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Intracellular activities of *Salmonella enterica* in murine dendritic cells

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Summary

Dendritic cells (DC) efficiently phagocytose invading bacteria, but fail to kill intracellular pathogens such as *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium). We analysed the intracellular fate of *Salmonella* in murine bone marrow-derived DC (BM-DC). The intracellular proliferation and subcellular localization were investigated for wild-type *S.* Typhimurium and mutants deficient in *Salmonella* pathogenicity island 2 (SPI2), a complex virulence factor that is essential for systemic infections in the murine model and intracellular survival and replication in macrophages. Using a segregative plasmid to monitor intracellular cell division, we observed that, in BM-DC, *S.* Typhimurium represents a static, non-dividing population. In BM-DC, *S.* Typhimurium resides in a membrane-bound compartment that has acquired late endosomal markers. However, these bacteria respond to intracellular stimuli, because induction of SPI2 genes was observed. *S.* Typhimurium within DC are also able to translocate a virulence protein into their host cells. SPI2 function was not required for intracellular survival in DC, but we observed that the maturation of the *Salmonella*-containing vesicle is different in DC infected with wild-type bacteria and a strain deficient in SPI2. Our observations indicate that *S.* Typhimurium in DC are able to modify normal processes of their host cells.

Introduction

Dendritic cells (DC) have a central role in the linkage between innate and adaptive immunity (Banchereau and Steinman, 1998). DC are able to phagocytose microbes, process the phagocytosed material and present microbial antigens to other cells of the immune system. These functions allow the organism to mount a specific and efficient immune response. An important feature of DC biology is the ability to migrate from the site of antigen uptake in the periphery to lymphatic organs, where the presentation of antigens can take place (for a review see Mellman and Steinman, 2001). As microbial pathogens are confronted with the activity of DC, the successful colonization of a host by a pathogen might require interference with DC functions or even subversion of DC functions for the progression of infection.

There is an increasing number of indications for interactions between microbial pathogens and DC (reviewed by Rescigno, 2002). However, only limited data are available on the activities of intracellular pathogens within DC and how normal functions of DC are affected by microbial virulence factors.

We are interested to understand the basic virulence mechanisms of *Salmonella enterica*, an important foodborne Gram-negative pathogen. In addition to localized gastrointestinal infections associated with diarrhoea, certain *S. enterica* serovars are able to penetrate the intestinal mucosa, spread via the reticuloendothelial system and cause an often fatal systemic bacteraemia. A form of salmonellosis in humans known as typhoid fever can be studied in a murine model after infection with *S. enterica* serovar Typhimurium (*S.* Typhimurium).

It is not fully understood how *S.* Typhimurium can spread systemically after oral infection, but phagocytic cells have been considered as possible vehicles for transport of *S.* Typhimurium from mucosal sites such as Peyer's patches to mesenteric lymph nodes and other organs (Rescigno *et al*., 2001; Cheminay *et al*., 2002). A large number of virulence factors are required for pathogenesis of *S.* Typhimurium, and many of these are clustered within pathogenicity islands or on the virulence plasmid. *Salmonella* pathogenicity island 1 (SPI1) encodes a protein translocation system referred to as a type III secretion system (T3SS), which is involved in invasion of non-phagocytic cells by *S.* Typhimurium and triggering of diarrhoeal symptoms. *S.* Typhimurium possesses a second T3SS that is encoded by SPI2. The SPI2 system is activated by intracellular bacteria and is required for the intra-

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cellular lifestyle of *Salmonella*. Mutant strains deficient in SPI2 functions are dramatically attenuated in systemic virulence and are reduced in proliferation in infected eukaryotic cells (reviewed by Hensel, 2000).

Salmonella Typhimurium is efficiently taken up by DC, and bacterial antigens are presented by DC harbouring *Salmonella* (Svensson *et al*., 1997; Marriott *et al*., 1999; Hopkins *et al*., 2000; Niedergang *et al*., 2000; Yrlid and Wick, 2002) (for recent reviews, see Wick, 2002; 2003). Although the intracellular fate of *S.* Typhimurium in macrophages has been studied in detail, and many virulence determinants for the intracellular survival and proliferation in macrophages were identified, only a little information is available on the fate of *S.* Typhimurium after uptake by DC and the intracellular activities of the pathogen in these cells. Previous observations (Garcia-del Portillo *et al*., 2000; Niedergang *et al*., 2000) indicated that the intracellular fate of *S. enterica* in DC is different from that in macrophages. Using an immortalized DC-like cell line, Garcia-del Portillo *et al*. (2000) observed that intracellular *Salmonella* reside in a compartment that is different from that described for *Salmonella* inside macrophages. In detail, the late endosomal/lysosomal membrane marker LAMP-1 was absent from the *Salmonella*-containing vacuole (SCV). There are experimental indications that *Salmonella* is not efficiently killed after uptake by DC. Surprisingly, *Salmonella* virulence factors important for invasion of host cells and intracellular survival and proliferation in macrophages are not required for the entry, survival and proliferation in DC (Niedergang *et al*., 2000).

We investigated the intracellular fate of *S.* Typhimurium in murine bone marrow-derived DC (BM-DC) and deployed molecular approaches to monitor the activities of the pathogen after uptake by DC. We also analysed the function of virulence factors for the intracellular phenotype of *S. enterica* in DC. These combined approaches provide novel insight into the activities of an intracellular pathogen within DC.

Results

Intracellular Salmonella *are a static population in infected DC*

Several experimental studies have demonstrated that the number of intracellular *S.* Typhimurium remains constant after uptake by DC (Marriott *et al*., 1999; Niedergang *et al*., 2000; Cheminay *et al*., 2002). We considered that, after uptake by BM-DC, intracellular *S.* Typhimurium either proliferate at a rate that is equal to the rate of killing by host cells or the bacterial population is static and not affected by antimicrobial activities. In both scenarios, the net number of intracellular bacteria will be constant over a given experimental interval. In order to investigate the intracellular fate of *S.* Typhimurium in DC, we performed infection studies with bacteria harbouring a segregative plasmid. Plasmid pHS422 (Hashimoto-Gotoh *et al*., 1981) has a temperature-sensitive origin of replication that allows replication of the plasmid at permissive temperatures of 30°C or below and results in segregation of the plasmid between dividing cells at 37°C or higher. The approach has been used previously to determine *Salmonella* proliferation in infected organs (Benjamin *et al*., 1990) as well as cultured cells (Clements *et al*., 2002).

BM-DC and the macrophage-like cell line RAW264.7 were infected with *S.* Typhimurium wild-type and mutant strains, each harbouring plasmid pHSG422, and the proportion of intracellular bacteria with or without pHSG422 was determined (Fig. 1). After infection of RAW264.7 macrophages, a rapid proliferation of wild-type *S.* Typhimurium was observed within an interval of 18 h. Selection for the plasmid-borne resistance markers revealed that a large proportion of the intracellular bacteria had lost the plasmid (Fig. 1C). Consistent with previous observations, a mutant strain deficient in SPI2 function did not replicate, and a large proportion of the recovered intracellular bacteria contained the plasmid. In contrast, the number of bacteria recovered from infected BM-DC was similar for wild-type *S.* Typhimurium and the SPI2 mutant strain, and no increase in the number of intracellular bacteria was observed. The ratio of bacteria harbouring pHSG422 to bacteria without plasmid remained similar within a period of 0.5–4 h (Fig. 1A) and within a longer period of 24 h (Fig. 1B).

These data indicate that, in BM-DC, intracellular *S.* Typhimurium represent a static, non-dividing population. Our data do not support a situation in which bacterial replication occurs at a rate that is similar to the killing of bacteria by the host cell. This situation is significantly different from that in the macrophage cell line, where wild-type *S.* Typhimurium can replicate rapidly.

