Effector proteins support the asymmetric apportioning of Salmonella during cytokinesis

Yaya Zhao, Jean-Pierre Gorvel, and Stéphane Méresse

Centre d’Immunologie de Marseille-Luminy, Aix Marseille Université UM2, Inserm, U1104, CNRS UMR7280, Marseille, France

ABSTRACT
Salmonella-infected cells are characterized by the presence of intra-cellular membranous tubules that emerge from bacterial vacuoles and extend along microtubules. The formation of Salmonella-induced tubules depends on the Salmonella pathogenicity island 2-encoded type III secretion system (T3SS-2) that translocates bacterial effector proteins inside host cells. Effector proteins have enzymatic activities or allow for hijacking of cellular functions. The role of Salmonella-induced tubules in virulence remains unclear but their absence is correlated with virulence defects. This study describes the presence of inter-cellular tubules that arise between daughter cells during cytokinesis. Inter-cellular tubules connect bacterial vacuoles originally present in the parent cell and that have been apportioned between daughters. Their formation requires a functional T3SS-2 and effector proteins. Our data establish a correlation between the formation of inter-cellular tubules and the asymmetric distribution of bacterial vacuoles in daughters. Thus, by manipulating the distribution of bacteria in cytokinetic cells, Salmonella T3SS-2 effector proteins may increase bacterial spreading and the systemic character of the infection.

Introduction
Salmonella Typhimurium (Salmonella enterica subsp. enterica serovar Typhimurium) is a Gram-negative bacterial pathogen and a prominent cause of human gastroenteritis and systemic infection in immunocompromised individuals. This is an intracellular bacterium that survives in a variety of tissues and cells. The capacity to replicate intracellularly is essential for Salmonella virulence. Intracellular Salmonella resides in a membrane bound compartment named the Salmonella-containing vacuole (SCV). It also replicates in the cytosol of epithelial cells but the physiological relevance of the presence of fast replicating cytosolic Salmonella in epithelia is not understood.

Growing within a SCV presents important challenges that are supported by Salmonella type 3 secretion systems (T3SS). T3SSs direct the secretion and translocation of effector proteins (effectors) from the bacterial to the eukaryotic cytosol. Effectors are capable of influencing host functions. T3SS-1 effectors manipulate the cortical actin cytoskeleton, trigger the formation of membrane ruffles and facilitate the uptake of bacteria by non-phagocytic cells. T3SS-2 translocates more than 20 effectors that are collectively required for intracellular replication and virulence in mice (for review see ref. 3).

Salmonella vacuoles have several unique morphological characteristics. A SCV contains a single bacterium and divides when the bacterium undergoes cell division. This trait requires the function of host proteins. Abnormal vacuoles enclosing several bacteria have been observed upon inhibition of dynein function or in the absence Plekhm1, a host protein that interacts with the T3SS-2 effector SifA. The SCVs are connected to each other by membrane tubules that elongate with the mechanical support of the microtubule cytoskeleton and motors. Tubules were originally described in infected epithelial cells but also form in macrophages though they are more difficult to observe. Different kinds of tubules have been described and are together referred to as Salmonella-induced tubules (SIT). At least 6 T3SS-2 effectors are involved in the formation (SifA, SopD2, SteA), elongation (PipB2) or structure (SseF, SseG) of SITs. The precise functions of SITs remain elusive but their absence is associated with a virulence defect.

We observed that Salmonella-infected cells, like non-infected cells, enter mitosis and apportion bacteria between

© 2016 Yaya Zhao, Jean-Pierre Gorvel, and Stéphane Méresse. Published with license by Taylor & Francis. This is an Open Access article distributed under the terms of the Creative Commons Attribution-Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0/), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited. The moral rights of the named author(s) have been asserted.
daughter cells. During the process of cytokinesis the clusters of bacterial vacuoles destined to one or the other daughter cell remain connected by a membrane tubule that passes through the midbody. The present study investigates the mechanism of formation and the function of these tubules in relation with the host cell division and bacterial spreading.

Results

Salmonella-infected cells are occasionally connected by inter-cellular membrane tubules

SITs are intra-cellular tubules frequently seen in Salmonella-infected epithelial cells. We observed in HeLa cells the presence of membrane tubules emerging from one infected cell and appearing in continuity with similar tubules present in the adjacent infected cells (Fig. 1A). These tubules were comparable to SITs as they were 1) not observed in non-infected cells nor in cells infected with a T3SS-2 deficient strain (ΔssaV); 2) connected to SCVs; 3) enriched in lysosomal glycoproteins such as LAMP1 (Fig. 1A), in various T3SS-2 effectors (Fig. S1) and in SCAMP3 (Fig. S2). We examined the cell culture and infection conditions favoring the formation of these inter-cellular tubules, which we will refer to as ICTs, and analyzed the kinetics of their formation (Fig. 1B-D). ICTs were first observed at 8 h post-infection (p.i.) and in a maximum of 15-20 % infected HeLa cells at 14 h p.i.

