Adhesin-dependent binding and uptake of *Salmonella enterica* serovar Typhimurium by dendritic cells

Aizhen Guo,^{1†} Melissa A. Lasaro,¹ Jean-Claude Sirard,^{2‡} Jean-Pierre Kraehenbühl² and Dieter M. Schifferli¹

Correspondence
Dieter Schifferli
dmschiff@vet.upenn.edu

¹University of Pennsylvania School of Veterinary Medicine, 3800 Spruce Street, Philadelphia, PA 19104, USA

²Swiss Institute for Experimental Cancer Research (ISREC), 1066 Epalinges, Switzerland

Salmonella enterica serovar Typhimurium can be internalized by immature dendritic cells (DCs). The interacting host and bacterial molecules initiating this process remain uncharacterized. The objective of this study was to investigate whether specific fimbriae are involved in the early step of binding and uptake of *Salmonella* by DCs. Type 1 fimbriated *S. enterica* serovar Typhimurium or recombinant *Escherichia coli* expressing the type 1 fimbriae showed a significantly greater ability to attach to murine bone-marrow-derived DCs than non-fimbriated bacteria. The FimH adhesin was required for efficient interactions with DCs, since fimbriated *fimH* mutants were impaired in both binding and internalization. Finally, the internalization involved a FimH-dependent process but did not require *sipB*, a gene essential for *Salmonella*-mediated invasion of mammalian epithelial cells. Collectively, these data suggest that the bacterial interaction of DCs through the type 1 fimbrial adhesin FimH is sufficient to target *S. enterica* serovar Typhimurium for cellular uptake.

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INTRODUCTION

Salmonella enterica serotypes are correlated with different types of human and animal disease syndromes such as bacteraemia, typhoid fever and enterocolitis. *S. enterica* serotype Typhimurium is a highly evolved pathogen that has adapted to colonizing and surviving for long periods in humans, mice and other animal hosts. The virulence is partially attributed to its capacity to invade host cells.

After oral infection, *S. enterica* serotype Typhimurium colonizes the small intestine and preferentially enters Peyer's patches M cells (Jepson & Clark, 2001). Thanks to the *Salmonella* type III secretion system (T3SS) encoded by *Salmonella* pathogenicity island 1 (SPI-1) (Galan, 2001; Lostroh & Lee, 2001), *Salmonella* induce their uptake by non-phagocytic cells, such as intestinal epithelial cells. How important epithelial cell invasion is *in vivo* for crossing the intestinal barrier remains questionable, SPI-1 deletion

mutants being still enteroinvasive (Murray & Lee, 2000). SPI-1 facilitates M cell invasion (Penheiter *et al.*, 1997), but is not essential for entry by this route (Clark *et al.*, 1998; Jepson & Clark, 2001). The invading *Salmonella* cells that have translocated through the intestinal epithelial layer reach the subepithelial compartment where they interact most efficiently with dendritic cells (DCs) that reside under M cells or in M cell pockets (Hopkins *et al.*, 2000). As a third uptake mechanism, CD18-expressing phagocytes were shown to transport *Salmonella* from the gastrointestinal tract to the bloodstream, suggesting a paracellular route of entry (Vazquez-Torres *et al.*, 1999; Vazquez-Torres & Fang, 2000). Such direct uptake has been visualized with intraepithelial DCs that send tight-junction-forming dendrites to the intestinal lumen (Niess *et al.*, 2005; Rescigno *et al.*, 2001), suggesting that CD18-expressing phagocytes are most probably DCs. DCs are found in the peripheral tissues, mainly as immature DCs that have high phagocytic and endocytic abilities. Immature DCs are widely spread in the lamina propria of the gut and can be recruited at the subepithelial site of infection through proinflammatory molecules, particularly chemokines expressed by the infected enterocytes (Didierlaurent *et al.*, 2002; Sierro *et al.*, 2001). Both host molecules and microbe-associated molecular pattern, such as lipopolysaccharide, peptidoglycan and flagellin, then participate in the maturation process of DCs. Maturing DCs lose their phagocytic properties and upregulate surface molecules, such as chemokine receptor

[†]Present address: National Key Laboratory of Agricultural Microbiology, Lab of Animal Virology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, 430070, PR China.

