Salmonella translocates across an *in vitro* M cell model independently of SPI-1 and SPI-2

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We have used an *in vitro* model of intestinal M cells to examine the mechanisms by which *Salmonella enterica* translocates across these specialized cells, which constitute a primary site of infection of the mammalian host. *S. enterica* can invade cultured cells by deploying a type III secretion system (TTSS) encoded within *Salmonella* pathogenicity island 1 (SPI-1) to translocate effector proteins into the host cell cytoplasm that trigger cellular responses, including prominent cytoskeletal rearrangements. After *Salmonella* enters the host cell, a second TTSS encoded in SPI-2 modulates intracellular trafficking and enables the bacteria to replicate within a modified vacuolar compartment. Within the host intestine, specialized antigen-sampling M cells, which reside in the epithelium overlying lymphoid tissues in the gut, are a preferential site of *Salmonella* invasion. The mechanisms of infection of M cells remain poorly defined and it is not known whether either SPI-1 or SPI-2 is required for infection of these cells. To address these questions we have employed an *in vitro* M cell model involving co-culture of polarized Caco-2 intestinal epithelial cells with Raji B cells. *S. enterica* serovar Typhimurium translocated across Caco-2/Raji co-cultures to a much greater extent than they cross native Caco-2 cell monolayers. *Salmonella* translocation was greatly reduced by heat treatment or fixation, suggesting that processes distinct from the sampling of inert particles are the main determinants of bacterial translocation. Translocation across both mono-cultured and co-cultured Caco-2 cells was partially inhibited by treatment with the dynamin inhibitor dynasore, but resistant to EIPA, an inhibitor of macropinocytosis. There was no difference between the abilities of wild-type *Salmonella* Typhimurium and mutants lacking multiple SPI-1 effectors to translocate across the M cell model, although the SPI-1 effector mutants were somewhat attenuated for translocation across native Caco-2 layers. There was also no difference between wild-type and SPI-2 mutants in M cell translocation. Together these data suggest that SPI-1 and SPI-2 are dispensable for rapid M cell translocation and that infection at these specialized epithelial sites involves distinctive mechanisms that are not reliably modelled using conventional cell culture infection models.

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INTRODUCTION

Salmonella enterica is a Gram-negative bacterium that causes severe infections ranging from gastroenteritis to typhoid fever (Ohl & Miller, 2001). The ability of *Salmonella* to cross the intestinal epithelium is the critical first stage in infection and so there is a need to understand the mechanisms by which the bacteria achieve this. In common with a number of other pathogenic bacteria, *Salmonella* can manipulate the cytoskeleton of cultured cells through the injection of an array of bacterial effector molecules into the host cytoplasm. Numerous studies have addressed the mechanisms by which *Salmonella* drives its entry into cultured cells by initiating membrane ruffling,

which facilitates bacterial internalization by a process thought to closely resemble macropinocytosis (Cossart & Sansonetti, 2004).

Many *Salmonella* virulence genes are clustered in *Salmonella* pathogenicity islands (SPIs). SPI-1 and SPI-2 encode type III secretion systems (TTSSs) that mediate the injection of effector proteins into the host cell cytoplasm (Galan & Wolf-Watz, 2006). Some of the effectors secreted by TTSS-1 regulate actin dynamics. For example, SipA promotes actin bundling and SopE and SopE2 activate members of the Rho GTPase family, leading to the formation of membrane ruffles (Galan, 2001). SPI-1 is primarily responsible for invasion and SPI-2 is required for intracellular survival and the systemic phase of infection, although the exclusivity of these functions has been questioned recently. For example, some SPI-1 effectors persist post-invasion and appear to contribute to modulation of the *Salmonella*-containing

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; EIPA, 5-(N-ethyl-N-isopropyl)amiloride; SPI, *Salmonella* pathogenicity island; TRITC, tetramethylrhodamine isothiocyanate; TTSS, type III secretion system.

vacuole and to bacterial replication at later stages of infection (Brawn *et al.*, 2007; Giacomodono *et al.*, 2007), while SPI-2 has also been found to be expressed in the intestinal lumen prior to invasion (Brown *et al.*, 2005).