ICTs do not form between independently infected HeLa cells

To define the conditions that lead to the formation of ICTs, we examined if these tubules could arise upon encounter of 2 infected cells. For this, we trypsinized and then co-cultured 2 batches of HeLa cells previously infected for 3 h with Salmonella strains expressing either GFP or DsRed. ICTs were
almost exclusively observed between cells enclosing *Salmonella* expressing the same color protein (Fig. 2A). ICTs connecting cells enclosing GFP and DsRed bacteria were very rare (less than 1% of infected cells presenting ICTs) and, in most cases, both cells contained the 2 types of bacteria, strongly suggesting a secondary infection. We conclude that ICTs do not form upon encounter of 2 infected cells.

### ICTs form during mitosis of an infected cell

Another possibility was that ICTs form during the cytokinesis of an infected cell. As *Salmonella* has been reported to perturb the cell cycle, we first evaluated the consequences of an infection on the capacity of HeLa cell to divide. We infected cells with GFP-expressing bacteria for 14 h and analyzed by flow cytometry the DNA content of the *Salmonella*-infected (GFP positive) and non-infected (GFP negative) cell populations (Fig. 2B). As compared to mock-infected and to non-infected cells we found a lower percentage of infected cells in the G0/G1 phase but small and non-significant increases for the S and G2/M populations (Fig. 2B). Thus, within our experimental time frame, *Salmonella* does not substantially affect the capacity of HeLa cells to go through their regular cell cycle. Next, we examined whether a correlation exists between the cell cycle and the formation of ICTs. Cytokinesis is the last step of the M phase and this
process immediately precedes the G1 phase. Therefore, we checked whether entry in G1 phase and the formation of ICTs were concomitant. We treated Salmonella-infected cells with the microtubule destabilizing agent nocodazole that arrests the cell cycle prior to mitosis. After 16 h treatment, more than 70% of HeLa cells had a G2/M-amount of DNA while the number of cells in the G0/G1 fraction was reduced to 14% (Fig. 2C). Following nocodazole washout, cells resumed their progression through the cell cycle. The number of cells in G0/G1 phase started to increase 2 h post-washout and peaked at 67% after 3 and 7 h (Fig. 2D). A parallel microscopic analysis of immunostained cells showed that the fraction of infected cells with ICTs followed a very similar profile (Fig. 2D). These results associate the cytokinesis that is observed at the transition between M and G0/G1 phases and the presence of ICTs, suggesting these tubules could form during cytokinesis.

The protein AIM-1 (Aurora and Ipl1-like midbody-associated protein) is concentrated in the midbody, which is the central region of the thin inter-cellular cytoplasmic bridge formed between daughter cells at the end of the cytokinesis. AIM-1 immunostaining of infected cells showed that ICTs are passing through the midbody (Fig. 2E and S1). We found 67.4 ± 2.8% of infected and cytokinetic cells with ICTs. These results strongly suggest that ICTs form during the cytokinesis of Salmonella-infected cells. We propose that these tubules are SITs still connecting the 2 groups of bacterial vacuoles upon their apportioning in daughter cells.

**Salmonella does not block cell cycle progression of macrophages**

Since macrophages are key players in salmonellosis, we investigated the cell cycle and the presence of ICTs in mouse macrophages. RAW 264.7 cells were infected with Salmonella for 14 h. AIM-1 immunostaining was used to identify dividing cells (Fig. 3A). We found similar percentages of mitotic cells in the infected and non-infected populations of macrophages (5.8 ± 0.7% and 5.2 ± 0.4%, respectively). It suggests that Salmonella do not alter the capacity of RAW 264.7 macrophages to go through mitosis. We also analyzed infected macrophages for the presence of tubules after immunostaining for LAMP-1 or PipB2-2HA, but we could not detect the presence of ICTs in these cells.

Next we followed the cell cycle of infected versus non-infected mouse macrophages. As compared to non-infected cells, we found 1.63 ± 0.15 and 2.04 ± 0.26 times more infected cells in the G2/M phase 30 min and 2 h p.i., respectively (Fig. 3B), indicating that Salmonella are preferentially taken-up by pre-mitotic and/or mitotic macrophages. Between 8 and 10 h p.i the fraction of infected G2/M cells returned to a normal level whereas cells in S phase were slightly under-represented. At 14 h p.i., the infected and non-infected populations were not statistically different (Fig. 3C).