[‡]Present address: INSERM, U801, Institut de Pasteur de Lille, Institut de biologie, Groupe AVENIR d'Immunité Anti-Microbienne des Muqueuses, 1 rue du Pr Calmette – BP 447, 59021 Lille Cedex, France.

Abbreviations: DC, dendritic cell; FCS, fetal calf serum; PE, R-phycoerythrin.

CCR7 that initiates their migration to the mesenteric lymph node (Cheminay *et al.*, 2002), as well as major histocompatibility complex and co-stimulatory molecules (e.g. CD80, CD86 and CD40) that serve to activate naïve T cells (Wick, 2002, 2003). Thus unique maturation steps empower DCs to become most effective antigen-presenting cells in their role as specialized messengers linking innate to adaptive immunity.

Although the uptake of *S. enterica* serotype Typhimurium in DCs remains an intensive field of study, very little is known about the bacterial molecules involved in the earliest recognition steps. *S. enterica* serotype Typhimurium produces a variety of fimbrial adhesins that have been shown to be involved in bacterial binding in murine intestines (van der Velden *et al.*, 1998). Fimbriae are surface-exposed hair-like structures that consist of helically arranged protein subunits. Their adhesive properties are most frequently mediated by a minor tip subunit. The fimbrial subunits are typically encoded by a gene cluster, together with export, assembly and regulatory proteins. The current study investigated whether *Salmonella* fimbriae are involved in the initial interaction between bacteria and DCs. Our data provide evidence that the type 1 fimbriae, thanks to their adhesive subunit FimH, mediate the attachment of *S. enterica* serotype

Typhimurium to DCs, leading to a significantly increased SPI-1-independent bacterial internalization process.

METHODS

Bacterial strains and plasmids. *S. enterica* serotype Typhimurium and *Escherichia coli* strains and plasmids used in this study are detailed in Table 1. Bacteria were routinely grown on LB medium supplemented with the appropriate antibiotics. All the transformants were prepared by high-voltage electroporation. Static broth cultures were performed at 37 °C for 48 h to enrich for type 1 fimbriae. Expression of type 1 fimbriae was determined with antibodies, and expression of FimH and mannose-sensitive binding was screened by yeast cell (*Saccharomyces cerevisiae*) agglutination (Hancox *et al.*, 1998). *Salmonella* type 1 fimbriae were also expressed as recombinant organelles on the surface of *E. coli* strains ORN103 or AAEC189, both lacking their own mannose-sensitive type 1 fimbrial genes (Table 1; designated *fim* or *pil*). *E. coli* ORN103(pKT303) was essentially non-fimbriated, suggesting a polar effect on the transcription of downstream regulatory genes (Table 1). Therefore, a new *fimH* deletion mutant was constructed according to the following strategy. Long PCR was carried out to amplify all the type 1 fimbrial genes encoding the structural and biogenesis proteins (*fimA*–*fimF*) using pISF101 as the template, according to the manufacturer's protocol (Expand Long Template PCR; Roche Applied Science). The oligonucleotides were derived from the 5' end of *fimA* and a sequence complementary to 3' end of *fimF*. *Nde*I and *Bam*HI sites were introduced into the upper and lower primer, respectively

