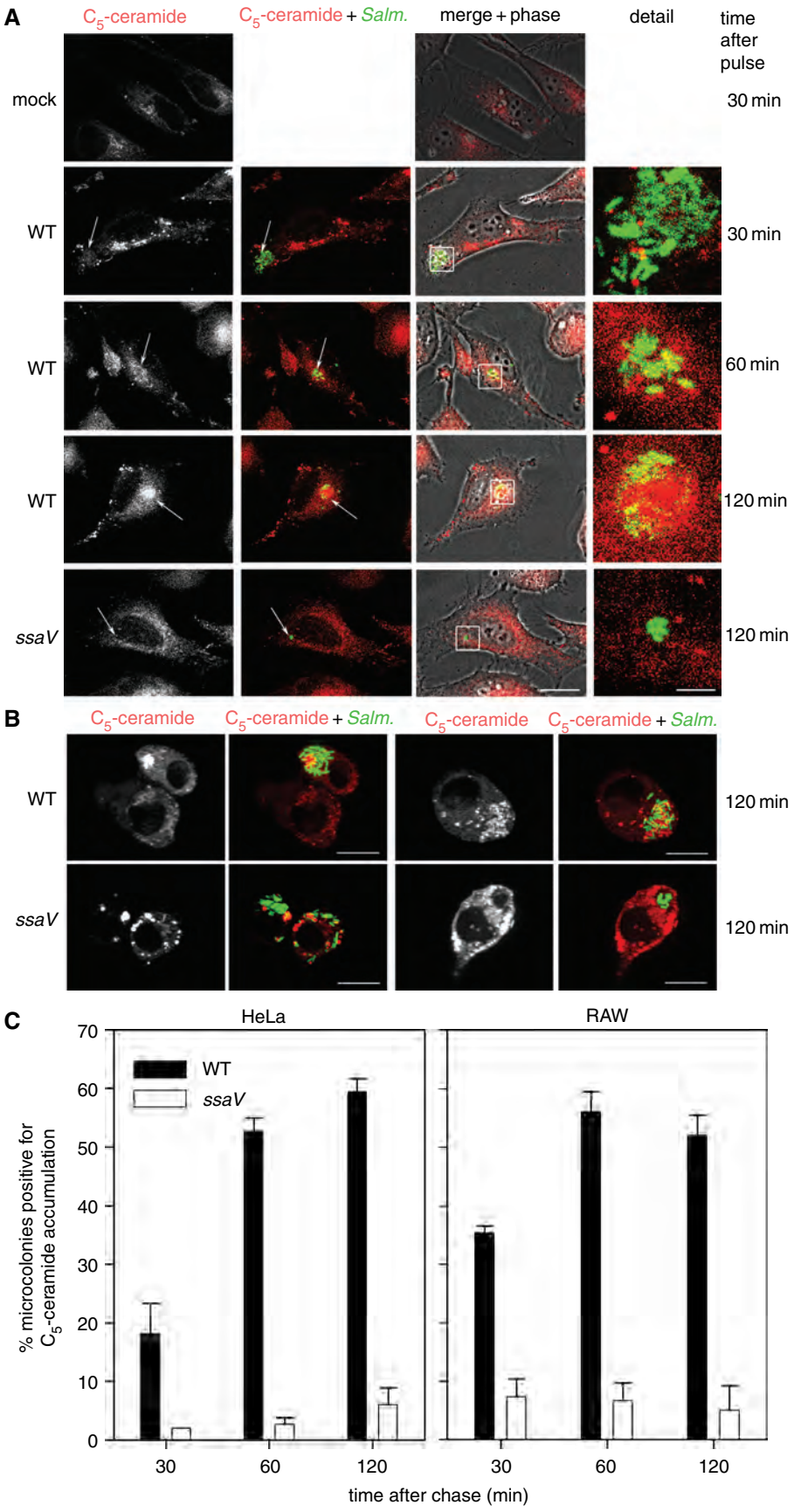

Figure 6: Redirection of C_5-ceramide-containing vesicles to *S. typhimurium* microcolonies. HeLa cells (A) and RAW 264.7 macrophages (B) were infected with *S. typhimurium* wild type (WT) or a SPI2-deficient mutant strain (*ssaV*) both expressing GFP (green). At 12 h (for HeLa) or 9 h (for RAW264.7) after infection, cells were pulsed with 2.5 μM Bodipy-TR-C_5-ceramide (red) for 15 min at 37 °C and subsequently washed to remove non-internalized tracer. At 30, 60, or 120 min after addition of tracer, cells were washed and fluorescence microscopy was performed with living cells. The position of *S. typhimurium* microcolonies is indicated by arrows. Scale bars represent 10 and 2 μm in overview and detail micrographs, respectively. (C) HeLa or RAW 264.7 cell infections were performed as above and the association of the SCV with C_5-ceramide was quantified for 50 infected host cells for each of the experimental conditions. The mean values of three independent experiments with SD are shown.

HeLa cells (Figure 6B). In both HeLa cell and RAW264.7 cell infection experiments, about 50–60% of the SCV harboring wild-type *Salmonella* recruited C_5-ceramide within 2 h after the pulse, while accumulation of the tracer to SCV harboring the *ssaV* mutant strain remained at the basal level of 5–10% (Figure 6C).

From these observations, we conclude that *Salmonella* can induce a general re-distribution of compartments involved in exocytotic transport. This effect is independent from bacterial invasion and requires the function of the SPI2-T3SS.

Discussion