**T3SS-2-expressing bacteria are preferentially taken up by mitotic macrophages**

A T3SS-1-dependent preferential invasion of G2/M HeLa cells has been previously reported. In this study, we used Salmonella grown in minimal medium that favors the expression of T3SS-2 rather than of T3SS-1 in order to decrease the T3SS-1-mediated killing of macrophages. Thus, T3SS-1 is probably not involved in the preferential uptake of Salmonella by G2/M RAW 264.7 cells. To verify this point, we incubated macrophages for 30 min with different types of live or inert particles, analyzed their DNA content by flow cytometry and determined the fractions of cells having taken particles in various phases of the cell cycle (Fig. 3D). Inert particles (latex beads and heat killed Salmonella) were preferentially taken-up by cells in S phase. E. coli and a ΔssaV mutant that lacks a functional T3SS-2 were equally taken-up by cells in either phase, while bacteria that do not express T3SS-1 (ΔpargH) showed a profile similar to that of wild-type Salmonella. All together these data indicate that Salmonella expressing T3SS-2 are preferentially phagocytosed by G2/M macrophages.

**T3SS-2 effectors support the formation of ICTs and the apportioning of SCVs between daughter cells**

During cytokinesis the content of the parent cell is divided in 2 parts. Daughter cells receive equal amounts of genetic material and a part of the cytoplasmic content. We observed variable situations regarding the distribution of bacteria between daughters. It was observed that one daughter did not contain bacteria (right panels in Fig. 3A and 4A). The existence of daughters with a complete absence of Salmonella was confirmed by the lack of signal after immunostaining with an anti lipopolysaccharide antibody (Fig. S3). This kind of cytokinesis was referred to as “negative.” Among the “positive” cytokinesis (bacteria are present in both phases, left panels in Fig. 3A and 4A), we sometimes observed that one daughter contained far more bacteria than the other. The cytokinesis was termed as “unbalanced” if one daughter contained more than 2/3 of total bacteria. Other positive cytokinetic situations were referred to as “balanced” (Fig. 4A). Having defined these observations, we asked whether ICTs could play a role in the apportioning of bacteria between daughter cells. As the formation of ICTs requires T3SS-2 function we infected HeLa cells with wild-type Salmonella or a ΔssaV strain. For each strain, 100 to
200 infected and mitotic cells were analyzed by microscopy for the presence of ICTs and the distribution of bacteria in daughter cells. ICTs were seen in 67.4 ± 2.8% cytokinetic cells infected by the wild-type strain and absent from DssaV-infected cells. We scored 77 ± 0.7% and 63.3 ± 1.4% positive cytokinesis for wild-type and DssaV infected cells, respectively. However, we observed, both in wild-type and DssaV infected cells a correlation between the number of bacteria and the percentage of positive cytokinesis (Figure S4A), indicating that the lower percentage of positive cytokinesis for the DssaV mutant may be directly linked to its replication defect (83% of DssaV infected cells contained less than 10 bacteria as compared to 13.8% for wild-type infected HeLa cells, Figure S4A). To get more insight, we analyzed a selection of strains deleted of genes encoding for a T3SS-2 effector. In Figure 4B we plotted positive cytokinesis as a function of cytokinetic cell with ICTs. This representation revealed the presence of 2 groups of bacteria. Mutant strains belonging to the first group (ΔsopD, ΔsifB, ΔsseJ, ΔpipB) were similar to wild-type Salmonella. Mutants of the second group (ΔsifA, ΔsopD2, ΔsseF, ΔsspH2, ΔpipB2) were, like the DssaV mutant, characterized by the presence of few or the absence of ICTs and a low number of positive cytokinesis. While the latter defect might also be attributed to an intracellular replication defect for ΔsseF22 and ΔsifA23 mutants, the deletions of pipB2, sspH224 or

**Figure 3.** Cell cycle analysis of Salmonella-infected RAW 264.7 macrophages. (A) Salmonella-infected RAW 264.7 macrophages were infected with GFP-expressing wild-type Salmonella for 14 h. Fixed cells were immunostained for AIM-1 and imaged for GFP (green), AIM-1 (red), and DAPI (white) by confocal microscopy. Infected macrophages divide and bacteria are distributed in both daughters (left image) or remain all in one of the daughters (right image). Bar, 10 μm. (B) Kinetic analysis of infected vs. non-infected RAW 264.7 cell cycle. RAW 264.7 cells were incubated with GFP-expressing wild-type Salmonella, fixed at different time points, DNA stained with propidium iodide and analyzed by flow cytometry (t = 0 corresponds to non-infected cells, t = 30 min corresponds to the end of the incubation period with bacteria). GFP fluorescence was used to define the gates corresponding to infected and non-infected cell populations. For each time point, the ratio between the percentages of Salmonella-infected versus non-infected cells in each phase of cell cycle was determined. Data (mean) are from 3 independent experiments. A two-way ANOVA test was used to determine whether a mean value was significantly different from one. Significant P values are indicated: *, P<0.05; **, P<0.01; ***, P<0.001. (C) Percentages of infected and non-infected RAW 264.7 cell in G0/G1, S and G2/M phases 14 h p.i. Data (mean ± SD) are from 3 independent experiments. Multiple t-tests were used to compare the mean values. P values: ns, not significant. (D) Salmonella are preferentially phagocytosed by pre-mitotic or mitotic RAW 264.7 cells. RAW 264.7 cells were incubated in the presence of fluorescent beads (Fluoresbrite® YG), heat-killed Salmonella (HK WT) or GFP-expressing bacteria (E. coli, or wild-type (WT), ΔsopD or ΔssaV Salmonella strains) for 30 minutes to allow phagocytosis. Cells were fixed and DNA stained with propidium iodide. For heat killed Salmonella, cells were immunostained for LPS. Normalized data were calculated by the ratio between the percentages of particle-containing vs. control cells in each phase of cell cycle. Data (mean ± SD) are from 5 to 9 independent experiments. A one-sample t-test was used to determine whether a mean value was significantly different from one. Significant P values are indicated: *, P<0.05; **, P<0.01.
sopD2\textsuperscript{12} do not impact the intracellular replication of Salmonella in HeLa cells.