Most studies that have addressed cellular roles for SPI-1 and SPI-2 have concentrated on non-polarized cell lines, whereas the principal site of infection is the polarized epithelium lining the intestinal tract, which has features not readily modelled in conventional cell culture experiments (Buda *et al.*, 2005). Studies of intestinal infection in intact animals have found that *Salmonella* invasion of villus enterocytes is accompanied by membrane ruffling that closely resembles SPI-1-dependent cellular responses seen during infection of cultured cells (Takeuchi, 1967; Frost *et al.*, 1997). Furthermore, recent studies revealed that translocation and dissemination of *Salmonella enterica* serovar Dublin in calves is dependent on SPI-1 but not SPI-2 (Pullinger *et al.*, 2007). Several studies have identified Peyer's patches of the distal ileum as preferential sites of *Salmonella* infection in at least some animals (Carter & Collins, 1974; Frost *et al.*, 1997; Jepson & Clark, 2001). Here, intercalated between the enterocytes overlying lymphoid tissue, is a specialized cell type, the microfold/membranous M cell, which has an antigen-sampling function (Brayden *et al.*, 2005), a specialization that appears to have been exploited by *Salmonella* and other pathogens to invade the epithelia and disseminate through the body (Clark *et al.*, 1994; Jepson & Clark, 1998; Jones *et al.*, 1994).

The interaction of *Salmonella* with M cells has mostly been investigated *in vivo* following oral or intragastric inoculation or infection of ligated gut loops. Some authors have reported, or assumed, that SPI-1 is necessary for M cell invasion (Jones *et al.*, 1994; Penheiter *et al.*, 1997; Vazquez-Torres *et al.*, 1999). Others have suggested that SPI-1 enhances, but is by no means essential for, invasion of murine M cells by *Salmonella* (Clark *et al.*, 1996, 1998; Jepson & Clark, 2001), a finding that correlates with the attenuated but significant virulence of SPI-1 mutants following oral infection (Galan & Curtiss, 1989). *In vivo* infection models have limited potential for elucidating molecular mechanisms underlying invasion due to inherent problems in their experimental manipulation. Recently, an *in vitro* M cell model has been described in which human intestinal epithelial cells (Caco-2 cells) acquire M cell-like characteristics after being co-cultured with Raji B cells (Gullberg *et al.*, 2000; Kerneis *et al.*, 1997). We have previously reported that *Salmonella* uptake and translocation across this co-culture model exceeds that observed with native Caco-2 cells (Martinez-Argudo *et al.*, 2007). It has yet to be determined whether M cell translocation involves SPI-mediated mechanisms triggered during invasion of cultured epithelial cells.

In this study, we investigated the mechanisms involved in M cell infection by *Salmonella* using the *in vitro* co-culture model of M cells. Translocation of viable *Salmonella* across Caco-2 cells was dramatically increased by co-culture with

Raji B cells, confirming the generation, and preferential infection, of M-like cells. Bacterial translocation was greatly reduced by heat treatment or formaldehyde fixation, was partially inhibited by the selective dynamin inhibitor dynasore, but was unaffected by the macropinocytosis inhibitor EIPA. Translocation of wild-type and mutant *Salmonella* strains across the M cell model indicated that neither SPI-1 nor SPI-2 effectors are essential for invasion of M cells by *Salmonella*.

METHODS

Bacterial strains and culture. *S. enterica* serovar Typhimurium strain IR715, a nalidixic acid-resistant derivative of wild-type strain 14028s, and isogenic mutants ZA20 (*sopABDE2*) and ZA21 (*sipA sopABDE2*) were obtained from the laboratory of Andreas Baumler (Zhang *et al.*, 2002). The wild-type strain NCTC 12023 (equivalent to ATCC 14028s), and the *ssaV* mutant identified in the original signature-tagged mutagenesis screen as P2D6 were obtained from the laboratory of David Holden (Hensel *et al.*, 1997). All strains were grown in Luria-Bertani (LB) broth and maintained on LB agar plates supplemented with appropriate antibiotics at 37 °C.