In this study, we describe the novel finding of an interference of intracellular *Salmonella* with exocytotic pathways of the host cell. Using VSVG-EGFP and a fluorescently labeled C_5-ceramide as markers of exocytotic transport, we demonstrated that wild-type *Salmonella* actively redirect exocytic cargo vesicles to the SCV and that these vesicles are retained in the vicinity of the SCV. The recruitment does not result in a gross fusion of the post-Golgi vesicles with the SCV and is dependent on the continuous bacterial protein biosynthesis, activity of the SPI2-T3SS and, more specifically, on the function of a subset of SPI2 effector proteins. The recruitment appears to be a dynamic, reversible process as the block of bacterial protein biosynthesis after establishing the SCV, as well as a temperature shift resulting in misfolding of ts-VSVG led to the release of VSVG-positive compartment from the SCV-associated localization.

These observations should revise current models for the nature of the SCV. The biogenesis of the SCV has been characterized in various studies that indicated a unique nature of this compartment. Due to the presence of lgp such as LAMP-1 in the membrane of the SCV, this compartment is commonly considered as a late endosomal vesicle that does not follow the default pathway of maturation. Recent studies, however, showed that *Salmonella* actively retain the SCV in a perinuclear position (15) and interfere with the function of microtubule motor proteins (11,13). The recruitment of post-Golgi vesicles suggests that the SCV can interact with the exocytic pathway of the host cell, an effect most likely mediated by the interference of *S. typhimurium* with microtubule-dependent trafficking events. The interchange with the secretory pathway may be important for the supply of the proliferating bacterial population with nutrients and to provide membrane material to the growing SCV. Yet experimental evidence for the access of *Salmonella* within the SCV to secretory material has to be provided by further studies.