Strains of the second group are deleted of genes known to be involved in the formation of SITs except for sspH2. Yet, we found that a \( \Delta \)sspH2 mutant is also defective for the formation of SITs in HeLa cells (Fig. S5). These results establish a correlation between the formation of ICTs and the apportioning of bacteria in both daughters.

Next, we tested if such a correlation exists in macrophages. SITs are barely observable in resting macrophages and we did not detect the formation of ICTs. Nevertheless, we analyzed the distribution of bacteria in daughters for a selection of T3SS-2 mutants and, as a control, a \( \Delta \)prgH strain that lacks a functional T3SS-1.

Like in HeLa cells, we observed less positive cytokinesis in macrophages infected with \( \Delta \)ssaV and \( \Delta \)sifA mutants.
than in those infected with the wild-type strain (Fig. 4C). Strains lacking SopD2 or SspH2 presented also a significantly lower number of positive cytokinesis (Fig. 4C). A \( \Delta \text{prgH} \) mutant was not different from the wild-type bacteria. Altogether, these results indicate that strains that do not form or produce altered SITs in HeLa cells are affected for the formation of ICTs and highlight an association between a reduced formation of ICTs in HeLa cells and a lower fraction of positive cytokinesis in HeLa and macrophages.

Lastly, we analyzed the influence of T3SS-2 on the even distribution of bacteria between daughter cells. As compared to a \( \Delta \text{ssaV} \) strain and to a selection of T3SS-2 effector mutants (\( \Delta \text{sifA} \), \( \Delta \text{sopD2} \), \( \Delta \text{sspH2} \)), we found less mitotic HeLa cells with a balanced number of wild-type bacteria. This phenotype was independent of the number of wild-type or \( \Delta \text{ssaV} \) intracellular bacteria (Fig S4B). We found in mitotic mouse macrophages a similar difference between wild-type and a \( \Delta \text{ssaV} \) mutant. However, the selected mutants (\( \Delta \text{sifA} \), \( \Delta \text{sopD2} \), \( \Delta \text{sspH2} \) and \( \Delta \text{prgH} \)) were not different from the wild-type strain. Together these data show that T3SS-2 sustains an asymmetric distribution of bacteria during cytokinesis.

**Discussion**

Bacterial effectors have a range of activities inside host cells that support pathogenesis.\(^{25}\) This study adds new functions to the actions covered by the T3SS-2: the uptake of *Salmonella* by pre-mitotic and/or mitotic macrophages and the asymmetric apportioning of bacteria during mitosis.

T3SS-1 is required to invade non-phagocytic cells.\(^{26}\) Invasion involves the translocon protein SipB that binds cholesterol.\(^{27}\) As cell surface cholesterol is more abundant during mitosis, *Salmonella* invade preferentially mitotic epithelial cells.\(^{19}\) The present work emphasizes a similar role for T3SS-2 in the uptake of *Salmonella* by G2/M macrophages. SseB, SseC and SseD are secreted by T3SS-2 and form a translocon.\(^{28}\) Whether these proteins bind cholesterol is unknown.

Within the time frame of our experiments *Salmonella* does not block the cell cycle of infected cells. This is evidenced by the fact that: i) ICTs form between mitotic cells; ii) nocodazole treatment blocks both infected and non-infected cells in G2/M phase and cell cycle resumes upon nocodazole wash out; iii) infected and non-infected cells have similar cell cycle profiles. Yet, recently published data found a T3SS-2- and SpvB-dependent arrest of *Salmonella*-infected HeLa cells in G2/M phase.\(^ {16}\) SpvB possesses an ADP-ribosyl transferase activity. Its ectopic expression leads to a G1/S or G2/M arrest of the cell cycle of tumor cells.\(^ {29}\) The influence of T3SS-2 on the cell cycle was detected at very late time points (> 20 h p.i.) while *spvB* is induced rather early during infection.\(^ {30}\) Therefore, It is possible that SpvB is expressed but not immediately secreted or is not sufficient to induce a modification of the cell cycle. Our experiments were performed in a narrower time frame and this might explain why, in our experimental context, *Salmonella* did not modify the host cell cycle.