Expression of virulence genes encoded by SPI2 is induced by intracellular S. Typhimurium *in BM-DC*

To elucidate further the role of bacterial virulence factors during interaction with BM-DC, we analysed the expression of genes in SPI2 during infection of DC. The specific intracellular activation of SPI2 genes has been reported previously (Valdivia and Falkow, 1997; Cirillo *et al*., 1998; Deiwick *et al*., 1999; Pfeifer *et al*., 1999) and is considered as a requirement for the adaptation of *S.* Typhimurium to the intraphagosomal environment.

To obtain a highly sensitive quantification of the intracellular gene expression, we used reporter strains of *S.* Typhimurium harbouring a fusion of the promoter of the SPI2 gene *sseA* to the green fluorescent protein (GFP) gene. In order to achieve regulation characteristics that

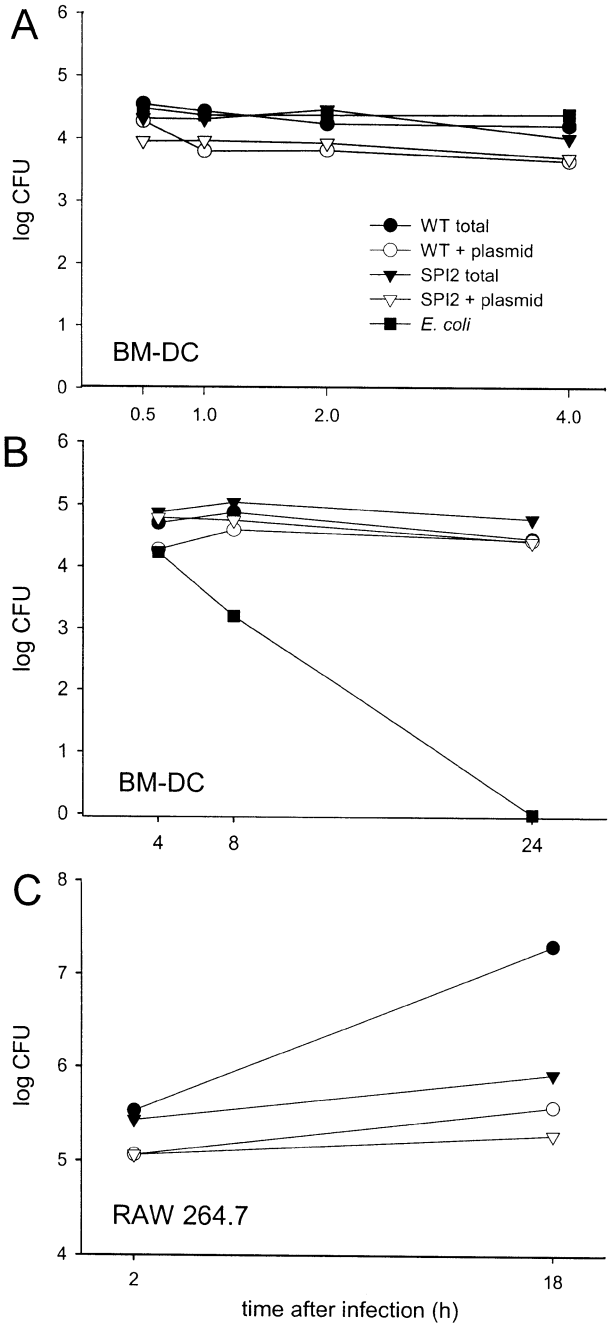


Fig. 1. Intracellular growth characteristics of *S.* Typhimurium in murine BM-DC and in the macrophage-like cell line RAW 264.7. Cell cultures were infected with opsonized *S.* Typhimurium or *E. coli* strains as indicated. After infection for 30 min, non-internalized bacteria were removed by washing, and gentamicin was added to kill residual extracellular bacteria. After various periods of time, the cells were washed and lysed with 0.5% Triton X-100 for 10 min. Serial dilutions of the lysates were plated on agar plates without and with antibiotics to enumerate the total number of intracellular bacteria, and the proportion that still harbours plasmid pHSG422 respectively.
A and B. Infection of sorted murine BM-DC.
C. Infection of RAW 264.7 cells.

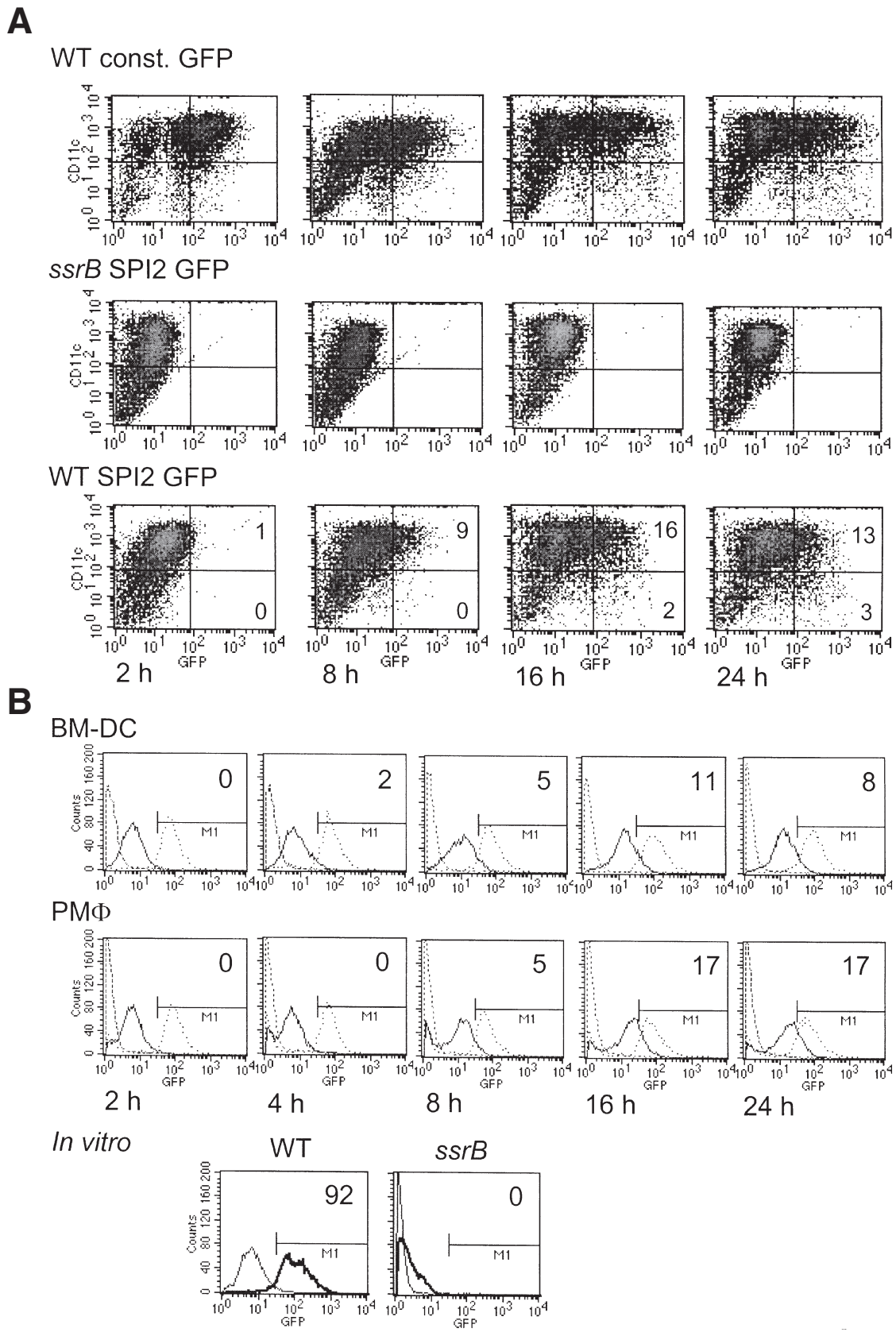
are similar to those of chromosomally encoded SPI2 genes (Hansen-Wester *et al.*, 2002), this fusion was transferred into the low-copy-number vector pWSK29 to generate pLS824. Expression analyses under defined *in vitro* conditions indicated that the expression of GFP under the control of Pro_{sseA} occurred under the same conditions observed for SPI2 genes, and that expression was dependent on the function of the two-component regulatory system SsrAB.