Cell culture model. Raji B cells were grown in RPMI 1640 medium (Gibco-BRL) supplemented with 10 % fetal calf serum, 1 % Glutamax (Gibco), 0.1 mg streptomycin ml⁻¹ and 100 units penicillin ml⁻¹ (Sigma) at 37 °C in 5 % CO₂. Caco-2 cells (3–10 passages since obtaining from ATCC) were grown in DMEM (Sigma) supplemented with 10 % fetal calf serum, 1 % non-essential amino acids, 1 % Glutamax and 12 µg gentamicin ml⁻¹ (Sigma) at 37 °C in 5 % CO₂. After trypsinization, 0.5 × 10⁶ Caco-2 cells were seeded onto 1.2 cm diameter Transwell (Corning) polycarbonate inserts (3 µm pore) and incubated at 37 °C in 5 % CO₂ for 14 days in order to allow full differentiation before addition of 0.5 × 10⁶ Raji B cells in the basal chamber. The co-cultures were maintained for a further 6 days. Monocultures of Caco-2 cells, cultivated as above (except that Raji B cells were replaced with Raji cell medium), were used as controls.

Measurement of microsphere translocation. Microsphere translocation was measured as described previously (Martinez-Argudo *et al.*, 2007). Briefly, Transwells were washed with serum-free medium prior to the addition of 500 µl of 0.46 µm diameter fluorescent polystyrene (YG) microspheres (7.7 × 10⁸ ml⁻¹; Polysciences) to the apical chamber, and then incubated at 37 °C. Basolateral samples were taken after 30 min and the number of transported particles measured using a Spectramax M2 fluorescent plate reader (Molecular Devices).

Measurement of bacterial translocation. *Salmonella* strains were grown overnight in LB then diluted 1 : 100 in fresh LB and incubated for 3.5 h with shaking at 37 °C. Cells were infected for 1 h by diluting 25 µl of the exponential-phase bacteria in 500 µl DMEM to give an average m.o.i. of 20–30 (approx. 10⁷ bacteria added per layer). Serial dilutions from the inoculum were plated to calculate the actual number of bacteria inoculated. The number of translocated bacteria at 30 and 60 min was determined and expressed relative to the number of bacteria inoculated.

To kill bacteria, *Salmonella* cultures were incubated at 70 °C for 20 min or diluted with 2 % paraformaldehyde (1 : 1), incubated at 37 °C for 45 min and washed with fresh LB prior to infection. Cells were infected as above and the number of translocated bacteria was quantified using the LIVE/DEAD BacLight bacterial viability kit from Invitrogen.

Where indicated, inhibitors EIPA [5-(*N*-ethyl-*N*-isopropyl)amiloride; 50 μ M] and dynasore (80 μ M) were added 30 min prior to infection and maintained during infection.

Visualization of M cells and *Salmonella*. Following *Salmonella* infection, Caco-2 layers cultured alone or with Raji B cells on Transwells were washed thoroughly in PBS and fixed in 2% paraformaldehyde at 4 °C for at least 45 min. After permeabilization of cells with Triton X-100 (0.3% in PBS for 20 min), Transwells were incubated with goat anti-*Salmonella* antibody (Kirkegaard & Perry Laboratories; 1:200 in PBS) for 45 min at room temperature and, after washing with PBS, with FITC-conjugated anti-goat antibodies (1:100 in PBS). F-actin was simultaneously localized by inclusion of phalloidin-TRITC (tetramethylrhodamine isothiocyanate) with the secondary antibody as previously described (Jepson *et al.*, 1995). Transwells were then washed thoroughly in PBS and mounted in Vectashield containing DAPI (4',6-diamidino-2-phenylindole; Vector Laboratories). Distribution of F-actin and *Salmonella* was then examined by confocal microscopy using a Leica TCS SP2 AOBs system (Leica Microsystems) attached to a Leica DM IRE2 inverted microscope, and equipped with a 543 HeNe laser for excitation of TRITC and an argon laser for FITC excitation at 488 nm. An oil-immersion objective lens (63 $\times$, NA 1.4) was used, and imaging parameters standardized to allow direct comparison between images. Stacks of images at 0.5 μ m intervals were acquired and maximum projections of these created with Leica confocal software.