We initially observed the presence in infected epithelial cells of tubules seemingly passing from one cell to a neighbor cell. Our data suggest that these tubules are remnants of SITs that form during mitosis. As tubules connect SCVs, one may assume they still exist after SCVs were apportioned in daughter cells. These tubules pass through the midbody and are probably closed at the time of abscission.

During cell division, the content of a mother cell is distributed between daughter cells. This mechanism has been extensively described for the genetic material. Organelles that are present in multiple dispersed copies will be randomly allocated more or less equally in each daughter cell. The Golgi apparatus, which is a single functional unit, fragments at the onset of mitosis thereby favoring its symmetrical random distribution in daughter cells. However the distribution is not necessarily symmetrical. For example mitochondria are asymmetrically distributed between daughters in human mammary stem-like cells. Old damaged mitochondria are asymmetrically distributed between daughters in human mammary stem-like cells. Old damaged mitochondria are asymmetrically distributed between daughters in human mammary stem-like cells. Old damaged mitochondria are asymmetrically distributed between daughters in human mammary stem-like cells. Old damaged mitochondria are asymmetrically distributed between daughters in human mammary stem-like cells. Old damaged mitochondria are asymmetrically distributed between daughters in human mammary stem-like cells. Old damaged mitochondria are asymmetrically distributed between daughters in human mammary stem-like cells. Old damaged mitochondria are asymmetrically distributed between daughters in human mammary stem-like cells. Old damaged mitochondria are asymmetrically distributed between daughters in human mammary stem-like cells. Old damaged mitochondria are asymmetrically distributed between daughters in human mammary stem-like cells.

In contrast to the previous phenotype, the asymmetric distribution of bacteria in daughters is independent of the number of intracellular bacteria. It is also influenced by the T3SS-2 in epithelial cells and macrophages. Yet, the absence of a single effector (SifA, sopD2, SspH2) is sufficient to perturb the asymmetric distribution of *Salmonella* in HeLa cells but not in macrophages. Overall, these processes reduce the bacterial burden of one of the daughters and thereby contribute to its survival. In *vivo*, this asymmetric apportioning may promote the dissemination of the infection. Ultimately it will be important to investigate the consequence of an infection on the cell cycle.
during a systemic infection and to determine whether the apportioning of bacteria between daughter cells favor the bacterial spreading and the dissemination of the infection.

**Materials and methods**

**Materials**

HA-tagged proteins were detected using a mouse monoclonal anti HA (Covance, clone 16B12). The mouse anti human LAMP1 H4A3 antibody developed by J. T. August was obtained from Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development (NICHD) and maintained by the University of Iowa (Department of Biology). The mouse anti AIM-1 (#611082) was obtained from Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development (NICHD) and maintained by the University of Iowa (Department of Biology). The mouse anti SCAMP3 antibody was generously provided by J. David Castle (University of Virginia, Charlottesville). Fluorescent Alexa secondary antibodies were obtained from Jackson ImmunoResearch. Fluo-sphere® YG Carboxylate Microspheres 1 μm were from Polysciences, Inc. (#15702-10). Propidium Iodide was from Sigma (P4170).

**Statistical analyses**

Statistical analyses were performed with Prism 6 software (GraphPad). with one-way ANOVA and Tukey post-test or 2-tailed unpaired Student’s t test. P-values: ns, not significant; *, P<0.05; **, P<0.01; ***, P<0.0005.

**Bacterial strains and growth conditions**

Bacterial strains used in this study are listed in Table 1. Strains were cultured in LB broth (Difco) or minimal medium (M9 pH 7.2, glycerol 0.2%, MgSO4 1 mM, CaCl2 200 mM, thiamine 1 mg/ml, casamino acids 1 mg/ml). Ampicillin (50 μg/ml), kanamycin (50 μg/ml), tetracycline (10 μg/ml) and chloramphenicol (50 μg/ml) were added when required.

