Dynamic Remodeling of the Endosomal System During Formation of Salmonella-Induced Filaments by Intracellular Salmonella enterica

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The infection by Salmonella enterica results in the massive remodeling of the endosomal system of eukaryotic host cells. One unique consequence is the formation of long tubular endosomal compartments, so-called Salmonella-induced filaments (SIF). Formation of SIF requires the function of type III secretion system and is a requirement of efficient intracellular proliferation of Salmonella. Using high-resolution live cell imaging approaches and electron microscopy, we report for the first time the highly dynamic characteristics of SIF and their ultrastructural properties. In the early phase of infection (4–5 h), SIF display highly dynamic properties in various types of host cells. SIF extend, branch and contract rapidly, and a stabilized network of SIF is formed later (≥8 h after infection). The velocities of SIF extension and contraction in the different phases of infection were quantified. Our observations lead to novel models for the modification of host cell transport processes by virulence factors of intracellular Salmonella.

Key words: intracellular pathogenesis, Salmonella-containing vacuole, Salmonella-induced filaments, type III secretion system

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The transport of vesicles in eukaryotic cells is a sophisticated process that requires the co-ordinated function of cytoskeletal transport routes, motor proteins and regulators (reviewed in 1–3). For example, endosomal vesicles containing internalized material undergo a canonical series of maturation steps that involve the directional transport on microtubules (MTs) (4).

The modification of this cellular machinery is of vital importance for intracellular pathogens. Salmonella enterica is a Gram-negative bacterium not only able to invade non-phagocytic cells but also able to survive after internalization by phagocytic cells (recent overview in 5). Regardless of the way of uptake, Salmonella remains located within a membrane-bound compartment termed Salmonella-containing vacuole (SCV). The SCV has special features such as the presence of late endosomal/lysosomal membrane proteins (e.g. LAMP-1), an acidic pH and the juxtaposition to the trans Golgi network, MT-organizing center and nucleus in epithelial cells. To control its intracellular fate, Salmonella actively manipulates the host cell, and a type III secretion system (T3SS) encoded by Salmonella pathogenicity island 2 (SPI2) is essential for this pathogenic lifestyle (reviewed in 6). The SPI2-T3SS is induced inside the SCV and translocates a set of 19 and possibly more effector proteins across the vacuolar membrane. While the initial steps of the biogenesis of the SCV do not appear dependent on the SPI2-T3SS, its function is later required to maintain the integrity of the SCV and its specific subcellular localization, to prevent the delivery of antimicrobial host factors to the SCV, to modify the organization of the host cell cytoskeleton and to alter vesicular transport.

A remarkable phenotype induced by intracellular Salmonella is the formation of so-called Salmonella-induced filaments (SIF) (7). SIF are tubular membrane structures that contain various late endosomal/lysosomal markers that are also characteristic for the SCV. The formation of SIF has been characterized in epithelial cell lines, and SIF were shown to form along MTs (8,9). Previous work indicated that the induction of SIF formation is dependent on the function of the SPI2-T3SS (10). Mutant strains defective in the SPI2-T3SS and unable to translocate effector proteins do not induce SIF formation. More specifically, a subset of SPI2 effector proteins is involved in SIF formation consisting of SifA, SseF, SseG, SopD2 and PipB2 (10–14).

Recent studies demonstrated the involvement of MT motor proteins in SIF formation (15,16). A model has been proposed in that the balanced activity of opposing motor proteins dynein and kinesin is required for the maintenance of the SCV in a perinuclear localization and the effective intracellular replication of Salmonella (17,18).

Despite these observations, the biogenesis of SIF is not well understood, and it is not clear how SIF formation correlate to the intracellular survival and proliferation of Salmonella. The involvement of host cell transport processes prompted us to investigate the dynamics of the host cell endosomal system in response to Salmonella infection using live cell approaches and ultrastructural analyses. Our observations indicate that SIF formation is
induced in various cell types infected with Salmonella and that SIF are highly dynamic structures.

Results

Live cell setups for endosomal remodeling by Salmonella

Endosomal remodeling and formation of SIF have been reported previously for Salmonella-infected epithelial cells (7), and it is now clear that this phenotype requires the function of the SPI2-T3SS and a subset of SPI2-T3SS effector proteins (10). So far, these studies have been performed with fixed cells that were analyzed for the extent of SIF formation, the presence of specific host cell markers and bacterial factors involved. For a more detailed understanding of SIF biogenesis, we developed live cell setups and investigated the dynamics of SIF formation.

A characteristic feature of the SCV as well as of SIF is the presence of highly abundant lysosomal glycoproteins such as LAMP-1. We transfected HeLa cells with a vector expressing LAMP-1-green fluorescent protein (GFP), followed by infection with S. typhimurium that constitutively expresses GFP or mCherry. SIF formation was observed in live host cells infected with S. typhimurium wild type (WT), but not in cells infected with strains defective in the SPI2-T3SS (sfaV) or a strain deleted in sifA, encoding the key effector for SIF induction (sifA) (Figure 1A, Figure S1). Because of the uniform size and shape of the intracellular bacteria, the bacterial GFP fluorescence in general was easily distinguishable from the GFP label on endosomal membranes. Infected cells were also fixed and immunostained for LAMP-1. SIF formation was observed in LAMP-1-GFP-transfected and non-transfected cells. A nearly complete colocalization of LAMP-1-GFP and endogenous LAMP-1 was confirmed in fixed and immunostained cells (Figure S2). The frequency of SIF-positive cells and the gross morphological appearance of SIF were similar in transfected and non-transfected HeLa cells (data not shown). Furthermore, the intracellular proliferation of Salmonella was not altered by LAMP-1-GFP transfection. These results indicated that LAMP-1-GFP is a useful marker for further analyses.

As alternative markers, we investigated the loading of host cells after infection with fluid-phase markers such as Alexa Fluor 568-conjugated dextran 10 000 (Alexa568-dextran) (Figure 1B,C) or 10-nm gold particles conjugated with BSA–rhodamine (Figure 1D). Labeling of the SCV and of SIF was observed with either tracer. Both approaches were combined, and the analyses of living cells indicated that infection with Salmonella WT resulted in the formation of SIF that were positive for LAMP-1-GFP and Alexa568-dextran added 5 h after infection with Salmonella (Figure 1C). The majority of SIF were positive for both markers, although some SIF were only labeled with the fluid tracer or LAMP-1-GFP. Note that LAMP-1-GFP labels the cytoplasmic face of the SIF membrane, while Alexa568-dextran labels the lumen of the SIF tubules. Experiments were performed with fluid tracers added 5 h after infection, and imaging was performed 2 h after addition of the tracer. Interestingly, we observed that in a portion of infected cells analyzed, the SCV as well as SIF were labeled with the tracer. The entire lumen of the SCV appeared positive for the tracer (Figure 1D). These observations are in accord with the recently reported continuous interaction of the SCV with the endosomal system (19). Because of its higher photostability, we preferentially used LAMP-1-GFP for further investigations in live cell experiments with HeLa cells.