Statistical analysis. All data are expressed as means $\pm$ standard error (SE). Significance of differences between mean values was assessed using two-tailed unpaired Student's *t* test with significance set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Salmonella translocation is increased in an *in vitro* M cell model and is dependent on bacterial viability

We used a previously described procedure to differentiate Caco-2 intestinal cells into M cells by co-culturing with

Raji B cells (Gullberg *et al.*, 2000; Maresca *et al.*, 2007; Martinez-Argudo *et al.*, 2007). The presence of cells displaying morphological and functional characteristics of M cells was confirmed by confocal microscopy following phalloidin-TRITC labelling of F-actin (Fig. 1), since M cells have reduced apical F-actin content (Martinez-Argudo *et al.*, 2007), and by measurement of polystyrene microsphere transcytosis, as M cells exhibit enhanced transport (Gullberg *et al.*, 2000; Martinez-Argudo *et al.*, 2007). In agreement with previous data, translocation of fluorescent microspheres (approx. 0.5 μ m) measured after 30 min was found to be increased by Raji co-culture on average 29.7 ± 7.3 -fold ($n=7$) ($0.5\text{--}2 \times 10^8$ particles per co-cultured cell layer of approx. 0.5×10^6 cells). These findings indicate the reproducibility of the co-culture model but also reveal some variability in the extent to which the cells are phenotypically distinct from native Caco-2 cells following co-culture.

Addition of exponential-phase *S. Typhimurium* to the apical compartment and plating of basal bathing medium revealed that translocation of wild-type *Salmonella* was increased 38.5 ± 6.5 -fold ($n=10$) in the M cell model when compared with the control Caco-2 monolayer ($1.5\text{--}3 \times 10^6$ bacteria per co-cultured cell layer of approx. 0.5×10^6 cells). Since M cells *in vivo* and in the co-culture model are able to translocate inert particles in large numbers, it was necessary to determine whether M cell translocation of *Salmonella* occurs by a passive or active process. To address this question we examined whether bacterial viability is required. Uptake of killed *Salmonella* was greatly reduced compared with that of live *Salmonella* irrespective of the method used to kill the bacteria (Fig. 1). Furthermore, translocation of killed bacteria across the M cell model (which was below detectable levels across native Caco-2 layers) was much lower than that of viable bacteria

Fig. 1. Confocal microscopy shows that bacterial viability is needed for bacterial uptake in the M cell model. Bacteria (labelled as described in Methods) appear green and M cells (indicated by arrows) can be recognized as they show minimal apical localization of apical F-actin (labelled red with phalloidin-TRITC). Scanning electron microscopy has previously confirmed the presence of large cells with minimal brush border. (a) Live bacteria were used to infect the M cell model. (b, c) Cells treated as in (a) but bacteria were first killed by heat-inactivation (b) or paraformaldehyde (c). Very few killed bacteria were observed associated with cells. All images are maximum projections. Field of view 238 $\times$ 238 μ m.

($25 \pm 11.2\%$ for viable bacteria, $0.6 \pm 0.5\%$ for heat-killed bacteria and $3 \pm 2.7\%$ for paraformaldehyde-fixed bacteria per co-cultured cell layer; $n=3$).