**Eukaryotic cells and culture conditions**

RAW 264.7 mouse macrophages and human HeLa cells were grown in DMEM (GibcoBRL) supplemented with 10% foetal calf serum (FCS; GibcoBRL), 2 mM nonessential amino acids, and glutamine (GibcoBRL) at 37°C in 5% CO2. Nocodazole (1 mg/ml stock solution in DMSO) was added as indicated.

| Name | Strain | Reference |
|------|--------|-----------|
| 12023 | Wild-type S. Typhimurium (Salmonella enterica, strain NCTC 12023) | Laboratory stock |
| AAG057 | ΔagvC::KmR | 32 |
| HH102 | Δssec::aphT | 33 |
| HH107 | Δssec::aphT | 33 |
| HH108 | Δssec::aphT | 33 |
| DH215 | ΔsflA::KmR | 34 |
| DH216 | ΔsflE::KmR | 34 |
| DH221 | ΔsopB::KmR | 34 |
| DH217 | ΔsopB2::KmR | 34 |
| ΔsflB::KmR | Provided by D. W. Holden |
| ΔsopD::KmR | Provided by D. W. Holden |
| ΔsopD2::KmR | Provided by D. W. Holden |
| ΔsopH::KmR | Provided by D. W. Holden |

**Bacterial infection**

HeLa cells were seeded in 6-well plates with or without 12 mm diameter glass coverslips at a surface ratio of 1:10 unless otherwise indicated, 24 h before infection. Bacteria were grown in LB broth overnight at 37°C with shaking, diluted 1:33 in fresh LB broth, and incubated in the same conditions for 3.5 h. Bacteria were pelleted by centrifugation, resuspended in Earle’s buffered salt solution (pH 7.4) and added to the cells at a multiplicity of infection of 100:1 unless otherwise indicated. The infection was allowed to proceed for 10 min at 37°C in 5% CO2. RAW 264.7 macrophages were routinely grown in Petri dishes normally used for microbiology. Twenty-four hours before infection, cells were resuspended, counted and seeded at a density of 10^5 cells per cm^2 in 6-well tissue culture plates with glass coverslips. Bacteria were cultured overnight in minimal medium at 37°C with shaking, pelleted by centrifugation and opsonised in DMEM containing FCS and 10% normal mouse serum for 30 min on ice. Bacteria were diluted in cell growth medium and added to the cells at a multiplicity of infection of 100:1. Plates were centrifuged at 500 g for 5 min at 4°C and incubated for 30 min at 37°C in 5% CO2. Cells were washed 3 times with growth medium containing 100 μg/ml gentamicin and incubated in this medium for 1 h, after which the gentamicin concentration was decreased to 5 μg/ml for the remainder of the experiment.

**Flow cytometry**

For cell cycle analysis, cells were fixed with 3% paraformaldehyde and permeabilized in ice cold 70 % ethanol. DNA staining was performed for 30 min at 37 °C with 10 μg/ml propidium iodide and 0.2 mg/ml RNase A (Sigma, R5503) in 20 mM HEPES, 160 mM NaCl, 1 mM EGTA. Cells were analyzed on a FACS LSR II UV apparatus (Becton Dickinson). Data analysis of the distribution of the different phases of cell cycle was performed using the FlowJo
8.3 software (Tree Star Inc.). Salmonella-infected GFP-positive cells and non-infected GFP-negative cells were gated based on the signal intensity in the FITC-A channel.

**Immunofluorescence**

Cells grown on coverslips were fixed with 3 % paraformaldehyde (pH 7.4) in PBS at room temperature for 10 min. Fixed cells were washed 3 times in PBS and permeabilized with 0.1 % saponin in PBS. Primary and secondary antibodies were diluted in PBS containing 0.1 % saponin and 5 % horse serum. Coverslips were incubated with primary antibodies for 60 min at room temperature, washed once in PBS containing 0.1 % saponin and then incubated with appropriate secondary antibodies. Coverslips were mounted onto glass slides using ProLong Gold with DAPI (Invitrogen). Cells were observed with an epifluorescence Axiosplan2 microscope (Zeiss) or a LSM780 confocal laser scanning microscope (Zeiss).

**Abbreviations**

ICT inter-cellular tubules
p.i. post-infection
SCV Salmonella-containing vacuole
SIT Salmonella-induced tubules
T3SS Salmonella type 3 secretion system

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

We thank Steve Garvis for the critical reading of the manuscript and David W. Holden for providing bacterial strains.

**Funding**

YZ was supported by the China Scholarship Council. We acknowledge financial supports from n° ANR-10-INBS-04-01 France Bio Imaging, LabEx INFORM, and from the Fondation pour la Recherche Médicale (FRM SC).

**References**

[1] Aussel L, Zhao W, Hébrard M, Guilhon A-A, Viala JPM, Henri S, Chasson L, Gorvel J-P, Barras F, Méresse S. Salmonella detoxifying enzymes are sufficient to cope with the host oxidative burst. Mol Microbiol 2011; 80:628-40; PMID:21362067; http://dx.doi.org/10.1111/j.1365-2958.2011.07611.x.

[2] Knodler LA, Vallance BA, Gelli J, Winfree S, Hansen B, Montero M, Steele-Mortimer O. Dissemination of invasive Salmonella via bacterial-induced extrusion of mucosal epithelia. Proc Natl Acad Sci USA 2010; 107:17733-8; PMID:20876119; http://dx.doi.org/10.1073/pnas.1006098107.