Intracellular Salmonella alter the overall endosomal organization

Using the live cell setup, we followed the intracellular fate of Salmonella and the organization of the labeled endosomes over time. We observed the fusion of LAMP-1-GFP-positive vesicles with SCV as early as 2 h after infection (Figure 2A; Movie S1).

As expected, the formation of SIF was observed in cells infected with Salmonella WT but not in cells infected with SPI2-deficient Salmonella. The formation of tubular extensions positive for LAMP-1-GFP emerging from the SCV was observed as early as 3 h after infection, and these tubules were rapidly changing in size (Figure 2B; Movie S2). In accord with previous studies (7,20), we noticed that the number of filaments increased over time, resulting in a complex meshwork of filaments visible after 10 h of infection with Salmonella. During this process, the increase of SIF was correlated with decrease of late endosomal/lysosomal vesicles (Figure 2C). The numbers of LAMP-1-GFP-positive vesicles in living cells were determined under various infection conditions (Figure 2D). We counted the total number of LAMP-1-GFP-positive, spherical structures per infected cell as indicated in Figure S3. In noninfected cells or cells infected with a SPI2 strain, the number of vesicles was not different from that of WT-infected cells observed 3–6 h after infection. In contrast, a highly decreased number of LAMP-1-GFP-positive vesicles was observed in cells infected for 8–9 h with WT Salmonella. These observations indicate that LAMP-1-GFP-containing membranes are reorganized into tubular SIF by the action of intracellular Salmonella.

Ultrastructural features of SIF

In all previous studies, the alterations of the endosomal system caused by Salmonella were analyzed using immunofluorescence microscopy techniques. In this study, we applied electron microscopy (EM) to investigate structural features of SIF with higher resolution. To correlate immunofluorescence microscopy to EM, we processed cells infected with Salmonella WT for immunogold labeling on cryosections. We found abundant labeling of the LAMP-1
on SCV as well as on membranes of adjacent vesicles (Figure 3A). In a portion of the sections analyzed for LAMP-1-GFP-expressing cells, tubular membrane structures with frequent anti-GFP labeling were observed (Figure 3B).

To reveal the spatial distribution of the SIF, we further analyzed vertical ultrathin sections from flat-embedded samples to preserve the polarity of adherent cells. We observed specific tubular membrane structures in Salmonella-infected cells that did not appear in mock-infected control cells (Figure S4) or cells infected with the SPI2 strain (data not shown). These membrane tubules were similar in proportion to prolonged tubular mitochondria but clearly distinguishable by lack of internal membrane cristae. Transmission EM analyses indicated typical diameters of SIF in the range of 160 (±39 nm), and we propose that these structures correspond to SIF observed in living cells or fixed immunostained cells. For some tubular membranes, a clear continuity between the SCV and the lumen of the SCV was observed as shown in Figure 3C,D, and occasionally, multiple membrane structures were observed for SIF and the SCV. Many tubular membranes were also found without connection to the SCV. This observation may be interpreted as cross-section through the three-dimensional structure because ultrathin sections represent merely 1% of the total cell volume. However, free SIF without connection to SCV were also observed by live imaging. Interestingly, closer inspection of the vicinity of the SCV (Figure 3C) and the tip of SIF (Figure 3D) indicated the presence of large amounts of small spherical as well as tubular vesicles. These might represent a pool of vesicles that interact with SCV and SIF and could contribute to the increase of SCV diameter and SIF length. However, because the fate of a vesicle cannot be followed by EM, it is also possible that these vesicles result from fission of the SCV or SIF.

Figure 1: Tracing of the endosomal system in living host cells infected with Salmonella. A) HeLa cells were transfected with LAMP-1-GFP (green) and infected with S. typhimurium WT and ssaV or sifA-deficient strains expressing GFP (green). Living cells were imaged 5 h after infection using a with Zeiss Axiovert 200M wide field microscope. B) HeLa cells were infected with S. typhimurium WT expressing GFP (green), and the fluid-phase marker Alexa568-dextran was added 5 h after infection. Living cells were imaged 2 h after addition of the marker. C) HeLa cells were transfected as in (A) and infected with WT Salmonella expressing mCherry, but in addition, Alexa568-dextran was added as in (B) at 5 h after infection. Note the strong colocalization of both markers in most of the tubular compartments. D) HeLa cells were infected with WT Salmonella, and the fluid-phase marker 10 nM gold–BSA–rhodamine was added 5 h after infection. After 2 h, the cells were washed and immediately imaged. Note the SCV that shows colocalization between intracellular Salmonella and gold–BSA–rhodamine (arrowhead). Scale bars, 5 and 2.5 μm for overview and inset, respectively.
Salmonella induces highly dynamic tubular endosomal aggregates

We next followed the formation of SIF in living HeLa cells after transfection with LAMP-1-GFP expression and infection with Salmonella WT or SPI2-deficient strains expressing GFP. Time-lapse series were acquired from 1 to 10 h after infection, and a representative example is shown in Figure 4 (Movie S3). We observed that the replication of intracellular bacteria was detectable starting at 3.5 h, and larger clusters of bacteria or microcolonies were observed from 6 h onwards. We also noted the formation of tubular LAMP-1-GFP-positive structures as early as 3 h after infection. The number and the extent of these tubular structures increased over time, and based on the appearance of the tubular structures, we considered them to be SIF. Closer analyses of the time-lapse movies indicated that individual SIF were highly dynamic with respect to not only extension but also contraction, branching or fusion with other SIF (Movie S3). We noted that these dynamics are most prominent shortly after induction of SIF. The velocity of growth and collapse or contraction as well as the frequency of branching and merging of SIF decreased over time, but SIF number and length increased. At the end of the observation period, a complex network of SIF was established similar to the appearance of SIF in fixed cells. At this time-point, the SIF were almost static and showed only little alteration in length.

Figure 2: Salmonella-induced changes to the host cell endosomal system. HeLa cells were transfected with a vector for LAMP-1-GFP expression and infected with Salmonella WT or SPI2-deficient strains expressing GFP. Imaging was performed using a PerkinElmer UltraView RS spinning disk confocal microscope. A) The fate of LAMP-1-GFP-positive vesicles (arrowhead) in the vicinity of the SCV (arrow) was followed 2 h after infection. Tracking of individual vesicles showed the fusion of these vesicles to the SCV (corresponding to Movie S1). B) Emergence of tubular structures from SCV 3 h after infection (Movie S2). Note the rapid growth of tubular structure (indicated by arrowheads) from an existing tubular compartment. C and D) Overall distribution of LAMP-1-GFP-positive membranes at early and late time-point of infection. C) Representative mock-infected cells or cells infected with WT or SPI2-deficient strain are shown 4 or 9 h after infection as indicated. Projections of five z sections with 0.5 μm spacing are shown. Scale bars, 5 μm. D) The overall number of LAMP-1-GFP-positive vesicles was determined in living HeLa cells at various time-points after mock infection or infection with WT or SPI2 strains. The mean number (±SD) of LAMP-1-GFP-positive spherical vesicles was determined for 20 cells per experimental condition, and the data shown are representative for three independent experiments.
These novel observations for a unique cellular structure induced by pathogenic bacteria prompted us to characterize SIF formation in living cells in more detail and in a quantitative manner.