BM-DC were infected with S. Typhimurium strains harbouring GFP reporter plasmids

At various time points after infection, host cells were fixed, immunostained for CD11c and analysed by flow cytometry (Fig. 2A) or fluorescence microscopy (Fig. 2C). Using a strain harbouring a plasmid pFPV25.1 for constitutive expression of GFP, we observed that about 40% of the cells were infected with *S.* Typhimurium and 90% of the infected cells were CD11c positive (Fig. 2A). Next, *S.* Typhimurium wild type and a strain deficient in the SPI2 regulatory system SsrAB, both harbouring pLS824 for GFP expression under the control of a promoter of SPI2, were used to infect BM-DC cultures. Induction of the SPI2 promoter fusion was observed in CD11c-positive cells 8 h after uptake of the wild-type strain. In contrast, this reporter fusion was not induced after uptake of a strain deficient in *ssrB*. Immunofluorescence analyses of individual infected cells also demonstrated the induction of the SPI2 promoter by *S.* Typhimurium within BM-DC (Fig. 2C). We observed that a proportion of intracellular *Salmonella* did not show detectable GFP fluorescence. This may result from low levels of expression or differences in virulence gene expression in individual intracellular bacteria.

We next released intracellular bacteria by lysis of host cells at various time points after infection and analysed GFP fluorescence of individual bacterial cells (Fig. 2B). The expression of the reporter fusion was observed 8 h after infection, and the levels of GFP increased at later time points after infection. The activation of the reporter fusion was dependent on the function of SsrB, as no induction was observed in the *ssrB* background. Comparison of the induction of the reporter gene fusion by *S.* Typhimurium in BM-DC with primary macrophages revealed similar kinetics of induction, but higher levels of GFP expression in primary macrophages.

These data suggest that specific signals inducing the expression of SPI2 genes are also present in DC. The molecular nature of these signals is not known to date. However, analyses of SPI2 gene expression under defined *in vitro* conditions indicated that nutritional limitation might be such a signal (Deiwick *et al.*, 1999). Furthermore, induction of SPI2 genes can be observed in various cells types (for a review, see Hansen-Wester and Hensel,



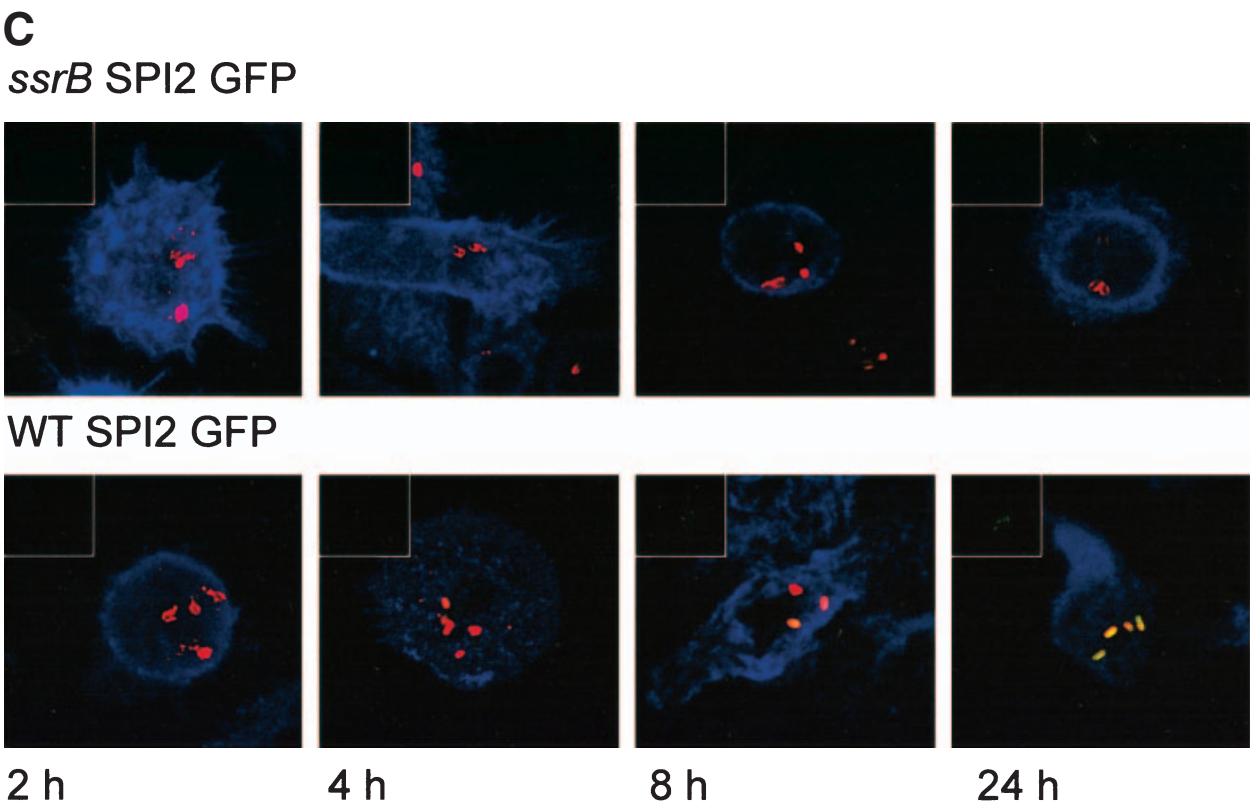


Fig. 2. *Salmonella* virulence gene expression of intracellular *S.* Typhimurium in BM-DC. BM-DC or peritoneal macrophages were infected with *S.* Typhimurium wild type (WT) or mutant strain P8G12 (*ssrB*::mTn*5*) deficient in the SPI2 regulatory system (*ssrB*). *Salmonella* strains harboured either plasmid pFPV25.1 for the constitutive expression of GFP (const. GFP) or plasmid pLS824 for the expression of GFP under the control of the regulatory system of SPI2 (SPI2 GFP). Non-internalized bacteria were removed by washing, and remaining bacteria were killed by the addition of gentamicin.
A. At various time points after infection, BM-DC were immunolabelled for CD11c on ice and subsequently fixed. Expression of CD11c and GFP was analysed by flow cytometry of about 20 000 cells.
B. At various time points after infection, BM-DC or peritoneal macrophages were lysed by the addition of Triton X-100, and intracellular bacteria were released. Samples were incubated with primary antibody against *Salmonella* O5 and a Cy5-conjugated secondary antibody to label bacteria. Approximately 10 000 LPS^{high}-positive, bacteria-sized particles were analysed for GFP expression. Solid line, *S.* Typhimurium wild type [pLS824]; dashed line, P8G12 [pLS824]; dotted line, wild type [pFPV25.1]. For *in vitro* controls of SPI2 induction, *S.* Typhimurium wild type [pLS824] and P8G12 [pLS824] were grown for 16 h in LB broth (non-inducing conditions, solid line) or PCN-P minimal media inducing SPI2 expression (bold line), and bacteria were analysed for GFP expression. The percentage of *Salmonella* wild type [pLS824] showing induction of GFP expression is indicated (gate M1).
C. As (A), but cells were fixed, immunostained for CD11c (blue) and *Salmonella* LPS (red) and analysed by confocal microscopy. Inserts show the green channel for GFP fluorescence.