Table 1. Strains and plasmids used in this study

Strain or plasmid	Parental strain and relevant properties*	Source or reference
<i>S. enterica</i> serotype Typhimurium		
AJB3	χ4252 (Nal ^R), derived from SR-11	Bäumler <i>et al.</i> (1996a, c); Lockman & Curtiss (1992b)
AJB4	χ4253 (Nal ^R), derived from SR-11 Δ <i>fim</i>	Bäumler <i>et al.</i> (1996a, c); Lockman & Curtiss (1992b)
ST100	Typhimurium C5 <i>sipB</i> ::Tn5 (Km ^R)	Hermant <i>et al.</i> (1995); Hersh <i>et al.</i> (1999)
DMS1508	AJB3 <i>sipB</i> ::Tn5	This study
DMS1509	AJB4 <i>sipB</i> ::Tn5	This study
LBH4	LB5010 <i>fimH</i> ::Km ^R , derived from LT2	Hancox <i>et al.</i> (1998)
<i>E. coli</i>		
BL21(DE3)	F <i>ompT hsdS_B</i> (r _B [−] m _B [−]) <i>gal dcm</i> (DE3)	Novagen, EMD Biosci.
SE5000	MC4100 <i>recA</i> (Fim [−])	Silhavy <i>et al.</i> (1984)
ORN103	<i>thr1 leu6 thi1 (argF-lac) U169 xyl7 ara13 mtl2 gal6 rpsL fhuA2 minA minB recA13 Δ(pilABCD FE hyp)</i>	Maurer & Orndorff (1987)
AAEC189	MM294 Δ <i>lac recA endA Δfim</i> (r _K [−] m _K ⁺)	Blomfield <i>et al.</i> (1991)
Plasmids		
pISF101	pACYC184 <i>fimAICDHFZYWU</i> (Cm ^R)	Thankavel <i>et al.</i> (1999)
pKT303	pISF101 Δ <i>fimH</i> (polar effects on downstream <i>fim</i> genes)	Thankavel <i>et al.</i> (1999)
pET16b	Expression vector with a T7 promoter	Novagen
pAZ24	pET16b <i>fimAICDHF</i>	This study
pAZ25	pAZ24 Δ <i>fimH</i> (non-polar 33 bp deletion)	This study
pAZ36	pACYC184 <i>fimAICDF</i>	This study
pISF204	<i>fimHFZ</i> in Ap ^R plasmid	Hancox <i>et al.</i> (1998)
pISF211	pISF204 Δ <i>fimH</i>	Hancox <i>et al.</i> (1998)
pKKGFP	pKK223.3 GFP (Ap ^R)	Hopkins <i>et al.</i> (2000)
pGFPmut3.1	pUC GFPmut3.1 (Ap ^R)	BD Biosciences, Clontech
pAZ8	pLG339 GFPmut3.1 (Tc ^R)	This study

*Ap, Ampicillin; Cm, chloramphenicol; Km, kanamycin; Nal, nalidixic acid; Tc, tetracycline.

(upper primer, 5'-GGAATTCCATATGAGACATAAATTAATGAC-3'; lower primer, 5'-CGGGATCCTTACTAATTGTAATTGATCAG-3'). The product was cloned into pET16b to obtain pAZ24. A 33-bp internal segment of *fimH* in pAZ24 was removed by *StuI* restriction and self-ligation to obtain pAZ25. The deletion was confirmed by checking for the loss of an internal *ClaI* site. Plasmid pAZ24 or pAZ25 was introduced into BL21(DE3) by electroporation, and *fim* and *fimH* gene expression was checked after IPTG induction. Both transformants produced type 1 fimbriae, but only BL21(DE3)(pAZ24) agglutinated yeast cells. The fimbrial gene cluster of pAZ25 was cloned as a *BglII*-*BamHI* DNA fragment into the *BamHI* site of pACYC184, in the opposite orientation to the tetracycline resistance gene, to obtain pAZ36. *E. coli* AAEC189(pAZ36) was shown to express fimbriae constitutively without agglutinating yeast cells.

Preparation of antiserum to type 1 fimbriae. The *Salmonella* type 1 fimbriae expressed by *E. coli* SE5000(pISF101) were purified as described by Dodd & Eisenstein (1982). SDS-PAGE and Coomassie blue staining confirmed the preparation's purity. Anti-fimbrial antibodies were raised in rabbits (Cocalico Biologicals) with resulting serum ELISA titres of >100 000. The antibodies were shown to agglutinate type 1 fimbriated *Salmonella* or recombinant *E. coli* expressing *Salmonella* type 1 fimbriae.