**Dynamic SIF are formed in various host cells**

Previous studies investigated SIF in epithelial cell lines such as HeLa. As the observation of SIF in cell lines raises some concerns about the broader biological relevance of the phenomenon, we set out to investigate SIF formation in other cell lines as well as in primary cells. One previous report showed the appearance of SIF-like tubular membranes in interferon-γ (IFN-γ)-stimulated macrophages infected with *Salmonella* (21). IFN-γ stimulation results in the activation of macrophages that coincides with changes in the gross cell morphology, most importantly the spreading of the cell and tight adherence to a substrate.

The murine macrophage-like cell line RAW264.7 was IFN-γ stimulated, pulse–chased with BSA coupled to 10-nm gold–rhodamine (Figure S5A) or Alexa568-dextran (Figure 5A,B) and infected with *Salmonella* WT, a SPI2 strain or mock infected. We observed that SIF were formed in cells infected with the WT strain but not after infection with the SPI2-deficient strain or mock infection (Figure 5A). The SIF showed rapid growth, branching or contraction similar to the phenotype observed in HeLa cells. In nonactivated macrophages, no SIF formation was detectable, and we consider the more spherical cell morphology as the main obstacle in visualization of SIF that may also be formed in nonactivated macrophages. Tubular endosomal compartments are commonly observed in living eukaryotic cells. Although short tubular compartments were found on SPI2 or mock-infected macrophages, the tubular compartments in WT-infected cells were clearly distinguishable with respect to their length and dynamic properties (Figure 5A; Movie S4). The quantification of the length of fluid tracer-labeled tubular endosomes demonstrates the unique properties of tubular endosomes induced by WT *Salmonella* (Figure 5B). The formation of
SIF and dynamic properties of SIF were similar in primary peritoneal macrophages from mice (Figure S5C) and in cell line macrophages (Figure S5B).

In contrast to epithelial cells, the formation of SIF in activated macrophages started at later time-points and was detected after 6 h of infection. Despite the delayed onset, SIF in macrophages were similar in their dynamic properties to SIF in HeLa cells. Rapid growth, contraction, branching and fusion of SIF were detected.

We also infected RAW cells with a *Salmonella* pathogenicity island 1 (SPI1)-deficient strain (data not shown). The bacteria were either grown to late log phase as for invasion

**Figure 4: Dynamics of LAMP-1 compartments and SIF in *Salmonella*-infected cells.** HeLa cells were transfected with LAMP-1-GFP and infected with a *S. typhimurium* WT strain constitutively expressing GFP. Time-lapse microscopy of GFP fluorescence was performed in intervals of 3 min, starting 1 h after the infection of the cells. Image acquisition was performed using the PerkinElmer UltraView RS spinning disk confocal microscope. The still images show projections of nine z sections with a spacing of 0.5 μm. The micrographs correspond to the time-lapse movie (Movie S3), and time-points of acquisition are indicated as h:min:seconds after infection. Three individual bacteria that developed into intracellular microcolonies during the course of the experiment are indicated by arrows (red, orange and yellow). The appearance of tubular vesicular structures positive for LAMP-1-GFP is indicated by arrowheads of different colors. Blue arrowheads indicate SIF that cannot be associated with one of the three microcolonies. Note the growth, shrinkage and disappearance of SIF starting at 03:30:00 and the formation of an extensive network of SIF starting at 06:50:00. Division of the intracellular bacteria was detectable starting at 02:40:00. The perimeter of one infected cell is indicated by a yellow line in the 01:00:00 image. Scale bars, 5 μm.
Figure 5: *Salmonella* induces highly dynamic SIF in various host cells types. Infection experiments were performed with the murine macrophage-like cell line RAW264.7 after activation by 5 ng/mL IFNγ (A and B), or murine BM-DC (C). Cells were mock infected or infected with *Salmonella* WT or SPI2 strains expressing GFP (green). IFNγ-activated RAW264.7 cells (A, Movie S4) were used for infection with *Salmonella* WT, and the fluid-phase marker Alexa668-dextran was used to follow SIF formation in living cells using a PerkinElmer spinning disk system. A) Left panels show the overview of the infected cells (scale bars, 5 μm), and boxes indicate positions in the periphery of the cells where representative stills show the details of dynamic alternations of SIF (scale bars, 1 μm). The relative time is expressed as h:min:seconds, and SIF growth is indicated by yellow arrowheads. B) IFNγ-activated RAW cells were infected as in (A), and the length of fluid tracer-labeled tubular compartments was determined. The average tubule length (±SD) of about 50 to 75 cells per condition is shown. C) For BM-DC, the fluid-phase marker 10-nm gold-BSA–rhodamine was added 1 h prior infection. Living cells were imaged 8 h after infection using a Zeiss Axiovert 200M wide field microscope. Scale bars, 5 μm.
of epithelial cells or to stationary phase as used for infection of macrophages with WT *Salmonella*. The growth phase of the bacteria had no effect on the time-point of onset of SIF formation, and also with exponentially growing bacteria, earliest SIF formation was observed 6 h after infection. The number, appearance and dynamics of SIF were indistinguishable in infected cells with *Salmonella* WT or the SPI1-T3SS-deficient strain. After invasion of non-phagocytic cells by *Salmonella*, the SPI1-T3SS remains active in translocation of effector proteins from *Salmonella* within the SCV. A recent study proposed that activity of SPI1 effectors contribute to the biogenesis of the SCV and might act synergistically with SPI2-T3SS effectors (22). Our observations suggest that, at least in macrophages, there is no requirement of the SPI1-T3SS and its effectors for the formation of dynamic SIF.

Dendritic cells are phagocytic and highly efficient antigen-presenting cells (23). We previously described that *Salmonella* is internalized by murine bone marrow-derived dendritic cells (BM-DC), where the bacteria persist as a non-replicating population (24). However, *Salmonella* actively translocates effector proteins by the SPI2-T3SS and affects antigen presentation by BM-DC (25). In this study, we used BM-DC in an experimental setup with fluid-phase tracers and observed extensive tubular structures in *Salmonella* WT-infected BM-DC (Figure 5C).