2001), suggesting that the SPI2-inducing signals are a general characteristic of vacuoles containing *S.* Typhimurium.

Intracellular Salmonella translocate a virulence protein into BM-DC and primary macrophages

To analyse whether, in addition to the expression of SPI2 genes, intracellular *Salmonella* assemble a functional T3SS in BM-DC, we investigated the SPI2-dependent translocation of an effector protein by intracellular *Salmonella* in BM-DC and primary macrophages. As a representative effector protein, we used an epitope-tagged derivative of SseJ, a protein that is expressed and translocated by intracellular *Salmonella* in epithelial and monocyte cell lines (Kuhle and Hensel, 2002). SseJ is associated with endosomal membranes after translocation (Kuhle and Hensel, 2002) and might modulate the biogenesis of the *Salmonella*-containing vesicle (SCV) (Ruiz-Albert *et al.*, 2002). Here, we infected BM-DC, murine peritoneal macrophages and the RAW264.7 cells with *S.* Typhimurium strains harbouring plasmid p2777 for the expression of haemagglutinin epitope (HA)-tagged *sseJ* under the control of its native promoter. Host cells were fixed 24 h after infection and processed for immunofluorescence analyses for the detection of SseJ-HA and intracellular *Salmonella*. SPI2-dependent translocation was observed in BM-DC and primary macrophages (Fig. 3) and, as reported previously (Kuhle and Hensel, 2002), in RAW264.7 cells (data not shown). The

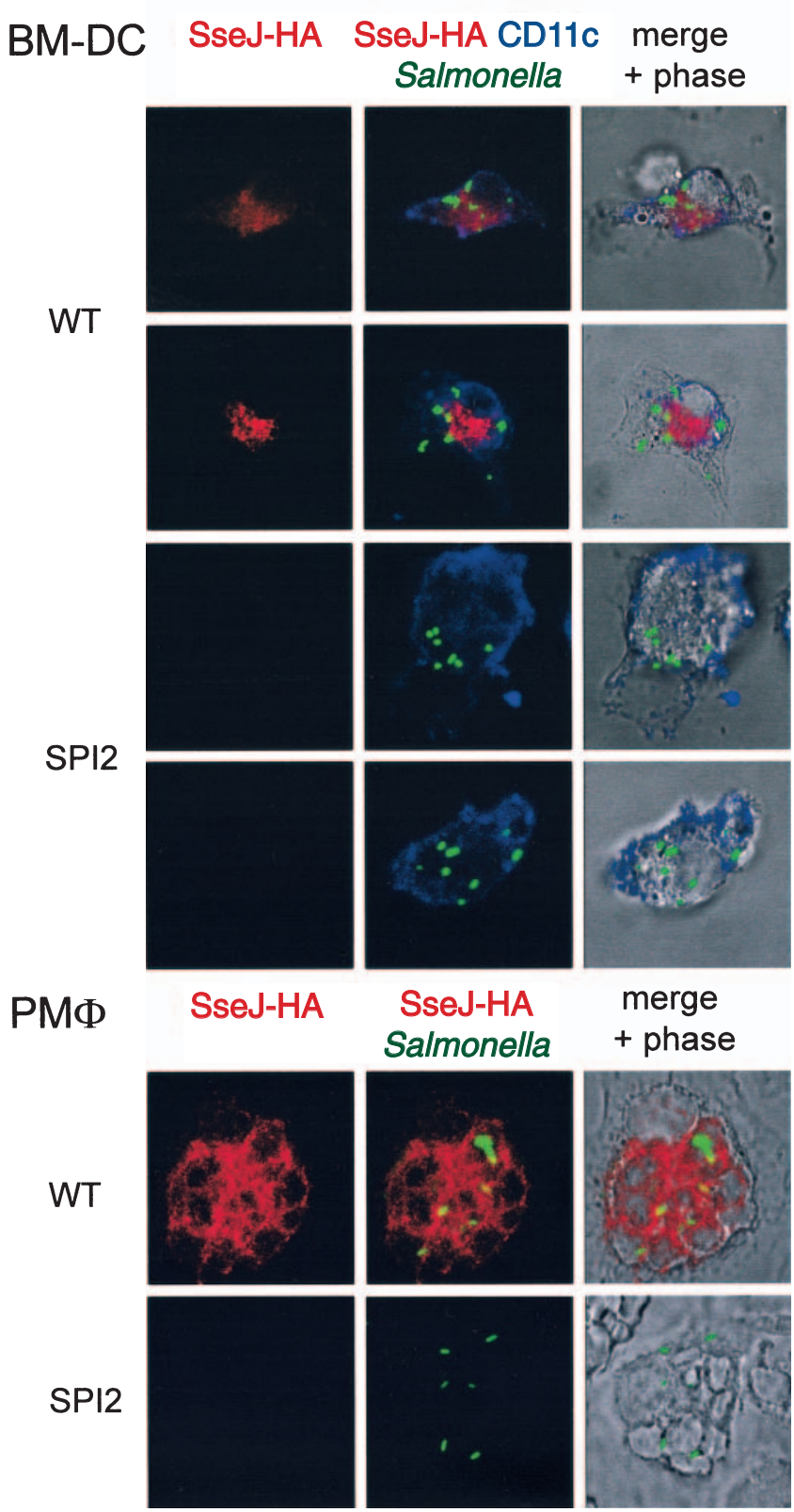


Fig. 3. An effector protein of the SPI2 system is translocated into BM-DC by intracellular *S.* Typhimurium. BM-DC and primary macrophages (PMΦ) were infected with wild-type *S.* Typhimurium (WT) or mutant strain P2D6 deficient in the SPI2-encoded T3SS (SPI2). Both strains harboured plasmid p2777 for the expression of a SseJ-HA fusion protein under the control of its native promoter and for the expression of GFP under the control of a constitutive promoter. Infection was performed for 1 h as described and, 24 h after infection, cells were processed for immunostaining. BM-DC were identified by immunostaining of CD11c (blue). Intracellular *Salmonella* were visualized by GFP fluorescence (green), and translocated SseJ-HA was detected using rat mAb against HA epitope and goat anti-rat Cy3 conjugate as secondary antibody (red). The phase-contrast image was overlaid to show the gross morphology of the cells.

HA-tagged effector protein SseJ was only detected in host cells infected with wild-type *Salmonella* harbouring p2777, but not in cells infected with mutant strain P2D6 [p2777] defective in the SPI2-encoded T3SS (Fig. 3). In general, immunostaining for SseJ-HA in infected primary macrophages resulted in stronger signals than in BM-DC. This observation is consistent with the higher induction of SPI2 genes and the intracellular replication of *Salmonella* in primary macrophages. The limit of sensitivity by immunostaining allowed the detection of translocated protein in host cells harbouring seven or more intracellular bacteria. Similar to the situation in infected macrophages or epithelial cells, translocated SseJ was not randomly distributed in the cytoplasm. A predominant localization in perinuclear regions of infected BM-DC was observed (Fig. 3). However, an association with filamentous host cell structures, as observed in *Salmonella*-infected epithelial cells (Kuhle and Hensel, 2002; Freeman *et al*., 2003), was not detectable in BM-DC. This is in line with the absence of filamentous aggregates of LAMP-1-positive endosomes referred to as *Salmonella*-induced filaments or SIF (see Fig. 5A).