The common understanding of exocytic transport from the Golgi to the plasma membrane has recently been challenged by novel observations. Alternative models propose that post-Golgi transport and sorting of newly synthesized proteins could intersect with endosomal compartments (25). For example, Ang *et al.* (26) used VSVG as a tracer protein and observed the transient presence of this protein in recycling endosomes. *Salmonella* resides in a compartment that has several characteristics of late endosomes, and in our model system, we only observed few events of direct co-localization of VSVG with the SCV or SIF as LAMP-1-positive compartments (see Figure 1B). Our observations of the recruitment of VSVG-containing compartments might also be in line with this alternative model of VSVG trafficking, and further work has to reveal whether recycling endosomes are enriched in the vicinity of the SCV. It has also been observed that overexpression of VSVG can result in mislocalization of Golgi to lysosome cargo such as LAMP-1 (27). A further alternative explanation for VSVG trafficking in *Salmonella*-infected cells could be the recruitment of VSVG already transported to the plasma membrane. We have not been able to experimentally distinguish vesicular VSVG from VSVG in the plasma membrane. However, the time-lapse microscopy of VSVG trafficking (Figure 3B, *Supplementary Movie 1*) and the trafficking of the tracer C_5-ceramide (Figure 6B) would support that vesicles recruited to the SCV originated from the Golgi rather than from the plasma membrane. As the redirection of transport was observed with VSVG and C_5-ceramide as tracers of rather distinct biochemical nature, we assume that our findings indicate a general interference of *Salmonella* with host cell transport processes. The implications of our observations for the physiology of intracellular *Salmonella* have to be defined in future studies.

How is post-Golgi transport redirected to and/or arrested at the SCV? We previously observed that intracellular *Salmonella* induce, in a SPI2-dependent manner, the bundling of microtubules (10). Such alteration of the microtubule cytoskeleton could block the exocytic transport and cause an accumulation of cargo vesicles in the vicinity of the SCV. The accumulation of dynein at the SCV was reported (11,12), and we observed a role of SseF and SseG in dynein recruitment to the SCV (GLA, VK, and MH, unpublished observations). Recent work by Boucrot *et al.* (14) indicated the interference with kinesin recruitment to the SCV by interaction of SifA with its target SKIP, a host cell protein that displaces kinesin on the SCV membrane. This activity was crucial to maintain an intact SCV that is a requirement for the intracellular replication of *Salmonella*. The distinct recruitment and displacement of motor proteins for the minus-end and plus-end-directed transport on microtubules might explain the redirection of microtubule-dependent exocytotic transport by virulent intracellular *S. typhimurium*, but further work has to clarify the roles of the various SPI2 effector proteins in these events.

Intracellular pathogens that proliferate within a membrane-bound compartment evolved very distinct strategies

to interfere with host cell transport processes and to modify host organelles. An interference of intracellular bacteria with the exocytic pathway beyond the TGN is rather uncommon (28) and so far only reported for *Salmonella* and *Chlamydia trachomatis* and *Chlamydia pneumoniae*. Cholesterol and Golgi-derived shingolipids are actively redirected to the *Chlamydia* inclusion, and this interference is required for intracellular proliferation of the pathogen (29,30). It was also shown that this pathogen interferes with the function of microtubule motor proteins to target the *Chlamydia* inclusion to the microtubule-organizing center (31). The integrity of microtubules and the Golgi is required for post-Golgi cargo to the *Chlamydia* inclusion (32). We observed that disruption of microtubules with nocodazole but not Golgi disintegration by brefeldin A reduced recruitment of VSVG-EGFP to the SCV, indicating differences in the intracellular strategy of both pathogens. However, as for *Salmonella*, the function of a T3SS was observed in *Chlamydia*. The contribution of the *Chlamydia* T3SS to the modification of host cell exocytosis awaits experimental investigation.