DC preparation. Murine bone-marrow-derived DCs were prepared as described by Inaba *et al.* (1992) and Lutz *et al.* (1999). Briefly, 6- to 8-week-old BALB/c mice were sacrificed, and the femurs and tibias were harvested. Bone marrow was flushed out of the shafts and cells seeded into Petri dishes with complete RPMI 1640 media (Glutamax; Gibco) supplemented with 20 ng murine recombinant granulocyte/macrophage colony-stimulating factor ml^{-1} (rGM-CSF; Pepro Tech Inc. or R&D Systems), 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 50 μM β -mercaptoethanol, 100 U penicillin ml^{-1} , 100 μg streptomycin ml^{-1} and 100 μg gentamicin sulfate ml^{-1} . For some experiments, bone marrow was depleted of red blood cells, T and B cells before differentiation of precursors into DCs (Niedergang *et al.*, 2000). The cells were incubated at 37 °C in a humidified incubator with 5% CO_2 for either 6–7 days (Inaba *et al.*, 1992) or 10 days with media changes every 3 days (Lutz *et al.*, 1999). The non-adherent cells were harvested, and DCs were defined by expression of the surface markers (CD11c, MHC-II, CD40, CD80, CD86) using antibodies conjugated to FITC, R-phycoerythrin (PE) or biotin (Pharmingen; BD Biosciences). Isotype controls were FITC- and PE-labelled non-specific antibodies, and streptavidine-conjugated FITC or PE were used to detect biotinylated antibodies. The immature dendritic cells were characterized by high-level expression of CD11c (CD11c⁺), low- to intermediate-level expression of MHC II, low-level expression of CD80 and CD86, and no expression of CD40.

Microscopy. Bacterial adhesion to DCs was examined by light microscopy. DCs (3.3×10^5 cells ml^{-1}) in RPMI medium without FCS and antibiotics were mixed with PBS-washed *S. enterica* serotype Typhimurium or recombinant *E. coli* grown statically for 48 h at an m.o.i. of 100. Bacteria and DCs were incubated at 37 °C on a rotating wheel at 15 r.p.m. At various time points, slides were prepared with a cytospin centrifuge (800 r.p.m., 5 min), air-dried, fixed in 4% paraformaldehyde and Giemsa-stained. Bacteria-cell interactions were recorded by counting the number of bacteria co-localizing with DC in ≥ 10 fields per slide. For some experiments, DCs were pretreated with cytochalasin D (5 μg ml^{-1}) for 10 min at 37 °C before the addition of bacteria.

Interactions of *Salmonella* with DCs or HeLa cells. The standard invasion assay by gentamicin resistance (Elsinghorst, 1994; Lee *et al.*, 1992) was modified as follows. The bacteria (m.o.i. = 12.5) were added to DCs (1×10^6 – 2×10^6 cells) and incubated for 1 h at 37 °C on a rotating wheel. The extracellular bacteria were removed

by washing three times with RPMI1640 medium containing 50 μg gentamicin ml^{-1} . The cells were further incubated for 1 h in RPMI1640 medium containing 50 μg ml^{-1} gentamicin at 37 °C. DCs were then washed three times with PBS, lysed with 0.5% Triton X-100 in PBS for 10 min and live *Salmonella* counted by a standard plating technique. The colony numbers for the wild-type strain AJB3 were normalized to 100% for comparisons with the numbers of the other *Salmonella* strains. The human HeLa epithelial cell line (ATCC CCL-2) was cultured in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% FCS and 1 mM non-essential amino acids (Gibco) at 37 °C in an atmosphere containing 5% CO_2 . Monolayers were prepared by seeding 2×10^5 cells into each well of a 24-well tissue culture plate and incubating for 48 h at 37 °C in a humidified atmosphere of 5% CO_2 in air. Bacteria were added to the cells at an m.o.i. of 20:1 to 200:1. Bacteria were incubated with cell monolayers for 1 h at 37 °C in 5% CO_2 before being washed three times with PBS. Extracellular bacteria were killed by incubating the cells in medium containing 50 μg gentamicin ml^{-1} for an additional 90 min. After three PBS-washing steps, the cell monolayers were lysed with cold 0.1% Triton X-100 and viable counts were determined as described above.