These observations indicate that the SPI2-encoded T3SS is assembled, and that *Salmonella* within BM-DC also encounter signals triggering translocation via this system.

Virulent Salmonella *modify their intracellular compartment in BM-DC*

We next analysed the intracellular localization of phagocytosed *S.* Typhimurium in BM-DC. Electron microscopy (EM) showed that the bacteria remain in a membrane-bound compartment (Fig. 4), and there was no difference in the localization of the wild-type strain and a mutant strain deficient in SPI2. Analysis by EM at 8–12 h after infection indicated that intracellular bacteria are intact without signs of disintegration of the bacterial envelope.

Furthermore, the co-localization of various host cell markers with the SCV was investigated by immunostaining and confocal microscopy (Fig. 5). In addition to *S.* Typhimurium wild type and the SPI2-deficient strain, we also analysed the intracellular fate of a *phoP* strain deficient in the PhoPQ global regulatory system. In contrast to previous data obtained with an immortalized cell line (Garcia-del Portillo *et al*., 2000), we observed that the SCV is predominantly co-localized with the lysosomal glycoprotein LAMP-1. A high frequency of co-localization was also observed with major histocompatibility complex (MHC) II-positive compartments. Regarding these markers, wild-type *S.* Typhimurium as well as SPI2 and PhoP mutant strains showed similar characteristics. We also used the monoclonal antibody (mAb) 2A1, which has been described as a marker for the maturation of murine DC (Inaba *et al*., 1992). 2A1 reacts specifically with DC; however, the antigen recognized by 2A1 has not been characterized. Staining of *S.* Typhimurium-infected BM-DC resulted in a granular staining pattern of 2A1 (Fig. 5), and co-localization of the marker with the SCV was frequently observed. The co-localization of the marker was

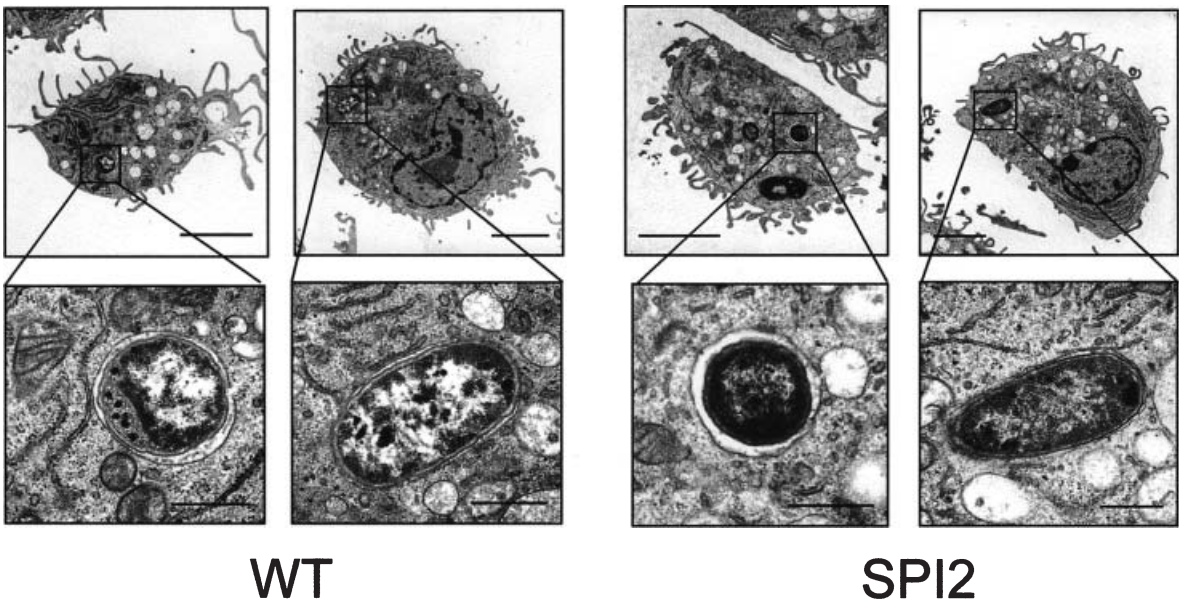


Fig. 4. Wild-type *S.* Typhimurium and a SPI2 mutant strain reside inside a membrane-bound compartment in BM-DC. BM-DC obtained after FAC sorting were infected with wild-type *S.* Typhimurium or a SPI2 mutant strain (P2D6, *ssaV*::mTn*5*). Eight hours after infection, the cells were fixed and processed for transmission microscopy. Scale bars: upper row = 3 μm; lower row = 500 nm.