**SIF rapidly extend, move and contract**

We next performed time-lapse microscopy of infected HeLa cells with a higher temporal resolution (one to eight images per second). The investigation of individual SIF showed the highly dynamic properties of SIF as shown in Figure 6A–D and Movie S5. Within few seconds, the appearance of SIF tips changes dramatically. Linear growth of SIF was observed as well as apparently random changes in the direction of SIF extension (Figure 6A). On individual SIF, phases of extension were directly followed by the complete contraction of the tubular extension (Figure 6B). Some short tubular vesicles pinched off from longer SIF, moved in various directions in the cell and could also contact other SIF (Figure 6C).

SIF were not always connected to SCV. Projections of Z stacks were generated and indicated that a proportion of the SIF had no detectable contact to any SCV present in the infected cell. Occasionally, SIF emerged from an SCV and later lost the connection to the SCV from which the tubule originated. Also, SIF without initial connection to SCV were observed that fused with SCV (data not shown).

For a subset of SIF, the presence of thinner, LAMP-1-GFP-positive tubular structures was observed that were followed by a tubular structure with the diameter typical for SIF (Figure 6D; Movie S6). During SIF extension and contraction, the thicker ‘trailing’ tubules always followed the thinner ‘tracking’ tubules. Because of the limitations in resolution, it was not possible to distinguish if two tubular structures of different diameter were colocalized or if the thinner structures temporarily increase or decrease in diameter. This phenomenon was only observed in cells early after the onset of SIF formations, that is 4–6 h after infection.

**SIF extend and contract with different velocities**

We first performed time-lapse analysis from 1 to 10 h after infection (Figure 4). The formation of SIF was detectable from 3 h after infection. At later time-points, the number of SIF per infected cell increased and bacterial replication led to the formation of microcolonies. The initiation of SIF already 3 h after infection was an unexpected observation because previous studies using fixed cells reported SIF formation not earlier than 5 h after infection. We quantified the appearance of SIF under the various experimental conditions and scored the appearance of SIF in about 100 infected cells per group. In living LAMP-1-GFP-transfected cells, 70% of the cells showed one or more SIF at 4 h after infection and at 8 h after infection 85% of the cells showed a complex network of SIF. In fixed cells, about 10% of the LAMP-1-GFP-transfected cells but none of the non-transfected cells were positive for SIF at 4 h after infection. Under both conditions, 65–70% of cells showed SIF if cells were fixed at 8 h after infection. The data show that SIF are formed early after infection, but these structures are only visible in living cells and most likely destroyed by fixation.

We set out to quantify the dynamics of SIF at various time-points of the intracellular life of *Salmonella*. For quantification, various time-lapse series were analyzed by recording the length of individual SIF over time using the EMBL IMAGE J plug in Kymograph (26). Kymographs were generated by determination of the length of selected SIF for each time-point in a time-lapse series (see Figure S6 for schematic representation and examples). Extremely dynamic alterations of SIF were observed at early time-points after infection (representative example shown in Figure 7A; Movie S7). The kymographs plotted for individual SIF showed areas of rapid increase of SIF length, followed by rapid contraction and repeated extension. Later in infection, that is 8–10 h after infection, a complex network of SIF extended through the entire cell. At this time-point, the speed of SIF growth and contraction was reduced (example shown in Figure 7B; Movie S8). The corresponding kymographs were often ‘flat’, indicating that only minor changes in SIF length occurred over the time of examination. Kymographs and velocity calculations were performed for a larger number of SIF in cells infected for 4–5 or for 8–9 h. The velocity of SIF growth and contraction was calculated and is displayed in Figure 7C. The average speed of SIF growth was 0.4 μm/second in cells at 4–5 h post-infection and reduced to 0.02 μm/second in cells at 8–9 h post-infection. Also, the average velocity of SIF contraction was substantially reduced from 0.5 to 0.08 μm/second at 4–5 and 8–9 h after infection, respectively (Figure 7C). As a control of our experimental
system, we followed the movement of lysosomes labeled with LysoTracker in living cells at various time-points. An average velocity of 1.52 and 1.01 ± 0.85 μm/second for lysosomes was recorded at 4 and 8 h after mock infection, respectively. These data demonstrate that about 10-fold reduction in SIF dynamics was not because of a general reduction in the velocity of vesicle motility over the time-course of the experiment.

We conclude that the dynamics of SIF is inversely correlated with the number of SIF and the extent of the intracellular replication of Salmonella.

Role of MTs in SIF dynamics

Previous studies highlighted the role of MTs for the biogenesis of SIF (7–9). The formation of SIF is dependent on the integrity of the MT network as MT depolymerization
by nocodazole prevented SIF formation. The interaction of MT with SIF was also addressed by ultrastructural analyses (Figure 8A). In this study, we show that a subset of SIF forms along MTs, which may act as guidance for the growth of SIF. In certain instances, SIF were closely associated with two MTs.

We analyzed the effect of pharmacological inhibitors of MT on SIF dynamics in live cells (Figure 8B; Movie S9). The addition of the MT-depolymerizing drug nocodazole resulted in the disintegration of the MT cytoskeleton (Figure S7). Nocodazole treatment at 5 h after infection did result in the loss of a large number of existing SIF (for example, SIF 02 in Figure 8C). However, about 10–20% of the SIF formed prior to nocodazole addition were maintained after drug treatment (for example, SIF 01 in Figure 8C). In contrast, most SIF were maintained if nocodazole was added 8 h after infection (data not shown), that is at a time-point when the dynamics of SIF was already reduced. SIF were entirely static in the presence of nocodazole, while SIF in mock-treated control cells were highly dynamic at this time-point. Similar results were obtained with Taxol, an inhibitor that results in stabilization of MT. To quantify the effects of nocodazole, we performed analyses on SIF in individual cells prior and after addition of the drug (representative example shown in Figure 8C). A kymograph obtained for a SIF prior to nocodazole addition indicated phases of rapid growth and contraction. After

Figure 7: Dynamics of SIF at various time-points after infection. Transfection and infection were performed as described in Figure 4, and time-lapse microscopy on the UltraView spinning disk system was performed with intervals of 250 milliseconds at various time-points after infection. Representative cells at 4 or 8 h after infection are shown for the different time-points of observation. For the quantification of the velocity of SIF extension, individual SIF were selected and analyzed using the EMBL IMAGEJ plug in Kymograph. A) The left panel shows an infected cell 4 h after infection. The region in the periphery of the cell with multiple SIF is indicated by the box and shown in detail in the right panel (also shown in Movie S7). The kymograph (lower left panel) corresponds to SIF 5 and includes the analyses of a 928 frames acquired over a period of 100 seconds. Detail micrographs (lower right panels) show the morphology of SIF 5 during phases of extension (green arrows) and contraction (red arrows) within the observation period of 100 seconds. B) A representative cell is shown 8 h after infection (also shown in Movie S8). The positions of SIF 1–4 are indicated in the upper right micrograph. The kymographs corresponding to SIF 1–4 are shown in the lower panels and have been generated by analyses of 928 frames acquired over a period of 100 seconds. C) The velocity of SIF extension and contraction was calculated from kymographs generated for cells after 4–5 or 8–9 h after infection, and the data are displayed as box and whisker plot. Velocities were determined for 50 to 70 events per category. p.i., post-infection.
addition of nocodazole, the dynamic changes of the specific SIF were completely blocked as early as 20 min after the addition of the inhibitor, and the SIF maintained their length without significant changes for the rest of the observation period. Finally, we analyzed the effect of the removal of nocodazole on SIF dynamics (Figure 8D; Movie S10). As expected, SIF dynamics ceased after addition of the drug, but rapid extension and retraction of SIF...
were restored by washing out nocodazole. At early time-points after nocodazole removal (Figure 8D, 10 min), we often found dynamic SIF with increased diameter, but a normal SIF appearance was restored at later time-point.