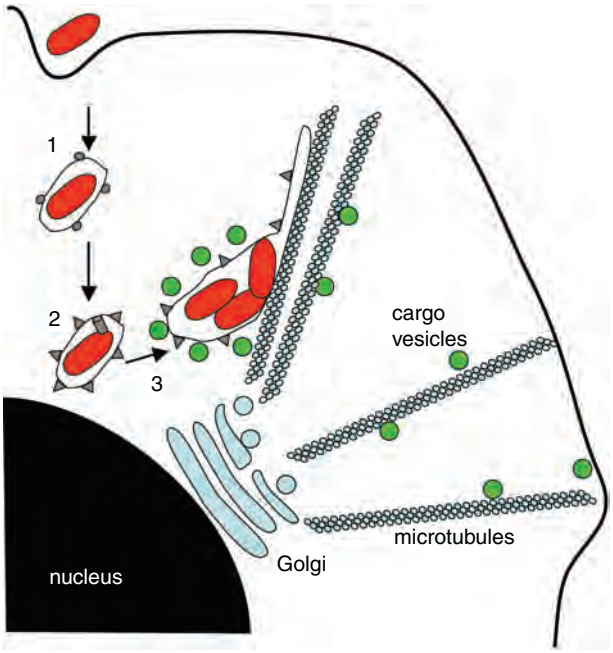

Figure 7: Model for the biogenesis of the SCV and the interaction of intracellular *Salmonella* with host cell trafficking. After invasion of epithelial cells, the SCVs transiently acquire early endosomal markers (1). Subsequently, the SCV contains marker molecules of the late endocytic pathway such as lgp. Due to unknown and SPI2-independent factors, intracellular *Salmonella* modify the normal endocytic pathway to direct the SCV to a perinuclear localization (2). The compartment remains in a perinuclear localization due to the activity of effector proteins of the SPI2-T3SS that interfere with the function of microtubule motor proteins. In this study, we show that SPI2 effector proteins modify exocytotic transport resulting in the recruitment of secretory vesicles to the vicinity of the SCV (3). Furthermore, the formation of SIF initiates along microtubules. The positioning of the SCV and modification of cellular transport might be prerequisites for the intracellular replication of *Salmonella*.

The effectors involved in VSVG recruitment and retention, namely SseF, SseG, and SifA, were previously characterized as being required to maintain the integrity of the SCV (6), targeting of *Salmonella* to the Golgi (15) and in modification of the microtubule cytoskeleton (10). Furthermore, the roles of SifA and, to a lesser extent, of SseF and SseG in intracellular replication and systemic virulence of *Salmonella* were demonstrated (6,33). We therefore propose that this subset of effector proteins and their effects on intracellular transport are crucial for the intracellular virulence of *S. typhimurium*. An earlier study reported the SPI2-T3SS-dependent interference of intracellular *Salmonella* with cellular transport (34). Although the assay did not distinguish between effects on endocytosis or exocytosis, our work supports the initial observation and specifically shows the modification of exocytotic processes. A model for the SPI2-mediated interference of intracellular *Salmonella* with host cell transport processes is shown in Figure 7. As the function of the SPI2-T3SS initiates with a delay of more than 1 h after entry into host cells, factors other than the SPI2-T3SS are required for the altered maturation of the SCV in epithelial cells (23) or macrophages (1). The activity of the SPI2-T3SS subsequently allows the transformation of the SCV to a compartment that has access to the exocytic pathway and allows proliferation of *Salmonella*.

Recent studies revealed a variety of cellular phenotypes related to the function of the SPI2-T3SS such as protection of intracellular *Salmonella* against reactive oxygen intermediates (5) and reactive nitrogen intermediates (4), the reduction of MHC II surface expression (35) or the reduced ability of dendritic cells to present antigens (36). As these cellular functions require cellular transport processes or exocytosis, it will be of future interest to correlate the various phenotypic readouts of SPI2 function with the modification of exocytosis reported here. The understanding of molecular interactions between *Salmonella* effector proteins and host cells molecules should shed light on this intriguing form of pathogenic interference.

Materials and Methods

Bacterial strains and growth conditions

S. typhimurium NCTC 12023 was used as wild type, and various isogenic mutant strains used in this study are listed in Table 1. *S. typhimurium* strains were transformed with pFPV25.1 or pRFP for the constitutive expression of GFP or DsRed, respectively. For infection of non-phagocytic cells, *S. typhimurium* strains were precultured in LB overnight and subsequently cultured for 3.5 h in LB to induce the expression of SPI1 genes. For infection of phagocytic cells, overnight cultures were used.

Mutant strains deficient in *pipB2* or *sopD2* were generated by one-step inactivation method as previously described (4,37).