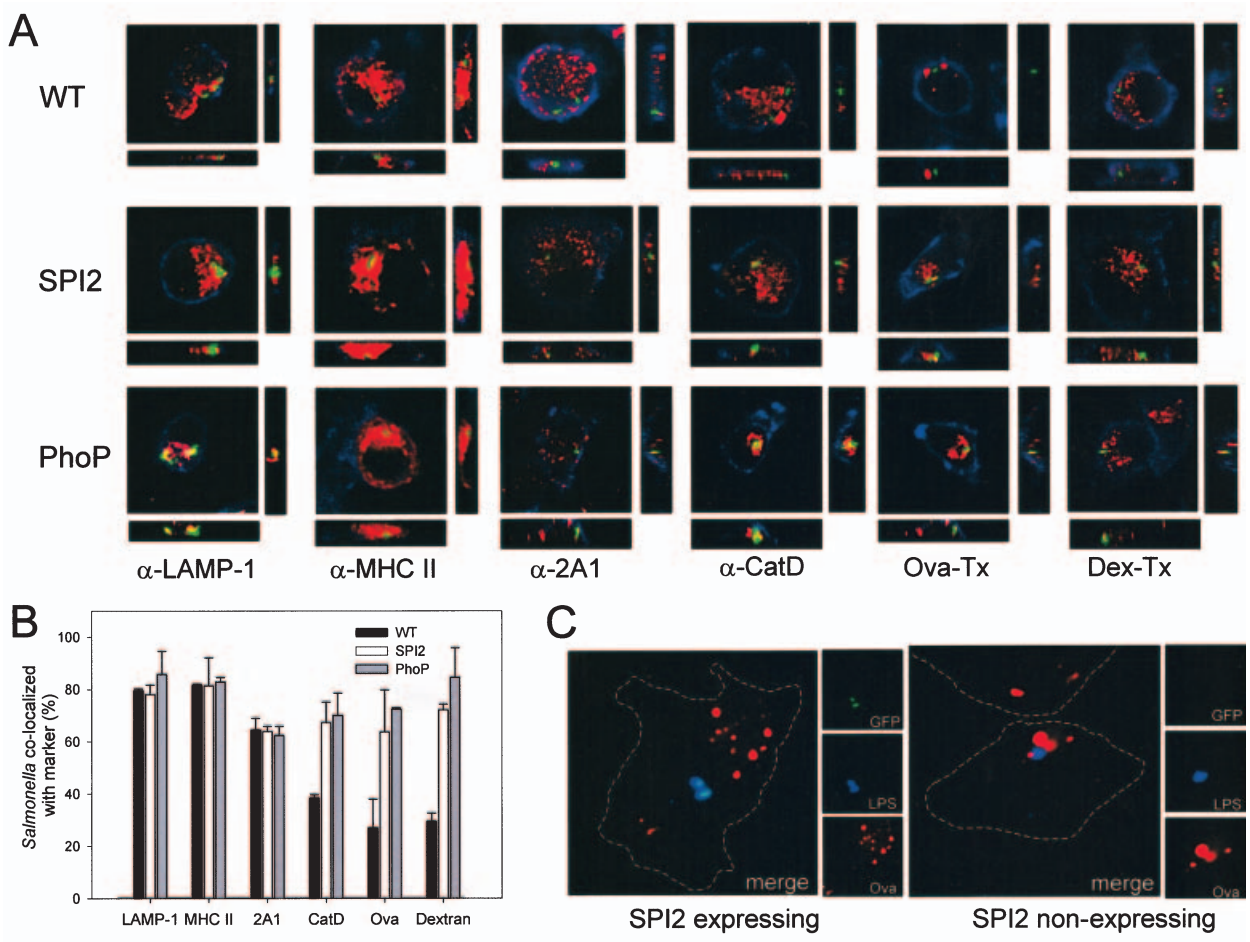


Fig. 5. Subcellular localization of intracellular *S.* Typhimurium in BM-DC. BM-DC were infected with *S.* Typhimurium wild type, the SPI2-deficient strain P2D6 (*ssaV*::mTn*5*) or the *phoP*-deficient stain CS015, each harbouring pFPV25.1 for constitutive expression of GFP (green). Eight hours after infection, BM-DC were fixed with paraformaldehyde and processed for immunostaining by permeabilization with 0.1% saponin. BM-DC were detected with an antibody against CD11c (blue). Various host cell markers were detected with the respective antibodies and visualized by staining with Cy3-conjugated secondary antibody (red). For part of the experiments, BM-DC were also pulse–chased 2 h before infection with the fluid tracers Texas red–ovalbumin (Ova) or Texas red–dextran (Dextran), each indicated by red fluorescence signals.

A. Confocal laser-scanning microscopy was used to analyse BM-DC infected with GFP-expressing *S.* Typhimurium wild type or the SPI2 or *phoP* mutant strains. Representative micrographs for the various markers are show with XY, YZ and XZ sections to demonstrate the intracellular localization of the bacterial cells.

B. Quantification of co-localization of various markers and *S.* Typhimurium strains. Eight hours after infection, CD11c-positive cells containing *S.* Typhimurium were located, and at least 50 intracellular bacteria were scored for co-localization with the indicated marker. Results shown are the means ± SE of two experiments performed on independent occasions. Similar observations were made 24 h after infection of BM-DC with *S.* Typhimurium.

C. Intracellular fate of SPI2 expressing and not expressing *S.* Typhimurium wild type. BM-DC were purified by MACS to obtain more than 90% purity. BM-DC were pulse–chased with Texas red–ovalbumin (red) 2 h before infection, washed and subsequently infected with *S.* Typhimurium wild type harbouring pLS824 for SPI2-dependent expression of GFP. Twelve hours after infection, BM-DC were fixed und processed for immunostaining of *Salmonella* LPS (blue). The gross morphology of BM-DC is indicated by dashed lines. Micrographs show representative SPI2-expressing and -non-expressing intracellular bacteria and their co-localization with the Texas red–ovalbumin.

similar to SCV containing wild-type *S.* Typhimurium or a SPI2-deficient strain. As further markers for the intracellular processing of phagocytosed bacteria, cathepsin D and endocytic fluid tracers were analysed. Cathepsin D co-localization was observed for about 67% or 70% of the SCV harbouring the SPI2 or the PhoP mutant strain, respectively, but only 38% of the SCV harbouring wild-type *S.* Typhimurium. BM-DC were pulse–chased with Texas red–ovalbumin or Texas red–dextran 2 h before infection with *S.* Typhimurium in order to load lysosomal compartments with fluid tracers. Both tracers co-localized with frequencies of 63–72% with the SPI2 mutant strain, 72–84% with the PhoP strain but only 27–29% with wild-type *S.* Typhimurium (Fig. 5B).

In BM-DC, the majority of wild-type *S.* Typhimurium reside in a compartment that has acquired late endosomal markers, but does not mature into a phagolysosome. In contrast, a larger proportion of SCV harbouring SPI2-

deficient *S.* Typhimurium acquired lysosomal markers, implying that maturation of these compartments can progress to later stages. A mutant strain deficient in the global regulatory system PhoPQ showed a trafficking pattern that was similar to that of a SPI2-mutant strain, i.e. the acquisition of late endosomal and lysosomal markers to the SCV. However, this maturation of the SCV is not sufficient to kill intracellular bacteria efficiently, as similar numbers of cfu were recovered after infection with wild-type or SPI2-deficent *Salmonella* (Fig. 1).

The data suggest that the SPI2 system is influencing the intracellular fate of *Salmonella* in BM-DC. SPI2-deficient strains are more frequently located in vesicles that have matured into phagolysosomes, whereas wild-type *S.* Typhimurium more frequently prevent or delay this maturation process. However, not all *S.* Typhimurium wild-type bacteria prevent the maturation of the SCV. Furthermore, as shown in Fig. 2, only a subset of intracellular wild-type *Salmonella* express SPI2 genes. To correlate the heterogeneity in the intracellular fate with the expression of SPI2 genes, we followed the trafficking of *S.* Typhimurium wild type harbouring the SPI2-regulated reporter described above. We observed that SPI2-expressing bacteria did not co-localize with Texas red–ovalbumin-loaded lysosomes, whereas non-expressing intracellular bacteria were frequently co-localized with Texas red–ovalbumin (Fig. 5C).

Discussion

To our knowledge, the data presented here describe for the first time the intracellular activities of a bacterial pathogen within DC. Although indirect evidence suggests modification of DC functions by viral, bacterial or eukaryotic pathogens (reviewed by Cutler *et al*., 2001), direct demonstration of the microbial activities was not accomplished before. Using a set of molecular and cellular microbiological approaches, we could demonstrate that virulence genes of *S.* Typhimurium are induced after uptake by DC, and that a functional T3SS is assembled by intracellular *Salmonella* residing in a parasitophorous vacuole within DC that translocates a representative virulence protein. We also observed that the intracellular trafficking of bacteria is influenced by functions of the SPI2-encoded T3SS, an important virulence factor for the intracellular survival and proliferation of *S.* Typhimurium in macrophages.

Previous studies suggested that *S.* Typhimurium persists but is unable to replicate in DC, and that virulence factors important for the intracellular phenotype in macrophages such as SPI2 are not important for intracellular *Salmonella* in DC. We confirm these observations but suggest that virulence factors activated by intracellular *Salmonella* affect the intracellular fate of *Salmonella* in BM-DC. Although *S.* Typhimurium wild type and the SPI2-deficent strain persisted to an equal extent in an experimental interval of 24 h, vesicles harbouring the SPI2-deficient strain acquired late endosomal and lysosomal markers to a higher extent than vesicles containing wild-type bacteria, indicating that the SPI2 virulence factor interferes with normal trafficking of a pathogen-containing vacuole in DC. These observations revise the view of the intracellular activities of bacterial pathogens in DC.

We observed that a certain proportion of intracellular *Salmonella* in BM-DC did not activate SPI2 genes (Fig. 2), and that did not prevent maturation of the SCV as indicated by co-localization with cathepsin D and fluid tracers (Fig. 5). Such heterogeneity in the intracellular phenotype of *S.* Typhimurium wild type has been observed before (for a review, see Holden, 2002). Different levels of virulence gene expression by individual bacteria may account for the different intracellular fate we also observed for *S.* Typhimurium wild type in BM-DC. A correlation between expression of SPI2 genes and maturation of the SCV was observed in BM-DC (Fig. 5C), suggesting a direct effect of SPI2 activity on maturation of the SCV. It would be of future interest to follow this correlation in detail using more homogeneous host cells, such as macrophage-like cell lines.