[3] LaRock DL, Chaudhary A, Miller SI. Salmonella interactions with host processes. Nat Rev Microbiol 2015; 13:191-205; PMID:25749450; http://dx.doi.org/10.1038/nrmicro3420.

[4] Eswarappa SM, Negi VD, Chakraborty S, Chandrasekhar Sagar BK, Chakravorty D. Division of the Salmonella-containing vacuole and depletion of acidic lysosomes in Salmonella-infected host cells are novel strategies of Salmonella enterica to avoid lysosomes. Infect Immun 2010; 78:68-79; PMID:19858305; http://dx.doi.org/10.1128/IAI.00668-09.

[5] McEwan DG, Popovic D, Gubas A, Terawaki S, Suzuki H, Stadel D, Coxon FP, Miranda de Stegmann D, Bhogaraju S, Maddi K, et al. PLEKHM1 Regulates Autophagosome-Lysosome Fusion through HOPS Complex and LC3/GABARAP Proteins. Mol Cell 2015; 57:39-54; PMID:25498145; http://dx.doi.org/10.1016/j.molcel.2014.11.006.

[6] Garcia-del Portillo F, Zwick MB, Leung KY, Finlay BB. Salmonella induces the formation of filamentous structures containing lysosomal membrane glycoproteins in epithelial cells. Proc Natl Acad Sci USA 1993; 90:10544-8; PMID:8248143; http://dx.doi.org/10.1073/pnas.90.22.10544.

[7] Knodler LA, Vallance BA, Hensel M, Jäckel D, Finlay BB, Steele-Mortimer O. Salmonella type III effectors PipB and PipB2 are targeted to detergent-resistant microdomains on internal host cell membranes. Mol Microbiol 2003; 49:685-704; PMID:12864852; http://dx.doi.org/10.1046/j.1365-2958.2003.03598.x.

[8] Rajashekar R, Liebl D, Seitz A, Hensel M. Dynamic remodeling of the endosomal system during formation of Salmonella-induced filaments by intracellular Salmonella enterica. Traffic 2008; 9:2100-16; PMID:18817527; http://dx.doi.org/10.1111/j.1600-0854.2008.00821.x.

[9] Krieger V, Liebl D, Zhang Y, Rajashekar R, Chlanda P, Giesker K, Chikkaballi D, Hensel M. Reorganization of the endosomal system in Salmonella-infected cells: the ultrastructure of Salmonella-induced tubular compartments. PLoS Pathog 2014; 10:e1004374; PMID:25254663; http://dx.doi.org/10.1371/journal.ppat.1004374.

[10] Schroeder N, Mota LJ, Méresse S. Salmonella-induced tubular networks. Trends Microbiol 2011; 19:268-77; PMID:21353564; http://dx.doi.org/10.1016/j.tim.2011.01.006.

[11] Stein MA, Leung KY, Zwick M, GarciadelPortillo F, Finlay BB. Identification of a Salmonella virulence gene required for formation of filamentous structures containing lysosomal membrane glycoproteins within epithelial cells. Mol Microbiol 1996; 20:151-64; PMID:8861219; http://dx.doi.org/10.1111/j.1365-2958.1996.tb02497.x.

[12] Jiang X, Rossanese OW, Brown NF, Kujat-Choy S, Galán JE, Finlay BB, Brunnell JH. The related effector proteins SopD and SopD2 from Salmonella enterica serovar Typhimurium contribute to virulence during systemic infection of mice. Mol Microbiol 2004; 54:1186-98; PMID:15554961; http://dx.doi.org/10.1111/j.1365-2958.2004.04344.x.

[13] Domingues L, Holden DW, Mota LJ. The Salmonella effector SteA contributes to the control of membrane dynamics of Salmonella-containing vacuoles. Infect
Knodler LA, Steele-Mortimer O. The Salmonella effector PipB2 affects late endosome/lysosome distribution to mediate Sif extension. Mol Biol Cell 2005; 16:4108-23; PMID:15987736; http://dx.doi.org/10.1091/mbc.E05-04-0367.

Kuhl V, Hensel M. SseF and SseG are translocated effectors of the type III secretion system of Salmonella pathogenicity island 2 that modulate aggregation of endosomal compartments. Cell Microbiol 2002; 4:813-24; PMID:12464012; http://dx.doi.org/10.1046/j.1462-5822.2002.00234.x.

Maudet C, Mano M, Sunkavalli U, Sharan M, Giacca M, Förstner KU, Eulalio A. Functional high-throughput screening identifies the miR-15 microRNA family as cellular restriction factors for Salmonella infection. Nat Commun 2014; 5:4718; PMID:25146723; http://dx.doi.org/10.1038/ncomms5718.

Terada Y, Tatsuka M, Suzuki F, Yasuda Y, Fujita S, Otsu M. AIM-1: a mammalian midbody-associated protein required for cytokinesis. EMBO J 1998; 17:667-76; PMID:9450992; http://dx.doi.org/10.1093/embj/17.3.667.