Flow cytometry binding and uptake assays. Bacterial and fimbrial adhesion to DCs, as well as bacterial uptake by DCs, was examined by flow cytometry. Bacteria transformed with a GFP-expressing plasmid and other appropriate compatible plasmids (Table 1) were grown statically. Initial experiments were undertaken with high-copy-number pKKGFP-transformed *S. enterica* serotype Typhimurium. However, the percentage of fluorescent bacteria grown statically for 2 days was frequently under 50%, suggesting plasmid loss. Thus, later experiments took advantage of the low-copy-number and stable plasmid construct pAZ8. Although the fluorescence intensity of *Salmonella*-associated DCs was lower with pAZ8- than with pKKGFP-containing bacteria, all bacteria associated to DCs were detectable. Bacteria were collected by centrifugation, washed and resuspended in 4 °C PBS. Bacterial numbers were estimated by OD₆₀₀ and checked by colony counts (c.f.u. ml^{-1}). DCs were harvested and cell viability ($\geq 90\%$) was tested by Trypan Blue staining. Bacteria and DCs were incubated at 4 °C for various times at an m.o.i. of 50 with gentle end-to-end agitation. Unbound bacteria were removed from DCs by three to five washes in PBS. DCs were further incubated with anti-CD11c-PE antibodies for 30–60 min in the dark at 4 °C with gentle end-to-end agitation. After three to five washing steps, cells were resuspended in 300–500 μl cold RPMI 1640 for flow cytometry (FACScan or FACSCalibur with CellQuest pro software; BD Biosciences). For the binding inhibition assay, bacteria were incubated with DCs in the presence of 100 mM α -methyl mannoside for 1 h. To study the binding of type 1 fimbriae to DCs, purified fimbriae of strain AJB3 were biotinylated with sulfo-NHS-LC-Biotin (Pierce), according to the manufacturer's instructions. Biotinylated fimbriae were incubated in 2% FCS-PBS with DCs at 4 °C for 1 h. PE-conjugated streptavidin was used to probe the biotinylated fimbriae that remained attached to DCs after three to five washes.

The uptake of bacteria by DCs was also examined by flow cytometry. Bacteria and DCs were incubated for 30 min at 37 °C on a rotating wheel (m.o.i. = 50), washed three to five times and the cells fixed with 4% paraformaldehyde. The cells were then divided into two groups, one group being permeabilized with 0.5% Triton X-100 at room temperature for 10 min. Cells were then labelled with rabbit anti-*Salmonella* O antigen factor 4 antiserum (Difco, BD Diagnostic Systems), sequentially followed by biotin-conjugated goat anti-rabbit IgG and FITC-conjugated streptavidin or Alexa Fluor488 anti-rabbit IgG (Invitrogen, Molecular Probes). The DCs were probed with PE-conjugated anti-CD11c IgG. The non-permeabilized cells were used to

determine the extracellular bound bacteria, while the permeabilized cells were interpreted as representing all the DC-associated bacteria, namely both the extra- and intracellular *Salmonella*.

Statistical analysis. Groups of data were compared with the unpaired Student's *t*-test, using an unequal variance *t*-test when needed, as determined by the F-test (Zar, 1974). Probability (*P*) values of less than 0.05 indicated that the groups were significantly different.

RESULTS

Adhesion of type 1 fimbriated *S. enterica* serotype Typhimurium to DCs

To characterize interaction of DCs with bacteria, cells were incubated at 37 °C, followed by extensive washing, Giemsa staining and analysis by light microscopy. *S. enterica* serotype Typhimurium grown statically interacted better with DCs than bacteria grown overnight or in exponential growth phase under aerated conditions (data not shown). Since *Salmonella* produce large amounts of type 1 fimbriae when grown statically, we hypothesized that the type 1 fimbriae were involved in bacterial association to DCs. The interactions of *S. enterica* serotype Typhimurium AJB3 and its non-fimbriated *fim* derivative AJB4 with DCs were compared over time (Fig. 1). The association of wild-type bacteria with DCs was low or not detectable at 5 min and increased gradually over time. The interaction of the fimbriated bacteria was about three- and fivefold more effective at 30 and 60 min, respectively, compared to the *fim*

mutant (Fig. 1b). Type 1 fimbriated *Salmonella* bound significantly better to cytochalasin D-treated DCs at 30 min [0.72 ± 0.10 *Salmonella* Fim⁺ per DC versus 0.10 ± 0.03 *Salmonella* Fim⁻ per DC (means $\pm$ SE, $P < 0.01$)] and 60 min (2.97 ± 0.54 *Salmonella* Fim⁺ versus 0.20 ± 0.06 *Salmonella* Fim⁻, $P < 0.01$), indicating that the interaction was independent of actin polymerization and phagocytosis. To specifically assess binding of bacteria to DC, flow cytometry assays were undertaken at 4 °C. Treatment at 4 °C impairs any internalization events and was observed to slow down DC aggregation. Bacteria-associated DCs were identified by using GFP-expressing bacteria and PE-anti-CD11c-labelled DCs. Moreover, to exclude any effect of the *Salmonella* type III secretion system SPI-1 in attachment, we constructed fimbriated and non-fimbriated bacteria deficient for SipB. After a 30 min bacteria/DC incubation, the type 1 fimbriated strain associated with most DCs (92 %), whereas no significant binding was detected with the non-fimbriated bacteria (Fig. 1c). Taken together, the flow cytometry data supported the microscopic observation that *S. enterica* serotype Typhimurium type 1 fimbriae are critical for bacterial adhesion to DCs.