The intracellular trafficking in BM-DC is in contrast to previous observations of the trafficking of *S.* Typhimurium wild type, SPI2 and PhoP mutant strains in RAW264.7 macrophages. Garvis *et al*. (2001) reported that the PhoP mutant strain, but not *S.* Typhimurium wild type or the SPI2-deficent strain, co-localize to lysosomal markers. However, both SPI2 and the PhoPQ regulatory system are important for the intracellular survival and replication in macrophage-like cell lines and primary macrophages (Miller *et al*., 1989; Ochman *et al*., 1996; Cirillo *et al*., 1998; Hensel *et al*., 1998). We have reported previously a requirement of PhoPQ function for SPI2 expression under *in vitro* conditions (Deiwick *et al*., 1999) and observed that a SPI2 reporter construct is not activated in the background of the PhoP strain after uptake by host cells (D. Jäckel, J. Jantsch and M. Hensel, unpublished observations). The similar intracellular characteristics of SPI2 and PhoP strains in BM-DC therefore, among other factors, might account for the virulence defect of the PhoP strain. Surprisingly, SPI2 as well as the PhoPQ regulatory system affect intracellular trafficking in BM-DC without influencing bacterial proliferation (Niedergang *et al*., 2000; this study). This paradoxical situation will require further detailed investigation.

There are different contributions of the SPI2 system to the intracellular phenotype of *Salmonella*. (i) SPI2 functions are required for the avoidance of antimicrobial activities of phagocytes, i.e. the reactive oxygen intermediates generated by NADPH-oxidase (Vazquez-Torres *et al*., 2000) and reactive nitrogen intermediates generated by iNOS (Chakravortty *et al*., 2002). (ii) Intra-

cellular *Salmonella* interfere with cellular trafficking by means of the SPI2 system, and one phenotype linked to this function is the appearance of tubular aggregates of endosomes in infected epithelial cells (Garcia-del Portillo *et al*., 1993; Beuzon *et al*., 2000). There are no indications that SPI2 function is important for avoidance of antimicrobial activities in DC, and the killing of phagocytosed microbes by oxygen or nitrogen radicals has not been observed in DC (unpublished observations). However, antigen processing and presentation by DC are processes that are highly dependent on intracellular trafficking. Recent studies demonstrated the role of retrograde vesicle transport for antigen presentation by DC (Chow *et al*., 2002) and the function of microtubules as cytoskeletal routes of vesicle trafficking (Boes *et al*., 2002). In addition to the effect of intracellular *Salmonella* on the maturation of the SCV, it might be considered that these specific functions of DC are also modified by intracellular *Salmonella* to suppress the formation of an adaptive immune response.

Analysis of the interaction of *Mycobacterium* spp. indicated a similar intracellular fate, although specific activities of this pathogen in DC have not been reported. In contrast to macrophages, DC are not permissive for mycobacterial proliferation, but fail to kill intracellular mycobacteria. Bodnar *et al*. (2001) observed persistence of *Mycobacterium tuberculosis* in murine DC and a role for iNOS in controlling intracellular replication. Jiao *et al*. (2002) reported that *Mycobacterium bovis* BCG can persist for several weeks in infected DC, and that antigen presentation of DC is strongly reduced after BCG infection. It was suggested that a specialized pathogen-containing compartment is formed that imposes bacteriostasis. It is not understood how this effect is mediated, but one factor for bacteriostasis might be nutritional limitation for the intracellular pathogen that could equally affect intracellular *Salmonella*, *Mycobacterium* and other bacteria after uptake by DC.

In a recent study, the intracellular fate of *Legionella pneumophila* in BM-DC was analysed (Neild and Roy, 2003). *L. pneumophila* is able to modify its intracellular fate by the formation of a unique, endoplasmic reticulum-derived compartment in macrophages as well as in BM-DC. As shown here for *Salmonella*, *Legionella* does not replicate in BM-DC but is metabolically active. However, a significant difference between both pathogens is the functional requirement for virulence genes. A *Legionella* strain deficient in *dotA*, a component of a type IV secretion system, is reduced in proliferation in macrophages and killed in BM-DC.

The combined data from our and previous studies suggest that the pathogen-containing vesicle in DC is a unique compartment distinct from that in other phagocytic cells. This compartment does not allow proliferation of intracellular *Salmonella*; however, it is permissive for the biosynthesis and secretion of virulence proteins. This specialized compartment may be a consequence of the function of DC to sample, transport and present antigens. We speculate that this specialized compartment is used by intracellular pathogens for their distribution in the host organism. Intracellular persistence in DC might be a requirement for the systemic spread of a pathogen (Cheminay *et al*., 2002). Future experiments involving *in vivo* models of infection are required to determine the role of DC as possible vehicles for pathogens and reservoirs. In this respect, approaches to deplete the DC population *in vivo* might be instrumental (Jung *et al*., 2002). We also need to understand the specific role of the SPI2 virulence system of *S.* Typhimurium in DC and whether the activities of SPI2 interfere with DC-specific functions.

Our observations could also have important implications for the use of *S.* Typhimurium as a recombinant live vector for vaccination. Promoters of SPI2 genes are efficient in controlling the expression of foreign antigens in macrophages but also in DC, as reported here. Previous work demonstrated that T3SS can be used to deliver vaccine antigens into antigen-presenting cells (APC) (Rüssmann *et al*., 1998). The SPI2-encoded system appears also be useful for this approach and might allow a more specific delivery of vaccine antigens to APC. We are currently investigating the potential of the SPI2-encoded system for the presentation of vaccine antigens.

In conclusion, we have shown that *S.* Typhimurium is able to activate specific virulence factors after uptake by DC, although the bacteria are unable to proliferate. Translocation of a virulence protein was observed, and the maturation of the pathogen-containing vesicle is influenced by the SPI2 system. These observations reveal a novel form of host–pathogen interaction.

Experimental procedures

Bacterial strains and growth conditions

S. Typhimurium NCTC 12023 was used as the wild-type strain, and all mutant strains are isogenic to this strain. SPI2 mutant strains P2D6 (*ssaV*::mTn*5*) and P8G12 (*ssrB*::mTn*5*) are deficient in the type III secretion system and the regulatory system that activates the expression of structural SPI2 genes respectively (Shea *et al*., 1996). *S.* Typhimurium CS015 is a *phoP*-deficient strain (*phoP*-102::Tn*10d*-Cm) described previously (Miller *et al*., 1989). *Escherichia coli* laboratory strain HB101 was used as a non-pathogenic control strain. Bacteria were grown in Luria–Bertani (LB) broth or on LB agar under selection for resistance for carbenicillin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}) or chloramphenicol (30 μg ml^{-1}) where appropriate. PCN-P minimal media inducing SPI2 gene expression has been described previously (Deiwick *et al*., 1999). Cultures were grown in glass test tubes with aeration in a roller drum at 37°C if not otherwise indicated.

DNA biochemistry

The plasmid pFVP25.1 (Valdivia and Falkow, 1997) was used to visualize the bacteria by constitutive expression of GFP. The segregative plasmid pHSG422 was kindly provided by Dr Hashimoto-Gotoh, Kyoto, Japan (Hashimoto-Gotoh *et al*., 1981).