The low levels of translocation of non-viable *Salmonella* across the M cell model confirmed the enhanced transport while indicating that translocation of *Salmonella* occurs via a selective process. This finding confirms previous data that the increased transport observed is not a result of loss of integrity of the cell layers, a finding we also confirmed previously by scanning electron microscopy and by measurement of transepithelial electrical resistance (Martinez-Argudo *et al.*, 2007). Further evidence of the specificity of the M cell model is also provided by data that not all bacterial species are translocated in greater numbers across Caco-2 cells following co-culture with Raji B cells. For example, translocation of wild-type enteropathogenic *Escherichia coli* (EPEC), *Clostridium difficile* and *Listeria monocytogenes* was not increased in the M cell model (Daniels *et al.*, 2000; Martinez-Argudo *et al.*, 2007).

***Salmonella* translocation by M cells involves dynamin and is distinct from macropinocytosis**

By analogy with growth factor-induced membrane ruffling, it has been concluded that *Salmonella* invades non-phagocytic cells by inducing a process akin to macropinocytosis (Cossart & Sansonetti, 2004; Francis *et al.*, 1993). As *Salmonella* preferentially enter M cells that also have a propensity for transport of inert particles and since translocation of polystyrene microspheres has previously been reported to involve macropinocytosis (des Rieux *et al.*, 2007), we sought to determine if *Salmonella* translocation follows a similar route. We analysed *Salmonella* translocation across the M cell model in the presence of two inhibitors: EIPA and dynasore. EIPA is commonly used to inhibit macropinocytosis (Amstutz *et al.*, 2008; Fallman & Gustavsson, 2005; Wadia *et al.*, 2004), although the selectivity of this inhibition has been questioned (Fretz *et al.*, 2006). Dynasore is a cell-permeable specific inhibitor of the GTPase activity of dynamin (Macia *et al.*, 2006), a molecule involved in clathrin-mediated endocytosis that may also have a role in the scission of the macropinosome from the plasma membrane (Amstutz *et al.*, 2008; Orth *et al.*, 2002). Initial experiments confirmed that short-term treatment with these inhibitors had no detectable effect on bacterial viability or motility (data not shown). Treatment with EIPA did not affect *Salmonella* translocation but when cells were pre-incubated with dynasore we observed a significant reduction in *Salmonella* translocation across both the Caco-2 monolayer and the M cell model with respect to untreated cells after 30 min (Fig. 2). The degree of inhibition of translocation associated with dynasore treatment was greater across native Caco-2 cells (64%) than across the Caco-2/Raji co-cultured layers (27.6%). The results obtained suggest macropinocytosis does not play a significant role in *Salmonella* translocation across M cells and that a minor proportion of rapid translocation is dynamin-dependent.

Fig. 2. Translocation of wild-type *Salmonella* across Caco-2 monocultures and the M cell model following treatment with inhibitors. Where indicated, EIPA or dynasore (or the vehicle DMSO) were added 30 min prior to infection and maintained during infection. Data show bacterial translocation 30 min after infection and are means $\pm$ SE from at least three independent experiments performed in duplicate. Asterisks indicate that differences between translocation of non-treated bacteria and that of inhibitor treated bacteria were statistically significant ($P<0.05$). Following each treatment, translocation was significantly greater across Caco-2 cell layers co-cultured with Raji cells ($P<0.001$).

Transcytosis of polystyrene microspheres across the Caco-2/Raji co-culture model has previously been shown to be inhibited by EIPA (des Rieux *et al.*, 2007) and our preliminary studies have also indicated that this inhibitor reduces translocation of 60 nm polystyrene nanoparticles across the M cell model (data not shown). The lack of effect of EIPA on *Salmonella* translocation thus supports our conclusion that this is mechanistically distinct from transport of inert particles. Our data are consistent with the conclusion that dynamin contributes to rapid transport across Caco-2 cells regardless of co-culture. This supports recently published data that cell invasion by some bacterial pathogens is dynamin-dependent (Veiga *et al.*, 2007). The authors concluded that *Salmonella* invasion was largely, though not completely, dynamin-independent in HeLa cells, suggesting that SPI-1-mediated invasion is dynamin-independent but that an alternative SPI-1-independent invasion mechanism may also operate. Our findings in Caco-2 cells thus give additional support to the hypothesis that alternative entry mechanisms operate in some epithelial cells. At later time points (60 min) the inhibition of transport associated with dynasore treatment was no longer evident although transport across the M cell model remained higher than across monocultured Caco-2 cells (data not shown), further supporting the operation of alternative entry and translocation mechanisms across these cells.