**Discussion**

The intracellular activities of *S. enterica* result in the remodeling of the host cell endosomal system and SIF formation. SIF are unique morphological alterations of endosomes that are only observed in *Salmonella*-infected cells (7) and dependent on the function of the SPI1-T3SS (10) and a subset of its effector proteins. In this study, we describe for the first time the biogenesis of SIF in living *Salmonella*-infected host cells and their highly dynamic properties.

Formation of tubular organelles is a common phenomenon in mammalian cells and observed, for example for transport of compartments from Golgi to plasma membrane (27) or sorting endosomes (28). Recently, the formation of tubular vesicular structures with a high content of major histocompatibility complex (MHC) II was observed in DC (29). Such tubules are formed without the involvement of intracellular bacteria. During our live cell analyses, we also observed shorter, motile LAMP-1-GFP-positive or fluid tracer-labeled tubules in HeLa cells, or RAW macrophages and BM-DC, respectively. The comparison of cells infected with WT and SPI2 *Salmonella* or mock-infected cells revealed the morphological difference between SIF induced by *Salmonella* and the intrinsic tubular compartments. The tubular endosomal structures observed in *Salmonella* WT-infected BM-DC had dynamic properties and because of their length were clearly distinguishable from shorter tubular endosomes that appeared in noninfected BM-DC or cells infected with a SPI2-deficient strain (Figure 5B).

Our observations indicate that dynamic properties of SIF are a general phenomenon associated with the intracellular life cycle of *Salmonella* in various host cell types. Uptake of *Salmonella* by macrophages is independent from bacterial invasion and function of effectors translocated by the SPI1-T3SS. Because SIF induction and dynamic properties of SIF were observed after uptake of noninvasive WT bacteria as well as of SPI1-T3SS-deficient strains but not with SPI2-T3SS-deficient *Salmonella*, we propose that the phenotypes described in this study are independent of SPI1 function. We found that highly dynamic SIF are formed in activated macrophages as well as in DC. While activated macrophages restrict the replication of intracellular *Salmonella*, the bacteria persist as a static, nonreplicating population in DC (24). These data clearly demonstrate that SIF formation is not *per se* linked to the rapid intracellular replication of *Salmonella* as previously proposed (20). A low number of bacteria in DC were sufficient to induce formation of dynamic SIF without a requirement for intracellular replication.

Dramatic differences in the velocity of SIF extension and contraction at different time-points after infection were recorded. Early after the onset of SIF formation (3.5–5 h after infection), the velocity and the variability in SIF appearance were most prominent, while at later points (>7 h after infection), a complex network of SIF was established that exhibited only little extension or contraction. The simplest explanation is the continuous integration of the endosomal membranes into SIF, resulting in a shortage of available membranes at later time-points of infection. We observed an inverse correlation between the number of globular LAMP-1-positive vesicles and the complexity of the SIF network (Figure 2). However, one should be aware that because of the transfection approach applied, only a subset of the endosomal vesicles in the host cells could be visualized. In addition, host cell molecules mediating the fusion of vesicle to extending SIF might be titrated by the increasing number of bacterial effectors that are translocated over time.

We have previously reported that intracellular *Salmonella* can induce, in a SPI2-T3SS-dependent manner, the bundling of MTs of infected host cells (9). Our ultrastructural analyses showed that SIF can form along MT and that SIF can be attached to two or more MTs (Figure 8A). The cross-linking activity might be mediated by effector proteins that are present in the membranes of SIF and bind directly to MTs or indirectly by MT-associated proteins.

SIF are considered as the result of continuous aggregation of endosomal membrane vesicles into tubules of uniform thickness. Our observation of the rapid extension and contraction or collapse of SIF during the early phase of infection would also support other models (Figure 9). During the early phase of intracellular life of *Salmonella*, SIF grow out from the SCV both by continual fusion of vesicles with the tip of SIF and by pulling force generated by MT motors associated with the tips of SIF. SIF growth can be directed toward minus ends or plus ends, depending on the proportion of dynein or kinesin motors, respectively, that are recruited. In this study, every fusion step will introduce membrane material into the SIF and partially and temporarily relax the internal stress. However, when the membrane tip of SIF is pulled by MT motors too far without fusion, the elastic stress in the membrane increases and reaches a critical threshold. If motors detach from MT or from SIF membrane, the SIF collapses rapidly, relaxing membrane elastic stress. The higher velocity of SIF contraction compared with extension would be in line with such model. Although SIF extension in both directions can be observed, extension toward the plus ends dominates over the time-course of infection, indicating a preferential recruitment of kinesin or cargo transported by kinesin. The appearance of a stabilized SIF network in later phase might indicate a consumption of vesicles available for fusion.

Furthermore, the growth is limited by the length of the MT transport track. In addition, the accumulation of SPI2...
Effector proteins leads to aggregation of MT that might limit the transport and stabilize a network of SIF. There is a clear role of MT-based motility, as indicated by the effect of nocodazole or Taxol on SIF dynamics, and the function of dynein and kinesin motors has been reported (15,16,30). The specific contribution of individual motor proteins in the dynamics of SIF extension and contraction remains to be clarified by future live cell studies. Also, our model cannot explain why SIF without connection to the SCV appear and move in infected cells.

Depolymerization of MT by nocodazole had different effects on SIF at early and later time-points after infection. In general, SIF dynamics was ablated by nocodazole inhibition. However, the less dynamic networks of SIF 8 h after infection and later were maintained, while most of the highly dynamic SIF in the early phase were lost after nocodazole treatment. This observation might indicate structural differences between ‘early’ dynamic SIF and ‘late’ non-dynamic SIF that we will investigate in subsequent studies.