Nix R, Altschuler S, Henson P, Detweiler C. Hemophagocytic Macrophages Harbor Salmonella enterica during Persistent Infection. PLoS Pathog 2007; 3:e193; PMID:18085823; http://dx.doi.org/10.1371/journal.ppat.0030193.

Santos AJM, Meinecke M, Fessler MB, Holden DW, Boucrot E. Preferential invasion of mitotic cells by Salmonella reveals that cell surface cholesterol is maximal during metaphase. J Cell Sci 2013; 126:2990-6; PMID:23687374; http://dx.doi.org/10.1242/jcs.115253.

Papezova K, Gregorova D, Jonuschies J, Rychlik I. Ordered expression of virulence genes in Salmonella enterica serovar Typhimurium. Folia Microbiol (Praha) 2007; 52:107-14; PMID:17575908; http://dx.doi.org/10.1007/BF02932148.

Brennan MA, Cookson BT. Salmonella induces macrophage death by caspase-1-dependent necrosis. Mol Microbiol 2000; 38:31-40; PMID:11029688; http://dx.doi.org/10.1046/j.1365-2958.2000.02103.x.

Abrahams GL, Müller P, Hensel M. Functional dissection of SseF, a type III effector protein involved in positioning the salmonella-containing vacuole. Traffic 2006; 7:950-65; PMID:16800847; http://dx.doi.org/10.1111/j.1600-0854.2006.00454.x.

Beuzón CR, Méresse S, Unsworth KE, Ruiz-Albert J, Garvis S, Waterman SR, Ryder TA, Boucrot E, Holden DW. Salmonella maintains the integrity of its intracellular vacuole through the action of SifA. EMBO J 2000; 19:3235-49; http://dx.doi.org/10.1093/emboj/19.13.3235.

Halici S, Zenk SF, Jantsch J, Hensel M. Functional analysis of the Salmonella pathogenicity island 2-mediated inhibition of antigen presentation in dendritic cells. Infect Immun 2008; 76:4924-33; PMID:18765734; http://dx.doi.org/10.1128/IAI.00531-08.

Dean P. Functional domains and motifs of bacterial type III effector proteins and their roles in infection. FEMS Microbiol Rev 2011; 35:1100-25; PMID:21517912; http://dx.doi.org/10.1111/j.1574-6976.2011.00271.x.

Galán JE, Curtiss R. Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells. Proc Natl Acad Sci USA 1989; 86:6383-7; http://dx.doi.org/10.1073/pnas.86.16.6383.

Hayward RD, Cain RJ, McGhie EJ, Phillips N, Garner MJ, Koronakis V. Cholesterol binding by the bacterial type III translocon is essential for virulence effector delivery into mammalian cells. Mol Microbiol 2005; 56:590-603; PMID:15819617; http://dx.doi.org/10.1111/j.1365-2958.2005.04568.x.

Nikolaus T, Deiwick J, Rappl C, Freeman JA, Schroder W, Miller SL, Hensel M. SeeBCD proteins are secreted by the type III secretion system of Salmonella pathogenicity island 2 and function as a translocon. J Bacteriol 2001; 183:6036-45; PMID:11567004; http://dx.doi.org/10.1128/JB.183.20.6036-6045.2001.

Mesa-Pereira B, Medina C, Camacho EM, Flores A, Santoro E. Novel tools to analyze the function of Salmonella effectors show that SvpB ectopic expression induces cell cycle arrest in tumor cells. PLoS ONE 2013; 8:e78458; PMID:24205236; http://dx.doi.org/10.1371/journal.pone.0078458.

Hautefort I, Thompson A, Eriksson-Ygberg S, Parker ML, Lucchini S, Danino V, Bongaerts RJM, Ahmad N, Rhen M, Hinton JCD. During infection of epithelial cells Salmonella enterica serovar Typhimurium undergoes a time-dependent transcriptional adaptation that results in simultaneous expression of three type 3 secretion systems. Cell Microbiol 2008; 10:958-84; PMID:18031307; http://dx.doi.org/10.1111/j.1462-5822.2007.01099.x.

Katajisto P, Doehla J, Chaffer CL, Pentinmikko N, Marjanovic N, Iqbal S, Zoncu R, Chen W, Weinberg RA, Sabatini DM. Asymmetric apportioning of aged mitochondria between daughter cells is required for stemness. Science 2015; 348:340-3; PMID:25837514; http://dx.doi.org/10.1126/science.1260384.

Zhao W, Moest T, Zhao Y, Guihon A-A, Buffat C, Gorvel J-P, Méresse S. The Salmonella effector protein SifA plays a dual role in virulence. Sci Rep 2015; 5:12979; PMID:26268777; http://dx.doi.org/10.1038/srep12979.