Table 1: Bacterial strains and plasmids used in this study

Designation	Relevant characteristics	Reference
Strains		
12023	Wild type	NCTC, Colindale, UK
P2D6	*ssaV*::mTn*5*	(38)
P3H6	*sifA*::mTn*5*	(6)
HH107	Δ *sseF*::*aphT*	(33)
HH108	Δ *sseG*::*aphT*	(33)
MvP373	Δ *sscB sseFG*::*aph*	(8)
MvP374	Δ *sifB*::*aph*	(4)
MvP375	Δ *sspH1*::*aph*	(4)
MvP376	Δ *sspH2*::*aph*	(4)
MvP377	Δ *sseJ*::*aph*	(4)
MvP378	Δ *sseI*::*aph*	(4)
MvP379	Δ *slrP*::*aph*	(4)
MvP498	Δ *pipB2*::*aph*	This study
MvP500	Δ *sopD2*::*aph*	This study
Plasmids		
pVSVG3-EGFP	Transfection vector for VSVG-EGFP	(20)
p2095	Pro $_{sseA}$ *ssc BsseF*::M45 in pWSK29	(39)
p2096	Pro $_{sseA}$ *sscB sseF sseG*::M45 in pWSK29	(39)
p2788	Pro $_{sseA}$ *sscB sseG*::M45 in pWSK29	This study
p2104	Pro$_{sifA}$ *sifA*::M45 in pWSK29	(39)
pFPV25.1	Constitutive GFP expression	(40)
pRFP	Constitutive DsRed expression	(41)

Plasmid p2788 for the complementation of the *sseG* deletion in strain HH108 was constructed as follows. Plasmid p2081 harboring Pro *sseA sscB sseFG*::M45 in pSK was used as template for PCR amplification with primers sscB-Rev-BglII (5′-gacagatctgttatttaagcaataagagtat-3′) and sseG-For-BglII (5′-gcgagatctcggggagaaccatgaaacctg-3′). The resulting product was digested with *Bgl*II, self-ligated, and electroporated in *Escherichia coli* XL1-Blue to generate p2730. The insert of p2730 consisting of Pro *sseA sscB sseG*::M45 was recovered as *Hind*III/*Xba*I fragment and subcloned in pWSK29 to generate p2788.

Cell culture

The murine monocyte cell line RAW264.7 and the human epithelial cell line HeLa were cultured in Dulbecco's modified Eagle medium (DMEM, PAA, Cölbe, Germany) containing 10% fetal calf serum (FCS) (Sigma, Taufkirchen, Germany) and 2 mM Glutamax (Invitrogen, Karlsruhe, Germany) at 37 °C in 5% CO_2. Cells with a passage number lower than 25 were used.

Bacterial infection of RAW264.7 macrophages and HeLa cells

For bacterial infection, RAW264.7 macrophages and HeLa cells were seeded in 24-well plates 28–40 h before infection with 1.5×10^5 cells/well and 0.4×10^5 cells/well, respectively. Bacteria were grown in LB broth containing the necessary antibiotics at 37 °C with agitation to stationary phase. For HeLa cell infection, cultures were subsequently diluted 1:30 with fresh LB broth and incubated for another 3.5 h at 37 °C with agitation to reach late logarithmic phase. The OD_{600} of the cultures was adjusted with phosphate-buffered saline (PBS; pH 7.3) to 0.2, and cells were infected with an MOI of 5 and 10 for RAW264.7 and HeLa cells, respectively. The bacteria were centrifuged onto the cells at $500 \times g$ for 5 min and incubated for 25 min at 37 °C in 5% CO_2. After infection, cells were washed three times with PBS and incubated for 1 h in medium containing FCS, Glutamax, and 100 μg/mL of gentamicin. The medium was replaced with medium containing FCS, Glutamax, and 10 μg/mL of gentamicin for the remainder of the experiment.