Plasmid p2777 for the expression of SseJ with a C-terminal fusion to the HA epitope tag and the constitutive expression of GFP was constructed as follows. Primers SseJ-Pro-For 5′-ccggaattcacataaaacactagcac-3′ and SseJ-HA-Rev2 5′-gcatcta gattaagcgtagtctgggacgtcgtatgggtattcagtggaataatgatgagc-3′ were used to amplify the *sseJ* gene with its promoter from genomic DNA of *S.* Typhimurium. The resulting product was digested with *Eco*RI and *Xba*I and subcloned into low-copy-number vector pWSK29 to yield p2684. Primers GFP-Pro-For-SacI 5′-gatgagctcgttcatgcgtgatgcaatg-3′ and GFP-Rev-SacI 5′-gat gagctccatttatttgtatagttc-3′ were used to amplify the GFP gene and a constitutive promoter from plasmid pFVP25.1. The product was digested with *Sac*I and cloned into the *Sac*I site of p2684 to yield p2777. p2777 was introduced into *S.* Typhimurium strains by electroporation. Regulated expression of the SseJ-HA fusion protein was analysed under *in vitro* conditions and was identical to previously described fusions proteins with the M45 epitope tag (Hansen-Wester *et al*., 2002; data not shown). The plasmid pLS824 is a pWSK29 derivative containing a fusion of the SPI2 promoter Pro_{sseA} to the GFP gene. *S.* Typhimurium [pLS824] expresses GFP under SPI2-inducing conditions *in vitro* (data not shown).

Preparation and culture of DC and macrophages

BM-DC were generated as described previously (Lutz *et al*., 1999; Cheminay *et al*., 2002) from C57BL/6 (Charles River Wiga). At day 8, cells were harvested, routinely yielding a population of more than 55% $CD11c^{high}$-positive cells. Cells were allowed to adhere to culture plates for at least 6 h. As described previously (Cheminay *et al*., 2002), fluorescence-activated cell sorting (FACS) on a MoFlo or the magnetic cell sorting (MACS) technique using CD11c MicroBeads (Miltenyi Biotech) was used in order to enhance purity of the CD11c-positive compartment and yielded a population of more than 90% $CD11c^{high}$-positive cells. The RAW 264.7 macrophage-like cell line was obtained from ATCC, and cells between passage number 8 and 25 were used for the experiments. Peritoneal macrophages (PMΦ) were prepared as described before (Chakravortty *et al*., 2002).

Antibodies and immunofluorescence

For immunostaining, the following antibodies were used at the indicated dilutions: rabbit α-mouse cathepsin D was a kind gift from Dr Pohlmann (Münster, Germany) and used at a dilution of 1:1000. Rat mAb α-mouse 2A1 was kindly provided by Dr N. Romani (Innsbruck, Austria) and was used at a dilution of 1:10. Rat mAb 1D4B against mouse LAMP-1 (CD107A) developed by Drs J. T. August and J. E. K. Hildreth was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA) and was used at a dilution 1:1000. Hamster mAB α-mouse CD11c (1:100) and biotinylated mAB α-mouse MCH II/1-AD (dilution 1:100) were purchased from BD Bioscience. Rat mAB α-HA epitope (Roche) was used at a dilution of 1:500.

Secondary antibodies (Dianova) were used as follows: goat α-rabbit conjugated to Cy3 was used at a dilution of 1:100, goat α-rat conjugated to Cy3 (dilution 1:100 and 1:1000), goat α-hamster conjugated to Cy5 (dilution 1:400), goat α-rabbit conjugated to Cy5 (dilution 1:100), and streptavidin conjugated to rhodamine Red-X was used at a dilution of 1:100.

For flow cytometry, the following antibodies were used according to the manufacturer's instructions (BD Bioscience) at the indicated dilutions or concentrations: α-FCRγII (CD16/CD32) (concentration 1 $\mu g \times 10^{-6}$ cells), hamster mAb α-mouse CD11c conjugated to PE (concentration 0.5 $\mu g \times 10^{-6}$ cells) and rat mAb α-mouse MHC II/I-Ad conjugated to fluorescein isothiocyanate (FITC; concentration 83 $ng \times 10^{-6}$ cells), rat mAb α-mouse CD86 (B7-2) conjugated to FITC (concentration 0.5 $\mu g \times 10^{-6}$ cells) and the isotype-matched controls hamster IgG group 1 conjugated to PE and rat IgG2a conjugated to FITC.

The fluid tracers Texas red–ovalbumin and Texas red–dextran (Molecular Probes) were used at concentrations of 150 $\mu g\ ml^{-1}$ and 50 $\mu g\ ml^{-1}$ respectively. Both endocytic markers were added to cells for 30 min, followed by washing for removal of non-internalized marker and incubation for 2 h before bacterial infection to allow the partitioning of markers to the compartments. The Live/Dead viability/cytotoxicity kit (Molecular Probes) was used according to the manufacturer's instructions to assay cell viability.

Bacterial infection of cultured cells

BM-DC and macrophages were infected with opsonized, stationary phase *S.* Typhimurium. Briefly, the multiplicity of infection (MOI) was adjusted by reading the culture density at the optical density of 600 nm, the bacteria were opsonized for 30 min with 20% normal mouse serum and added to the BM-DC. The MOI was confirmed by plating serial dilutions onto Mueller–Hinton plates. In order to synchronize the infection, the bacteria were centrifuged onto the BM-DC for 5 min at 500 *g*. The cells were incubated for 30 min at 37°C, 5% CO_2 if not otherwise stated. After infection, cells were treated with 100 $\mu g\ ml^{-1}$ gentamicin for 1 h, washed twice with PBS and incubated for the indicated periods of time in the presence of 10 $\mu g\ ml^{-1}$ gentamicin in complete RPMI medium for BM-DC or complete DMEM medium for RAW264.7 cells.

Analysis of intracellular GFP expression by FACS

Host cells were infected as described above with the indicated *S.* Typhimurium strains harbouring pLS824. After the indicated incubation times, the cells were lysed in PBS containing 0.5% Triton X-100 and incubated for 10 min with agitation to support lysis. Intracellular bacteria released by this method were immunostained using a primary antibody against *Salmonella* O5 and a secondary antibody. The samples were subjected to FACS analysis on a BD FACsCalibur.

Gentamicin protection assays of pHSG422 harbouring bacteria

Wild-type *S.* Typhimurium and mutant strain P2D6 harbouring pHSG422 were grown overnight in the presence of 50 $\mu g\ ml^{-1}$ carbenicillin at 30°C. To obtain stationary phase cultures containing less than one copy of pHSG422 per bacterial cell, a

procedure described by Benjamin *et al*. (1990) was modified. Briefly, 1 ml of a stationary phase culture grown at 30°C adjusted to an optical density at 600 nm of 1 was inoculated into 9 ml of LB, then grown for 2.5 h at 37°C. From this culture ($\approx 2 \times 10^8$ cfu ml^{-1}), 250 µl was opsonized in 1 ml of medium containing 20% normal mouse serum for 30 min. The infection was performed as described above. The infected cells were incubated at 39°C in an atmosphere of 5% CO_2. Cultivation at 39°C affected neither the viability of host cells as determined by the Live/Dead assay (Molecular Probes) nor their activation status as measured by the levels of MHC II and CD 86 expression (data not shown).

Immunofluorescence and transmission electron microscopy

For immunofluorescence, cells were fixed in paraformaldehyde, stained, mounted and analysed by confocal laser-scanning microscopy as described previously (Chakravortty *et al*., 2002). Images were processed using COREL DRAW 8.0. For transmission electron microscopy, infected cells were fixed with 2.5% glutaraldehyde for 2 h, washed twice with PBS, resuspended with PBS containing 2% gelatine and gently scraped off the wells, post-fixed with reduced osmium and prepared as described previously (Rittig *et al*., 1998).

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