A role of SPI2-T3SS effector proteins in controlling the MT motor protein activities acting on the SCV has been reported (31,32). According to current models for the function of SPI2-T3SS effector proteins, the SCV has to maintain a balance between opposing activities of motor proteins (17,18). Such balance would allow the proper intracellular positioning of the SCV and the sufficient supply of endosomal membranes to maintain the SCV with an increasing bacterial population. These models might have to be reconsidered in the light of our observations of the highly dynamic nature of SIF. For example, the proposed function of SifA as an effector that interacts with SKIP to prevent the kinesin motor protein activity on the SCV (31) might not be sufficient to explain the induction of SCV tubulation and the rapid extension or contraction of SIF. Future studies have to reveal the contribution of MT motor proteins to the dynamic features of SIF and how these activities are manipulated by the function of a subset of SPI2-T3SS effector proteins. The function of the SPI2-T3SS has been linked to various intracellular events such as the avoidance of antimicrobial activities, modification of the MT and actin cytoskeleton, interference with antigen presentation and many others (reviewed in 5,6).

The biological role of the induction of SIF and the remodeling of the endosomal compartment of the host cell of *Salmonella* is not completely understood. A clear requirement for intracellular replication is the recruitment of membranes for the extension of the SCV (10). We applied various methods for the labeling of the luminal content of SIF as well as the membranes, and both approaches resulted in the appearance of SIF with similar morphology and dynamic properties. Interestingly, we could observe that the lumen of SIF as well as that of the SCV were accessible to fluid-phase markers in various *Salmonella*-infected host cells. A recent study by Drecktrah et al. (19) used similar labels and a live cell setup, and these authors observed the continuous interaction of the SCV with endocytosed material. The data reported in this study support the model of Drecktrah et al. and stand against the currently prevailing model that the SCV is separated from the endosomal system. We propose that *Salmonella* within the SCV experiences nutritional limitations but temporarily gains access to external material by induction of SIF. Future work has to reveal whether this also leads to an increased availability of nutrients that may otherwise be

Figure 9: Models for dynamic extension and contraction of SIF. A) *Salmonella* within the SCV translocate effector proteins (E) of the SPI2-T3SS. B) By activity of effector proteins, membrane vesicles transported on MT are recruited to and fuse with the SCV. These events not only allow the enlargement of the SCV and delivery of luminal content to the SCV (indicated by blue shading) but also lead to accumulation of motor proteins on the SCV. C) Increased accumulation of motor proteins results in a pulling force on the SCV membrane and formation of tubular extension. D) If pulling forces are too high, motor proteins could lose contact to SIF membrane or MT tracks, resulting in contraction of SIF. Dependent on the nature of the motor protein recruited, SIF extend toward the minus (−) or plus (+) end of MT. The extension toward the plus end appears to be dominant.
limited within the SCV. We will also investigate if this recruitment contributes to the nutritional status of *Salmonella* in the SCV and could explain the unique intracellular lifestyle of the pathogen.

Materials and Methods

**Bacterial strains and culture conditions**

*S. enterica* serovar Typhimurium (*S. typhimurium*) NCTC 12023 was used as WT strain. Experiments were performed in parallel with *S. typhimurium* LT2A, a strain that shows attenuated virulence in vivo. We found no difference in the intracellular survival and replication or the SIF induction between 12023 and LT2A (Figure S1). For live imaging, strains were used harboring plasmid pFPV25.1 (33) or pFPV-mCherry/2 (kindly provided by L. A. Knodler) for the constitutive expression of enhanced GFP or mCherry, respectively. Mutant strains invC, P2D6 and P3H6 defective in the SPI1-T3SS, SPI2-T3SS or effector gene *sifA* respectively, have been described before (10,34). Bacterial strains were routinely cultured in Luria-Bertani (LB) medium without antibiotics prior to bacterial infection. For the activation of RAW264.7 cells, IFN-γ was added in concentrations between 2 and 10 ng/mL for 24 h. Typically, 5 ng IFN-γ/mL were used and resulted in about 90% activated macrophages as judged from the cell morphology. For the generation of primary peritoneal macrophages, BALB/c received an intraperitoneal injection of 4% thioglycolate in PBS and after 4 days, about 90% activated macrophages were isolated from the spleen. For infection of RAW264.7 and murine peritoneal macrophages, as described before (35). Murine macrophages were isolated basically as described before (35). Murine BM-DC were generated from C57BL/6 mice essentially as described before (24). BM-DC were further enriched by sorting on magnetic antibody cell sorting columns (Miltenyi Biotech), and a purity of 90% was routinely obtained. The BM-DC were in an immature state (CD11c high and MHCII low) as confirmed by fluorescence-activated cell sorter analyses.

**Cell culture**

Human epithelial cell line HeLa cells (American Type Culture Collection, ATCC no. CCL-2) and the murine macrophage-like cell line RAW264.7 (ATCC no. TIB-71) were cultured in DMEM with 10% fetal calf serum (FCS), penicillin and streptomycin and grown at 37°C with 5% CO₂. Cells were cultured in medium without antibiotics prior to bacterial infection. For the activation of RAW264.7 cells, IFN-γ was added in concentrations between 2 and 10 ng/mL for 24 h. Typically, 5 ng IFN-γ/mL were used and resulted in about 90% activated macrophages as judged from the cell morphology. For the generation of primary peritoneal macrophages, BALB/c received an intraperitoneal injection of 4% thioglycolate in PBS and after 4 days, macrophages were isolated basically as described before (35). Murine BM-DC were generated from C57BL/6 mice essentially as described before (24). BM-DC were further enriched by sorting on magnetic antibody cell sorting columns (Miltenyi Biotech), and a purity of 90% was routinely obtained. The BM-DC were in an immature state (CD11c high and MHCII low) as confirmed by fluorescence-activated cell sorter analyses.

**Host cells infection**

For infection of HeLa cells, bacterial strains were grown overnight in LB broth. Overnight cultures were diluted 1:30 in fresh LB broth and subcultured for 3.5 h. At this time-point, the cultures reached the late log phase and were invasive. HeLa cells were infected with a multiplicity of infection (MOI) of 50. For infection of RAW264.7 and murine peritoneal macrophages, as well as for BM-DC, the strains were grown for 14–18 h. Such overnight cultures were diluted and used directly for infection of macrophages or BM-DC at an MOI of 10 or 50, respectively. After an incubation of 30 min to allow bacterial internalization the cells were washed thrice with PBS to removed non-internalized bacteria. Subsequently, DMEM containing 10% FCS and 100 µg gentamicin/mL was added to kill non-internalized bacteria. After incubation for 1 h, the medium was replaced by medium containing 10 µg/mL gentamicin for the rest of the incubation time.

**Transfection**

The plasmid for the eukaryotic expression of LAMP-1-GFP was kindly provided by Patrice Boquet. HeLa (2 × 10⁵) cells were seeded in an eight-chamber glass slide (Nunc-LabTek) and allowed to adhere overnight. Transfection was routinely performed by the calcium phosphate method (36). In this study, 500 ng of plasmid DNA (LAMP-1 for single transfections) was mixed with the transfection reagent and added to cells in eight-chamber slide with DMEM with 10% FCS. Cells were incubated for 4–5 h, and later, medium was changed and fresh DMEM with 10% FCS was added, and cells were later used for infections 16–18 h after transfection.

**Live cell imaging**

Cells were cultured in chamber slides and transfected or stimulated with IFN-γ if required. Infection with *Salmonella* strains was performed as described above, and extracellular bacteria were killed by incubation with medium containing 100 µg/mL gentamicin. After 1 h, this medium was replaced by imaging medium containing 10 µg/mL gentamicin. Imaging medium is Eagles MEM without l-glutamine, phenol red and sodium bicarbonate, containing 30 µM HEPES, pH 7.4.

The chamber slide was then taken for imaging at required time-points after infection. The chamber slide was mounted on the microscope stage equipped with a humidified environment chamber maintaining 37°C and 5% CO₂.

For inhibitor studies, nocodazole (Sigma) or plactaxel (Calbiochem) were used at the indicated concentrations from stock solutions in dimethyl sulfoxide. The effect of the inhibitors on MT was controlled by immunofluorescence (Figure S6).

**Fluid-phase marker and pulse–chase**

For tracing the endocytic pathway, various fluid-phase markers were used. Alexa668-dextran MW 10,000 was obtained from Molecular Probes (Invitrogen; D-22912). A 10 nm gold–BSA conjugate was generated as previously described (37). Subsequently, gold–BSA was fluorescently labeled with *N*-hydroxysuccinimide (NHS) rhodamine succinimidyl ester (Molecular Probes, Invitrogen). For use as tracer in live cell experiments, the solution was adjusted to an OD₅₉₀ of 0.1, and aliquots of 100 µL were added to the wells of chamber slides at the indicated time-points. For some experiments, HeLa cells were transfected prior infection with a LAMP-1-GFP vector; otherwise, non-transfected cells were used. HeLa cells were infected by invasive *Salmonella* strains as described, and 5 h post-infection, cells were incubated with 200 µg/mL Alexa668-dextran or gold–rhodamine for 3 h and subsequently washed. Cells were incubated for the rest of the experiment with label-free media and subjected to live imaging. Macrophages were infected, and 6- to 7-h post-infection, cells were incubated with 100 µg/mL Alexa668-dextran for 30 min, washed thrice with PBS and then incubated with DMEM with 10% FCS without tracer for 30–40 min. Subsequently, the cells were used for live imaging. For BM-DC, the tracers were added prior infection, incubated for 30 min, followed by washing of the cells and infection with *Salmonella*. For labeling of lysosomes, HeLa cells were then washed once with PBS and loaded with 500 nM LysoTracker Red DND-99 (Molecular probes; L-7528) in DMEM with FCS for 1 h. After pulsing, the cells were washed thrice with PBS, and 500 µL of imaging medium was added to each well and the cells were imaged.

**Microscopy and imaging**

Imaging studies were performed using two spinning disk confocal microscopes (UltraView RS and ERS; PerkinElmer). The spinning disk head (Yokogawa) of both systems was mounted to an inverted microscope (Axiovert200; Zeiss) that was equipped with a humidified environment chamber maintaining 37°C and 5% CO₂. All acquisition settings were adjusted by using the UltraView Software (PerkinElmer).

At various time-points, post-infection images were taken of cells infected with *Salmonella*. GFP fusion proteins were excited with the 488 nm line of an argon ion laser. Alexa668-dextran and mCherry fusion proteins were imaged using either the 568 nm line of a krypton gas laser (UltraView RS) or a 560 nm diode laser (UltraView ERS). The exposure time and the Z spacing were adjusted individually for each cell/condition and are given within the legend of the figures.

Additionally, some of the time series were also recorded using an inverted fluorescent microscope [Zeiss Axiosvert 200M equipped with Axiocam MRm (Zeiss)] and ×100 Plan Neofluar objective (Zeiss).
which maintained 37°C, 5% CO2, and humidity during live imaging. The microscope has a mercury lamp of 100 W (Zeiss) as fluorescence light source and a motorized filter turret to select the filter set for the corresponding fluorochrome (GFP, Cy5, DAPI, FITC, etc.).

All acquisitions and settings for the live imaging were using the ADOBE PHOTOSHOP 8.0 software (Zeiss, with various modules). The resulting movie series were corrected for background fluorescence and bleaching using bleach correction and background subtraction plugins available at EMBL IMAGEJ (URL: http://www.embli-heidelberg.de/ExternalInfo/almf/htdocs/almf_website/html/EMBL_I mageJ.html). Majority of the image analysis (to calculate the velocity of SIF growth and contraction) was carried out using the IMAGEJ plugin Kymograph (26) (available at http://www.embli.de/almf/html/downloads.html) and also manual tracking.

**EM and immunolabeling**

To prepare samples embedded in EPON Epoxy Resin (EPON 812, Serva), cells were grown on glass coverslips in plastic culture dishes in complete DMEM medium. Cells were infected with *S. typhimurium* at an MOI 50 and incubated for 10 h prior fixation. All samples were fixed 10-h post-infection in 2% glutaraldehyde in 0.1 M cacodylate buffer, post-fixed in 2% osmium tetroxide, dehydrated in ethanol series and propylene oxide, flat-embedded and polymerized in EPON812 (Serva). Glass coverslips were removed in liquid nitrogen, and blocks with cell monolayer were embedded and polymerized again to enable cutting perpendicular sections parallel to the z-axis. Sections (40 nm) were cut with diamond knife on Reichert Ultracut S and contrasted in uranyl acetate and lead citrate.

For cryosections, HeLa cells grown on plastic culture dishes in complete DMEM medium were transfected (or mock transfected) with the LAMP-1-GFP vector. During transient expression, cells were infected (or mock infected) with *S. typhimurium* expressing GFP and incubated for 10 h prior fixation. Samples were fixed with 4% paraformaldehyde and 0.05% glutaraldehyde (GA) in 0.1 M Sorensen phosphate buffer, pH 7.4, scraped gently with a rubber policeman, washed and embedded in low-melting 10% sucrose–methylcellulose and transferred on carbon-coated CuPd EM grids with parlodion film. For immunolabeling, we used mouse anti-LAMP-1 antibody (clone H4B8; DSHB), followed by goat-anti-mouse secondary antibody conjugated to 10 nm gold (BioCell Int.) or with rabbit anti-GFP (Molecular Probes), followed by protein A coupled with 15 nm gold (CMC Utrecht). Grids were contrasted and embedded in mixture of uranyl acetate–methylcellulose.

Samples of plastic sections or immunolabeled cryosections were observed with a Phillips Morgagni EM 268D operating at 100 kV. Pictures were taken using CCD camera and ANALYSIS 3.2 software. Final figures were edited with ADOBE PHOTOSHOP 8.0.