Table 2: Antibodies used in this study

Specificity	Working dilution	Source
Bacto® rabbit *Salmonella* anti-*O*-testsera	1:500	BD (Heidelberg, Germany)
Mouse anti-human Lamp-1 (A4H3)	1:100	DSHB (Iowa City, IA, USA)
Mouse anti-human Golgin97	1:300	Invitrogen (Karlsruhe, Germany)
Rabbit anti-human Giantin	1:300	Antonietta De Matteis (Chieti, Italy)
Rabbit anti-human GM-130	1:300	Antonietta De Matteis
Mouse anti-β-tubulin Cy3	1:200	Sigma (Taufkirchen, Germany)
Goat anti-rabbit Cy3	1:500	Jackson (Dianova, Hamburg, Germany)
Goat anti-mouse Cy3	1:500	Jackson
Rabbit anti-mouse Cy3	1:500	Jackson
Goat anti-rabbit Cy5	1:300	Jackson

Transfection of HeLa with pVSVG3-EGFP

HeLa cells were seeded approximately 20 h before transfection in 24-well plates on glass coverslips at a density of about 4×10^4 cells/well. Immediately before transfection, cells were washed with PBS and 600 μL fresh media was added. Per well, 0.2 μg of pVSVG3-EGFP was diluted to a final volume of 20 μL in serum-free DMEM medium before addition of 3 μL of Polyfect transfection reagent (Qiagen, Hilden, Germany). The solution was briefly mixed and incubated for 5–10 min at room temperature before addition of 120 μL of DMEM + 10% FCS + 2 mM Glutamax. After mixing by pipetting up and down, the solution was added immediately to the cells. Cells were incubated at 37 °C in 5% CO_2 for different periods of time. Media were changed generally after 8–10 h of incubation.

Synchronization of VSVG-EGFP transport

HeLa cells transfected with VSVG-EGFP and infected with *S. typhimurium* harboring p2071 were shifted to DMEM/Hepes (PAA) + FCS + Glutamax + 10 μg/mL of gentamicin + 14 μg/mL of cycloheximide (Sigma) 12 h after infection and incubated at 19 °C without CO_2 (on a heating block) for 2 h to block VSVG-EGFP in the TGN. Medium was exchanged against DMEM + FCS + Glutamax + 10 μg/mL of gentamicin + 14 μg/mL of cycloheximide, and cells were incubated for the remainder of the experiment at 37 °C/5% CO_2, which led to transport of VSVG-EGFP to the plasma membrane. For inhibition of microtubule-dependent processes and secretion events, nocodazole (Calbiochem, Darmstadt, Germany; 10 μM in MeOH) or brefeldin A (Calbiochem, 5 μg/mL) was added to the cell culture directly after the 19 °C incubation step for the remainder of the experiment. For live cell imaging, cycloheximide was omitted.

Pulse/chase experiment with Bodipy-TR C_5-ceramide

Infected RAW and HeLa cells were incubated with 2.5 μM Bodipy-TR C_5-ceramide complexed with BSA (Molecular Probes, Invitrogen, Karlsruhe, Germany) for 15 min at 37 °C/5% CO_2 at 9 h and 12 h post-infection, respectively. Cells were subsequently washed three times with DMEM + FCS + Glutamax + 0.34% BSA and incubated for the remainder of the experiment in DMEM + FCS + Glutamax + 0.34% BSA + 10 μg/mL of gentamicin at 37 °C/5% CO_2. At different times after addition, cells were washed 3× with PBS and incubated for 15–30 min at 4 °C. The coverslips were mounted on Fluoroprep (bioMèrieux, Nürtingen, Germany) and sealed with Entellan (Merck, Darmstadt, Germany) and were directly imaged using the Zeiss Axiovert with Apotome and Axiovision version 4.3.

Immunofluorescence

For immunofluorescence, cells were grown in 24-well tissue culture plates on glass coverslips. After transfection and/or infection and incubation for different times, the cells were fixed with 3% para-formaldehyde in PBS for 15 min at room temperature and then washed three times with PBS. Antibodies were diluted in a blocking solution consisting of 10% goat serum, 1% bovine serum albumin (BSA), and 0.1% saponin (Sigma) in PBS (Table 2). The coverslips were incubated for 1 h at room temperature with the different antibodies. After each incubation step, the coverslips were washed three times with PBS. The coverslips were mounted on Fluoroprep (bioMèrieux) and sealed with Entellan (Merck). Micrographs were taken using the Zeiss Axiovert with Apotome and Axiovision version 4.3. Counting was performed with the Zeiss epi-fluorescence microscope using a double bandpass filter for detection of GFP and RFP fluorescence.