Fimbriae-specific binding to DCs

To confirm that the binding of bacteria to DCs was mediated by type 1 fimbriae, experiments were performed with an *E. coli* *fim* operon deletion mutant (ORN103) that was complemented with either plasmid pISF101 harbouring *Salmonella* type 1 fimbrial genes or pKT303, which is

Fig. 1. Type 1 fimbriae-dependent binding of *S. enterica* serotype Typhimurium to DCs. (a) Giemsa-stained DCs and *S. enterica* serotype Typhimurium AJB3 and AJB4 (*fim*) incubated at 37 °C for 60 min at an m.o.i. of 100. Bars, 40 μ m. (b) Bacteria were incubated with DCs at 37 °C for 5, 30 and 60 min. The numbers of bacteria associated with DCs were counted microscopically. The bars represent the mean $\pm$ SE values for AJB3 (filled bars) and AJB4 (shaded bars) of three experiments. There were statistically significant differences for the 30 and 60 min values ($P < 0.01$). (c) Binding of type 1 fimbrial *S. enterica* serotype Typhimurium DMS1508 (*sipB*). Bacteria were transformed with the GFP-expressing plasmid pAZ8. Only CD11c⁺-gated cells (DCs) are shown. DCs and bacteria were incubated for 30 min at an m.o.i. of 50 at 4 °C. The histograms represent control non-infected DCs (dotted line), DCs with DMS1509(pAZ8) (*fim sipB*) (thin grey line) or DMS1508(pAZ8) (*sipB*) (thick black line). The data are from one experiment and represent reproducible data from three experiments.

pISF101 with a deletion that eliminates fimbriation. *E. coli* expressing the *Salmonella* type 1 fimbriae interacted significantly better with DCs than the non-fimbriated *E. coli* after 30 or 60 min incubation at 37 °C (Fig. 2a). Binding assays with GFP-expressing *E. coli* showed that 93 % of the DCs were associated with fimbriated *E. coli* (Fig. 2b). In contrast, DCs incubated with *E. coli* (pKT303) did not bind any bacteria (Fig. 2b). Biotinylated purified type 1 fimbriae were found to bind in a dose-dependent manner to DCs (data not shown). Altogether, type 1 fimbrial expression was needed for efficient bacterial adhesion to the DCs.

FimH-dependent binding to DCs

The type 1 fimbriae of *Salmonella* bind to mannosylated glycoproteins on mammalian cells, and this interaction is mediated by their minor adhesive subunit FimH (Hancox *et al.*, 1998). To determine whether the adhesin FimH mediated bacterial binding to DCs, *fimH* mutants producing wild-type strain levels of fimbriae were studied. Whether *S. enterica* serotype Typhimurium *fimH*⁻ bound to DCs was evaluated by flow cytometry. As described by Hancox *et al.* (1998), *S. enterica* serotype Typhimurium LBH4, which has an insertionally inactivated *fimH* gene, produced type 1 fimbriae and agglutinated yeast cells only when complemented with pISF204 carrying *fimHFZ*. When complemented with pISF211, which contains *fimFZ*, only type 1 fimbriation was restored. Most of LBH4(pISF204) bound to DCs, as shown by flow cytometry, whereas the binding of LBH4 or LBH4(pISF211) was not significant and similar to the bacteria-free control (Fig. 3, top panel). The role of FimH in recognizing DC was also investigated with recombinant *E. coli* AAEC189(*fim*) complemented with plasmids pISF101(*fim*⁺) or pAZ36(*fimH*). Both *E. coli* AAEC189(pISF101) and AAEC189(pAZ36) produced similar amounts of type 1 fimbriae, but only the former bacteria agglutinated yeast cells and bound significantly to DCs, as shown in Fig. 3 (bottom panel). