Research Article

Host cell responses of *Salmonella typhimurium* infected human dendritic cells

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**Summary** Live attenuated *Salmonella* are attractive vaccine candidates for mucosal application because they induce both mucosal immune responses and systematic immune responses. After breaking the epithelium barrier, *Salmonella typhimurium* is found within dendritic cells (DC) in the Peyer’s patches. Although there are abundant data on the interaction of *S. typhimurium* with murine epithelial cells, macrophages and DC, little is known about its interaction with human DC. Live attenuated *S. typhimurium* have recently been shown to efficiently infect human DC *in vitro* and induce production of cytokines. In this study, we have analysed the morphological consequences of infection of human DC by the attenuated *S. typhimurium* mutant strains designated PhoPc, AroA and SipB and the wild-type strains of the American Type Culture Collection (Manassas, VA, USA), ATCC 14028 and ATCC C53, by electron microscopy at 30 min, 3 h and 24 h after exposure. Our results show that genetic background of the strains profoundly influence DC morphology following infection. The changes included (i) membrane ruffling; (ii) formation of tight or spacious phagosomes; (iii) apoptosis; and (iv) spherical, pedunculated membrane-bound microvesicles that project from the plasma membrane. Despite the fact that membrane ruffling was much more pronounced with the two virulent strains, all mutants were taken up by the DC. The microvesicles were induced by all the attenuated strains, including SipB, which did not induce apoptosis in the host cell. These results suggest that *Salmonella* is internalized by human DC, inducing morphological changes in the DC that could explain immunogenicity of the attenuated strains.

**Key words:** dendritic cell, immunity, *Salmonella*.

**Introduction**

Over the recent years, several bacterial and viral live vaccine vectors have been developed and shown to induce local and systemic immune responses. Among these, attenuated *Salmonella typhimurium* expressing heterologous antigens is one of the best studied, in particular, for mucosal vaccination.1,2 *S. typhimurium*, a facultative intracellular pathogen of mice that can also infect human beings, has a number of attributes that make it a potent vaccine vector.3,4 One particular property of *Salmonella* vaccine vectors is their ability to induce antigen-specific MHC class I restricted CD8+ cytotoxic T lymphocytes,5 despite the fact that they remain confined to the endosomal compartment of the host cells, the loading site for MHC class II molecules.6 Another important feature of *Salmonella* is their ability to induce their uptake by non-phagocytic cells, such as cells of the intestinal epithelium.7 The latter property enables *Salmonella* to cross epithelial barriers and to target the APC, such as dendritic cells (DC) in the surrounding tissues.

During infection of the host, *S. typhimurium* encounters several different host cell types, including the lining epithelial cells, macrophages and DC. The entry of *Salmonella* into the epithelial cells and macrophages is associated with prominent morphological changes of the host cell, such as (i) formation of filamentous structures in epithelial cells;8 (ii) formation of spacious phagosomes in both macrophages9 and epithelial cells;10 and (iii) apoptosis in macrophages.11 Whereas considerable work has been carried out in elucidating the interaction between *Salmonella* with epithelial cells and macrophages, the morphological effects of *Salmonella* infection on the human DC has not been determined.12 DC are professional APC, which are crucial for generating primary T-cell immune responses.13,14 They are particularly abundant in organs that provide an environmental interface, such as the skin, the respiratory system (e.g. sentinel of the immune surveillance in the airways15) and the gastrointestinal tract.13 Understanding their interaction with *Salmonella*, a candidate live vaccine bacteria may explain the ability of this bacteria to induce both MHC class I and MHC class II responses. The nature of the response of the DC after antigen uptake is an important consideration in determining the type and the magnitude of the
immune response generated. We have recently shown that the human DC16,17 and macrophages,18 when infected by
*S. typhimurium,* respond by releasing cytokines whose patterns depend on the genetic background of the bacteria. A better understanding of the interaction of DC with attenuated *S. typhimurium* mutants may help explain the ability of these bacteria to induce mixed Th1 and Th2 responses and perhaps yield more efficient vaccine vectors by genetic design.

In this study, we have characterized the morphological consequences of interaction between *Salmonella* and human DC, by exposing human blood monocyte-derived immature DC to both attenuated and virulent strains of *S. typhimurium* and examining the effects of infected cells by electron microscopy. Our results show that the dramatic morphological changes that DC undergo in response to the infection depend on the genetic background of the strains.

**Materials and methods**

*Culture of immature DC from peripheral blood monocytes*

Human immature DC were cultured from peripheral blood monocytes.16,19 Briefly, human PBMC from healthy donors were isolated by Ficoll–Hypaque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation ofuffy coats as previously described.20 After adherence for 1 h, culture dishes were rinsed with HBSS and adherent cells were incubated overnight in RPMI-1640 medium (Life Technologies, Rockville, MD, USA), supplemented with 10% FCS, 2 mmol/L glutamine, 100 U/mL streptomycin and 100 U/mL penicillin (complete culture medium, CCM). Loosely adherent monocytes were recovered in three rinses of HBSS. Immature DC were obtained from the loosely adherent monocytes by culture for 7 days in CCM in the presence of GM-CSF (Immugenex, Los Angeles, CA, USA) and IL-4 (R&D Systems, Minneapolis, MN, USA) at a final concentration of 10 ng/mL each.

**Salmonella strains and growth condition**

The wild-type strains of *S. typhimurium* used are the American Type Culture Collection strain ATCC 14028 and strain ATCC C53, which are both highly virulent in BALB/c mice.21 Three mutant strains designated SipB for *S. typhimurium* C53; PhoPc for *S. typhimurium* ATCC 14028 and AroA, all of which have a strongly attenuated virulence in mice and are under consideration as human vaccine vectors, were used.1 The strain PhoPc bears a point mutation in the PhoP virulence gene regulatory locus, whereas the AroA strain harbours a mutation in the gene encoding an enzyme in the prechorismate biosynthetic pathway and is therefore incapable of growing in the absence of precursors of aromatic compounds.22 SipB is a derivative of C53 and bears the insertion in the sipB gene that prevents the expression of functional SipB protein.23 The bacteria were grown overnight in aerated culture at 37°C, transferred to fresh media and grown for an additional 3–4 h under oxygen-limiting conditions in Luria–Bertini broth containing 30 mmol/L potassium chloride and 0.5% potassium nitrate. These growth conditions have been found to provide optimal efficiency of *Salmonella* invasion into mammalian host cells.24 Before infection of DC, the bacteria were washed in HBSS and suspended in prewarmed RPMI-1640 medium.

**Infection of DC**

Before infection with *S. typhimurium,* the DC were washed and resuspended in complete culture media without antibiotics. The DC were seeded for 1 h at a concentration of 2 × 10⁶/mL in six-well tissue culture plates and bacteria added to the DC culture to achieve a multiplicity of infection of 25 bacteria per cell. The infection was allowed to proceed for 30 min and the extracellular bacteria were killed by the addition of 60 mg/mL of gentamicin (Sigma, St Louis, MO, USA). The cells were harvested for microscopy after 30 min, 3 h and 24 h postinfection.

**Analysis of apoptosis of DC after infection by Salmonella**

Analysis of apoptosis was carried out as described by Dreher et al.16 Apoptotic cells were labelled with annexin V, which detects the translocation of phosphatidylserine to the outer layer of the cell membrane in early stages of apoptosis.25 Cells were washed and exposed for 10 min at room temperature to phycocerythrin-labelled annexin-binding buffer (10 μg/mL; PharMingen, San Diego, CA, USA) immediately before analysis. Flow cytometry was carried out on a FACScan (Becton Dickinson, Mountain View, CA, USA).

**Processing cells for electron microscopy**

The infected DC were washed in HBSS, fixed in 2.5% phosphate-buffered glutaraldehyde solution, postfixed in 1% osmium tetroxide in 0.1 mol/L sodium cacodylate buffer and contrasted in 0.5% uranyl acetate. This was followed by dehydration in a graded series of ethanol (70%, 80%, 96% and twice in 100%) and gradual replacement of ethanol with propylene oxide before infiltrating and embedding in epoxy resin.

Ultrathin sections of epon blocks were cut using a Reichert ultramicrotome (Reichert-Jung, Vienna, Austria). The sections were picked on 200-mesh carbon-coated copper grids, stained with uranyl acetate, counterstained with lead citrate and observed under a Philips 300 transmission electron microscope under an accelerating voltage of 60 kV. For scanning electron microscopy, cells were coated with palladium and examined with a Philips XL 30 FEG scanning electron microscope operating at 17 kV.

**Statistical analysis**

Parametric data were determined using F-test and were compared using the t-test. Differences were considered significant when *P* < 0.05. Results are expressed as mean ± SD.

**Results**

As previously determined, both wild-type strains and mutant strains of *Salmonella* efficiently infected the immature human DC.16 However, an observation of the infected cells showed definite morphological changes on the DC that were characteristic of the strain and of the duration of infection. At 30 min post-infection, scanning electron micrographs of DC showed generalized ruffles on the surface of the plasma membrane appearing in the form of microspikes. Figure 1A shows the uninfected cells. The wild-type strains induced characteristically deeper and more prominent ruffles (Fig. 1B) than the mutant strains (Fig. 1C,D). The ruffles were not observed at 24 h postinfection.

At 24 h postinfection the internalized bacteria were contained in either of two types of phagosomes: tight phagosomes (Fig. 2A,B) or spacious phagosomes (Fig. 2C) as defined on the bases of the association between the phagosomal membrane and the wall of the phagocytosed bacteria. The membrane of the tight phagosomes closely followed the contours of the bacteria that mainly occurred as single profiles (Fig. 2A), although occasional dividing bacteria were observed (Fig. 2B). However,
**Figure 1** Scanning electron micrograph of human dendritic cells (DC) at 30 min after infection with *Salmonella typhimurium*. (A) Uninfected, (B) infected with wild-type strain ATCC 14028, (C) infected with AroA and (D) infected with PhoPc. Note the prominent ruffling of the plasma membrane (arrows) of the DC after infection with the wild-type strain and compare with moderate ruffling after infection by the mutant strains. Bar, 1 μm.

**Figure 2** Transmission electron micrograph of human dendritic cells at 24 h after infection with attenuated strain PhoPc (A and B) and the wild-type strain ATCC 14028 (C) of *Salmonella typhimurium*. Note the tight phagosomes (arrow heads) associated with the attenuated strain in contrast to the large spacious phagosome (SP) resulting from infection by the virulent strain. The phagosomal membrane follows the contours of the bacterium even during bacterial division (B). Some of the bacteria are undergoing degradation as indicated by loss of integrity (stars). *S. typhimurium* (S). Bar, 2 μm.
the spacious phagosomes contained several bacteria that freely floated in an amorphous substance (Figs 2C, 3A). The spacious phagosomes were exclusively induced by the virulent strains, whereas the attenuated strains were contained in tight phagosome. Over time, the attenuated bacteria were degraded after internalization, as depicted by the loss of integrity of the bacteria profiles within the phagosomes (Fig. 2A).

The virulent Salmonella strains, in particular C53, characteristically induced apoptosis (Fig. 3A) to infected cells. Apoptotic changes were observed as early as 3 h after infection, but were maximal at 24 h. Mutation of the virulence gene sipB completely abolished the ability of this strain to induce apoptosis in DC (Fig. 3A–C).

Surprisingly, the cells infected by mutant strains lost their characteristic dendritic morphology on prolonged infection (24 h postinfection). Instead of showing the long dendritic processes that are characteristic of DC (Fig. 4A), their plasma membrane showed small spherical pedunculated microvesicles that measured approximately 0.2–0.7 \(\mu\)m in diameter. These microvesicles were observed after infection by both virulent strains (Fig. 4B) and mutant strains studied: SipB (Fig. 3B), AroA (Fig. 4C), Phopc (Fig. 4D). These vesicular structures appear to pinch off from the plasma membrane and to lie free from the cell and usually contained no prominent organelles. Scanning electron micrographs of cells infected by a strain-inducing apoptosis (C53; Fig. 5A) and with a mutant strain (AroA; Fig. 5B) showed that the apoptotic bodies and the microvesicular bodies were clearly distinct structures. To determine whether these microvesicular structures were characteristic of DC, we also infected human alveolar macrophages with S. typhimurium. Transmission electron microscopy showed that the macrophages were infected by all the different strains of Salmonella used in this study, but, in contrast to the DC, retained their characteristic microvilli and did not produce microvesicles on their plasma membrane (Fig. 6).

**Discussion**

S. typhimurium infection occurs naturally by oral route. Penetration of the intestinal epithelium by these pathogens occurs by preferential bacterial invasion of M cells.26 After passing through the M cells, the bacteria reach the Peyer’s patches and encounter a network of resident macrophages and DC.27 We have shown that on infection by S. typhimurium, DC respond by releasing cytokines, such as IL-10 and IL-12.16 Our current results show that, in addition to cytokine release, the infection is associated with dramatic changes in DC morphology. These changes include plasma membrane ruffling, cytoplasmic vacuolization, spacious phagosome formation and apoptosis. The morphological changes are similar to what has been reported in macrophages and epithelial cells.27 However, in contrast to macrophages and epithelial cells, DC were also found to lose their characteristic dendritic morphology and show small pedunculated spherical membrane-bound vesicles on their plasma membrane.

Electron microscopy has provided the morphological basis of understanding the sequence of events that takes place when Salmonella infects intestinal epithelium.28 Following close contact of the bacteria with the epithelium, the plasma membrane undergoes dramatic changes that are exemplified by localized membrane ruffles.28,29 In contrast, macrophages

![Figure 3](image_url)

**Figure 3** Transmission electron micrograph of human dendritic cells at 24 h after infection with (A) virulent Salmonella typhimurium strain C53 resulting in apoptosis and (B) SipB strain that induces expression of microvesicular bodies. Microvesicles hanging loosely from the plasma membrane (stars). Bacteria profiles (arrows). Apoptotic bodies (arrow heads) and condensed nuclear chromatin (N). Bar, 2 \(\mu\)m. (C) Flow cytometry with annexin-V-phycocerythrin (PE) detecting apoptosis after 24 h of infection with Salmonella strain C53 and the SipB mutant strain, which does not cause apoptosis. Data are expressed as a percentage of gated cells. Bars are mean ± SD of nine independent experiments. \(*P < 0.05\) compared with media (cells alone).
show generalized membrane ruffling similar to DC.\textsuperscript{7} Our previous finding showed that the mutant strain PhoPc is internalized better than the wild-type strain ATCC 14028.\textsuperscript{16} The current understanding is that bacteria-mediated membrane ruffling and macropinocytosis are important for \textit{Salmonella} entry into the host cell, because the mutants that are unable to trigger such ruffling are impeded in their ability to enter cultured epithelial cells and macrophages.\textsuperscript{30} Both the attenuated and the wild-type strains caused membrane ruffling to some extent. However, the virulent strains induced much more prominent ruffles when compared with the mutants. This apparent dissociation between uptake of \textit{Salmonella} by the DC and ruffling suggest that ruffling plays a minor role in the uptake of \textit{Salmonella} by immature DC. Moreover, macrophages and epithelial cells that do not show constitutive macropinocytosis\textsuperscript{31} show reduced uptake of mutants that do not induce ruffling.\textsuperscript{7,32} Our preliminary studies have shown that alveolar macrophages take up both wild-type strains and mutant strains of \textit{Salmonella} less efficiently than DC.\textsuperscript{33} Furthermore, a recent study reported that immature DC are capable of internalizing a wild-type bacteria and mutant strain, which is defective in the type III secretory system to the same extent.\textsuperscript{34} Although DC were not initially described as phagocytic cells, several reports have now shown that immature DC endocytose particulate antigens, such as bacteria, yeast and even polystyrene particles very efficiently.\textsuperscript{35–38} Our present work corroborates the notion that the internalization of \textit{Salmonella} in human DC \textit{in vitro} is not dependent on the ability of the bacteria to actively invade the cells.

Virulent \textit{S. typhimurium} strains induce massive uptake of extracellular fluid in epithelial cells\textsuperscript{10} and macrophages\textsuperscript{9} in the form of macropinosomes. These macropinosomes result in the formation of spacious phagosomes whereby the bacteria swim freely within the enclosed space.\textsuperscript{9} Similar to macrophages and epithelial cells, invasion of human DC by \textit{S. typhimurium} resulted in the formation of spacious phagosomes. However, spacious phagosomes were only observed in cells infected by wild-type strains of \textit{S. typhimurium}. Although the significance of the spacious phagosomes in host cells has not been fully elucidated, it has been suggested that these may delay acidification of the phagosomes and therefore create a tolerable intracellular environment allowing \textit{Salmonella} to survive in macrophages and epithelial cells.\textsuperscript{9} Consistent with this suggestion, our previous studies clearly showed that the wild-type strain survives longer inside the human DC than the attenuated strains.\textsuperscript{16}

DC are sentinels of the immune system that function by surveying peripheral tissues for antigen and stimulating naive T-lymphocyte responses to invading pathogens.\textsuperscript{39,40} Recent studies suggest that intracellular \textit{Salmonella} inhibit antigen presentation by DC and that T-cell proliferation is inhibited by direct contact with the bacteria \textit{in vitro}.\textsuperscript{41} These suggestions are surprising because of the many reports from experiments carried out \textit{in vivo} indicating strong B-cell responses and T-cell responses from \textit{Salmonella} infection, which have given impetus to \textit{Salmonella} being considered as a candidate vaccine.\textsuperscript{3,4,42} On prolonged infection (24 h postinfection) by \textit{Salmonella}, DC lost their characteristic dendritic morphology and showed small membrane-bound spherical pedunculated

\textbf{Figure 4} Transmission electron micrograph of human dendritic cells (DC) at 24 h after infection with \textit{Salmonella typhimurium}. (A) Uninfected, (B) wild-type strain ATCC 14028, (C) AroA and (D) PhoPc. Bar, 1 µm. Note the small membrane bound spherical bodies (arrows) that hang loosely from the membrane of all the infected cells. The noninfected cell displays the typical dendritic processes associated with DC. Spacious phagosome (SP). Inset: close-up view of the membrane bound microvesicles. Bar, 0.5 µm.
microvesicles hanging loosely from the plasma membrane. A recent proteomic study on microvesicles produced from human CD4D T cell line (CEM) T-lymphocyte identified 390 proteins comprising 34% from plasma membrane, including HLA class I molecules and 54% that are cytoplasmic in origin. Although the role of these microvesicles from human DC was not investigated, based on published reports, their observation raises the possibility of transfer of antigens from infected DC to bystander DC, thus circumventing the inhibitory effects of intracellular bacteria and potentially amplifying T-cell activation. In fact, it has been shown that DC have the unique ability to capture antigens from apoptotic cells by a and CD36 and cross-present antigens to cytotoxic T lymphocyte. In a seminal paper by Inaba et al. it was reported that DC efficiently present phagocytosed cellular fragments on MHC class II products, thus showing the unique ability of DC to acquire antigen from nonconventional sources. In our own cultures, some DC that had phagocytosed cell debris were occasionally observed.

We have described the interaction of S. typhimurium with immature human DC, a cell type that has a primary role in the immune response. The hallmark of this interaction is the generation of pedunculated membrane-bound microvesicles by the DC. A role of these vesicles in the transfer of antigens to bystander DC is suggested. Given the ability of Salmonella bacteria to inhibit T-cell-mediated immunity when located within DC and the strong immunogenicity of these bacteria in vivo, the development of bacterial vaccines must include more knowledge on the host cell response.

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References


Figure 5 Scanning electron micrographs of dendritic cells (DC) at 24 h after infection with (A) Salmonella typhimurium mutant strain (AroA) and (B) wild-type strain (C53). Note the large and smooth apoptotic bodies (B) that emerge from a cell undergoing apoptosis and the small pleiomorphic bodies projecting from the cell membrane (B) in the cell infected by mutant strain (arrows). Bar, 1 μm.

Figure 6 Transmission electron micrograph of a human alveolar macrophage at 24 h after infection with the Salmonella typhimurium mutant strain SipB. The microvilli on the cell surface are conserved (arrows head) and no microvesicles are displayed from the plasma membrane. Bacteria profile (arrow), and nuclear (N). Bar, 4 μm.