Live cell imaging

For live cell imaging, cells were cultured and infected in 8-well chamber slides (Nunc, Wiesbaden, Germany). Time-lapse microscopy was performed using a Zeiss Axiovert 200 microscope equipped with an incubation chamber for temperature control at 37 °C in a humidified atmosphere containing 5% CO_2.

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Supplementary Material

Figure S1. Effect of temperature shift on intracellular replication of *Salmonella* and expression of SPI2 genes. (A) HeLa cells were infected with *S. typhimurium* wild type at an MOI of 1. The infection was performed essentially as described for the experiment shown in Figure 3C and the cells were incubated for 12 h at 37 °C. Subsequently, one set of samples was incubated for 2 h at 19 °C (shift), while the other set of samples was incubated for 2 h at 37 °C (no shift). Finally, all samples were incubated for additional 2 h at 37 °C. For the determination of the intracellular replication, cells were washed and lysed by addition of PBS containing 0.1% Triton X-100. Serial dilutions were plated on agar plates in order to determine the number of colony-forming units (CFU) of intracellular *Salmonella*. (B) Cells were infected at MOI of 10 with the following *S. typhimurium* strains: wild type harboring reporter plasmid pLS824 with a fusion of the promoter of *sseA* to GFP (WT reg), a mutant strain deficient in the SPI2 regulatory system gene *ssrB* harboring pLS824 (ssrB reg), wild type harboring plasmid pFVP25.1 for constitutive expression of GFP (WT const), and wild type without a plasmid (WT). Incubation was performed with and without temperature shift as described for (A), and 16 h after infection, intracellular bacteria were released by lysis with Triton X-100. Labeling of *Salmonella* and flow cytometry for quantification of the mean GFP fluorescence of the bacterial population were performed as previously described (Jantsch J, Cheminay C, Chakravortty D, Lindig T, Hein J, Hensel M. Intracellular activities of *Salmonella enterica* in murine dendritic cells. Cell Microbiol 2003;5:933–945). The means ± SD of three independent experiments are shown.

Figure S2. Effect of nocodazole and brefeldin A on organelle organization. HeLa cells were treated for 2 h with 10 μM nocodazole, 5 μg/mL of brefeldin A, or the solvent MeOH (mock) as described for Figure 3C. Subsequently, the cells were fixed and processed for immunostaining for β-tubulin (red) or TGN marker Golgin97 (red) for inhibition by nocodazole or brefeldin A, respectively. Merged epi-fluorescence and phase contrast micrographs of representative cells are shown.

Figure S3. Micrographs showing the merged epi-fluorescence and phase contrast images of HeLa cells expressing VSVG-EGP (green) and infected with wild-type *Salmonella* (red). The TGN (blue) was immunostained with antisera against Giantin (A) or GM-130 (B). The yellow frame indicates the section selected for 3D reconstruction shown in supplementary Movies 2 and 3.

Movie 1: HeLa cells were transfected with pVSVG3-EGFP (green) and subsequently infected with *Salmonella* wild type harboring

pRFP (red) as described for Figure 3B. Images were taken in intervals of 2 min over a period of 2 h. The time-lapse movie shows one image per second.

Movie 2: The spatial organization of the TGN (immunostained for Giantin, blue), intracellular *Salmonella* (red), and VSVG-EGFP (green) containing compartments is shown in a 3D reconstruction. HeLa cell infection and immunostaining were performed as described for Figure 4. The 3D reconstruction was performed with 14 Z sections using the Inside 4D module of Axiovision 4.3.

Movie 3: As Movie 2, but immunostaining of TGN marker GM-130 (blue) was performed. This material is available as part of the online article from http://www.blackwell-synergy.com

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