SPI-1 effectors are not required for M cell translocation by *Salmonella*

In order to examine the role of SPI-1 effectors in M cell invasion by *Salmonella*, we infected the M cell model with

wild-type *S. Typhimurium* IR715 and two strains lacking the effector genes that have been shown to contribute to *Salmonella* invasion of epithelial cells. It was previously reported that the two mutant strains examined, ZA20 (*sopABDE2*) and ZA21 (*sipA sopABDE2*), are essentially non-invasive in non-polarized and polarized T84 cells (Raffatellu *et al.*, 2005).

Over a 30 min infection period, strains ZA20 and ZA21 exhibited a level of translocation across the M cell model that was not significantly different from wild-type (Fig. 3a). This suggests SPI-1 effectors that are required for *Salmonella* invasion of most epithelial cell lines (Clark *et al.*, 1996; Galan & Curtiss, 1989; Raffatellu *et al.*, 2005) are dispensable for *Salmonella* translocation across M cells. Furthermore, an *invA* mutant that lacks an intact SPI-1 TTSS was also found to translocate across the M cell model in similar numbers to its parent strain (data not shown). These data are broadly in agreement with *in vivo* studies, which have shown that SPI-1 mutants exhibit reduced but significant virulence after oral infection, and can also enter

murine M cells, though in reduced numbers under conditions that optimize invasion (Clark *et al.*, 1996, 1998; Galan & Curtiss, 1989). Although we cannot directly compare the present data with previous studies because here we are replicating translocation across human M cells rather than measuring intracellular bacteria in murine M cells, our findings add strong support to previous conclusions that the M cell route is an important factor in the remaining virulence of SPI-1 mutants. Of course this conclusion does not exclude the possibility that other SPI-1-independent routes of translocation exist, such as the proposed carriage by CD18-expressing phagocytes (Vazquez-Torres *et al.*, 1999). Since M cells constitute a tiny proportion of the intestinal epithelium, it is also likely that SPI-1-dependent invasion of enterocytes is a major factor in *Salmonella* virulence. For example, in calf ligated gut loops, translocation of *S. Dublin* from lumen to mesenteric lymph nodes was recently shown to be SPI-1 dependent (Pullinger *et al.*, 2007). However, the existence of alternative translocation routes that operate regardless of SPI-1 expression appears likely to be a significant factor in the success of *Salmonella* as a pathogen.

Over the 30 min period examined, translocation across control Caco-2 layers was significantly lower for ZA20 (13 %) and ZA21 (21 %) than for wild-type *S. Typhimurium* (Fig. 3b). While these data support previous findings that SPI-1 is essential for invasion, the significant translocation of the mutants indicates that a rapid, SPI-1-independent transport route also operates in these cells. Recently it was suggested that co-culture with Raji cells amplifies an existing pool of M-like cells within the normal Caco-2 population (Blanco & DiRita, 2006). Although this idea has yet to be proven, it is consistent with the observation that a small proportion of Caco-2 cells have a poorly differentiated brush border (Blanco & DiRita, 2006), a finding we have confirmed (data not shown). The observation that rapid transport of *Salmonella* across Caco-2 cells is partially SPI-1-independent is also consistent with the hypothesis that a small proportion of Caco-2 cells have functional properties reminiscent of M cells. The precise nature of this cell subpopulation requires further investigation.