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**Supporting Information**

Additional supporting information may be found in the online version of this article:

**Figure S1:** Intracellular replication of various *Salmonella enterica* strains and SIF induction. A) The intracellular replication of *S. typhimurium* strains LT2A and 12023 was compared. HeLa cells were infected with WT or SPI2-deficient strains (ssALF) and non-internalized bacteria were killed by addition of Genticain. The number of viable intracellular bacteria or colony-forming units (CFU) was determined at 2 h and 16 h after infection by lysis of host cells and plating of lysates onto agar plates. The --fold intracellular replication was determined by the ratio of CFU counts at 16 and 2 h after infection. B) LAMP-1-GFP-transfected HeLa cells were infected with GFP-expressing *Salmonella* WT 12023 or LT2A as indicated. Live cell imaging was performed 7 h after infection and representative stills of a time-lapse series are shown. Scale bar, 10 μm.

**Figure S2:** Localization of LAMP-1-GFP and endogenous LAMP-1 in *Salmonella*-infected cells. HeLa cells were transfected with the LAMP-1-GFP construct (green) and infected with *Salmonella* WT expressing GFP (green). 6 h after infection, the cells were fixed and processed for immunostaining of LAMP-1 (red). The position of intracellular *Salmonella* and SIF is indicated in the merged image by arrows and arrowheads, respectively. Scale bar: 5 μm.

**Figure S3:** Quantification of LAMP-1-positive vesicles. HeLa cells were transfected with the LAMP-1-GFP construct. A representative still is shown from a time-lapse series. The quantification of the number of spherical, LAMP-1-GFP-positive vesicles was performed manually using the Edit mode of EBML IMAGEJ. Scored compartments were marked by yellow dots and the total number of events is indicated. This approach was used for the quantification shown in Figure 2B.

**Figure S4:** Ultrastructure of the endosomal system in mock-infected cells. HeLa cells were mock infected in parallel to infection with WT *Salmonella* and processed as described for Figure 3C. Representative mock-infected cells are shown. Scale bars, 1 μm.

**Figure S5:** *Salmonella* induces highly dynamic SIF in various host cells types. Infection experiments were performed with the murine macrophage-like cell line RAW264.7 after activation by 5 ng/mL IFNγ (A). Cells were mock infected or infected with *Salmonella* WT or SPI2 strains expressing GFP (green). The fluid phase marker 10 nm gold–BSA–rhodamine was added 5 h after infection. Living cells were imaged 8 h after infection using a Zeiss Axiovert 200M wide field microscopy. Scale bars, 5 μm. IFNγ-activated RAW264.7 cells (B, Suppl. Movie 11) or primary murine macrophages (C, Suppl. Movie 11) were used for infection with *Salmonella* WT and the fluid phase marker Alexa688-dextran was used to follow SIF formation in living cells imaged 8 h after infection. Left panels show the overview of the infected cells (scale bars, 5 μm) and boxes indicate positions in the periphery of the cells where representative stills show the details of dynamic alternations of SIF (scale bars, 1 μm). The relative time is expressed as hh:mm:ss and SIF growth and SIF contraction is indicated by yellow and orange arrowheads, respectively.

**Figure S6:** Analyses of SIF dynamics by kymographs. A) Schematic representation of the analysis of SIF extension and contraction. Individual SIF were identified in time-lapse series and analyzed by the EMBL IMAGEJ plugin Kymograph. Kymograph (also called time-space plot) is a graphical method of displaying and analyzing moving structures. To create such a plot the trajectory of the moving object has to be found. Once this is defined, the pixel values of this trajectory are copied to a new image. This procedure is...
repeated for each frame of the image stack. If the moving structure is resulting from a fluoroscently labeled particle it is represented by a bright line in the kymograph. The slope of this line is proportional to the velocity of the moving particle and also dependent on the directionality of the particle. Non moving particle can be identified by vertical or horizontal lines (depending on the plotting method). Thus it is possible with this method to analyze the speed and directionality of particles. B) Kymographs for individual SIF recorded at 4–5 or 8–9 h after infection of LAMP-GFP-transfected HeLa cells with Salmonella WT. These are examples of the kymographs used to calculate the data shown in Figure 7C.

**Figure S7:** Effect of addition of Nocodazole on the microtubule cytoskeleton. HeLa cells were treated with a final concentration of 10 μg/mL nocodazole. The cells were fixed at the indicated time-points and processed for immunostaining of β-tubulin.

**Video S1:** Corresponding to Figure 2A. Movement and fusion of a LAMP-1-GFP-positive vesicle to the SCV. An overview of an infected cell is shown with a LAMP-1-GFP-positive vesicle docking to an SCV (indicated by a circle). Scale bar, 5 μm.

**Video S2:** Corresponding to Figure 2B. Appearance of tubular extensions from an SCV. Scale bar, 5 μm.

**Video S3:** Corresponding to Figure 4. A long time lapse from 1 to 10 h after infection show the formation of Salmonella microcolonies, highly dynamic SIF at early time-points after infection and the appearance of a complex SIF network later after infection. Images were taken with intervals of 3 min. Scale bar, 5 μm.

**Video S4:** Corresponding to Figure 5A. IFN-γ-stimulated RAW264.7 macrophages were infected with Salmonella WT or a SPI2 mutant strains expressing GFP or mock infected. The cells were pulsed with Alexa647-dextran 5 h after infection and imaged 2 h later. Detail sections are shown as indicated by the box in the overview still. Scale bar, 1 μm.

**Video S5:** Corresponding to Figure 6A and B. Growth, branching and collapse of SIF. HeLa cells were infected with LAMP-1-GFP and infected with Salmonella WT expressing GFP. The event shown was observed 4 h after infection. Scale bar, 2 μm.

**Video S6:** Corresponding to Figure 6D. The dynamic variations in SIF diameter were recorded a setup described for Figure 6A.

**Video S7:** Corresponding to Figure 7A. Dynamics of SIF formation shown for a representative cell at 4 h after infection.

**Video S8:** Corresponding to Figure 7B. Dynamics of SIF formation shown for a representative cell at 8 h after infection.

**Video S9:** Corresponding to Figure 8B. HeLa cells were transfected with the LAMP-1-GFP construct and infected with GFP-expressing Salmonella WT. At 4–6 h after infection, the solvent (mock) or inhibitors nocodazole or taxol were added and live imaging of infected cells was performed.

**Video S10:** Corresponding to Figure 8D. The effect of nocodazole treatment and removal on the dynamics of vesicle and SIF movement is shown.

**Video S11:** Corresponding to Figure S5 B and C. RAW264.7 cell (B) or primary murine macrophages were infected with Salmonella WT and the fluid phase marker Alexa647-dextran was used to follow SIF formation in living cells.

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**References**