Fig. 3. Translocation of wild-type, SPI-1 and SPI-2 mutants across the M cell model (a) and Caco-2 monocultures (b). Data show bacterial translocation 30 min after infection and are means $\pm$ SE from at least three independent experiments performed in duplicate. Data are normalized with respect to translocation of parent strains (IR715 for ZA20 and ZA21; 12023 for P2D6). Translocation of strain IR715 across the M cell model and Caco-2 monocultures reached $20.1 \pm 2.2\%$ and $1.0 \pm 0.4\%$, respectively. Translocation of strain 12023 across the M cell model and Caco-2 monocultures was $17.5 \pm 4.6\%$ and $0.6 \pm 0.3\%$, respectively. Asterisks indicate where differences between translocation of wild-type and mutant strains were statistically significant ($P < 0.01$).

SPI-2 does not contribute to rapid translocation of *Salmonella* across the M cell model

Having shown that SPI-1 is not required for M cell translocation, we next examined whether the other TTSS encoded by SPI-2 plays a significant role in the rapid translocation of *Salmonella*. We tested the ability of a SPI-2-deficient mutant P2D6 carrying a transposon insertion in *ssaV*, which encodes an essential component of the SPI-2-encoded TTSS (Hensel *et al.*, 1997; Shea *et al.*, 1996), to cross the M cell model. When compared with the parent wild-type strain, the *ssaV* mutant showed similar levels of translocation across the M cell model and mono-cultured Caco-2 cells, suggesting SPI-2 effectors are not required

Fig. 4. Confocal microscopy reveals distribution of wild-type (a) and SPI-2 mutant (b) in the M cell model. *Salmonella* were labelled with specific polyclonal antibodies and FITC-conjugated secondary antibodies (green), and F-actin in Caco-2 cells was localized with phalloidin-TRITC (red). M cells (indicated by arrows) exhibit minimal apical F-actin. Wild-type and SPI-2 mutants exhibit similar intracellular distribution. All images are maximum projections. Field of view 238×238 μm.

(Fig. 3). To establish if there was a difference in intracellular distribution of the two strains, bacteria were localized by immunofluorescent labelling and imaged by confocal microscopy. The numbers and distribution of intracellular bacteria were similar between the two strains, with bacteria preferentially associated with cells lacking an F-actin-rich brush border (Fig. 4). It thus appears that neither of the SPI-encoded TTSSs contributes to the rapid translocation route we are modelling.

It is well established that SPI-2 effectors contribute to the regulation of intracellular trafficking that is required for *Salmonella* to establish a niche within host cells that is conducive for its replication (Waterman & Holden, 2003). The finding that SPI-2 does not play a role in the rapid translocation process measured here is consistent with this TTSS being primarily involved in stages of infection that follow the initial translocation step.

Concluding remarks

Our data have confirmed that *Salmonella* can rapidly translocate in large numbers across intact Caco-2 monolayers, following their co-culture with Raji B cells. This rapid translocation route is dependent on bacterial viability, independent of SPI-1 and SPI-2, and appears to partially involve dynamin but not macropinocytosis. Rapid translocation of *Salmonella* thus appears to be a separate process from the invasion of cultured epithelial cells that has been most extensively studied.

Although the *in vitro* M cell model is amenable to experimental manipulation to address mechanistic questions, it is not without limitations. We saw significant variability in the extent of translocation across both co-cultured and mono-cultured Caco-2 cells, which probably reflects variation in the proportion of cell layers that functionally resemble M cells. We also found that Caco-2 cells had to be used at low passage numbers (i.e. passages 3–10) or significant phenotypic drift appeared to affect reliability of the Raji-induced differentiation (data not shown). By adopting a stringent policy of limiting passage numbers, the M cell model has proved sufficiently

reproducible for us to address key questions regarding the mechanisms utilized by *Salmonella* to cross the intestinal epithelial barrier. Our data strongly support previous evidence that SPI-1 mutants retain significant virulence when delivered orally and that this is at least in part due to their ability to enter and translocate across M cells (Clark *et al.*, 1996, 1998; Galan & Curtiss, 1989). We have also shown for the first time that dynamin-dependent endocytic mechanisms appear to contribute to *Salmonella* translocation.

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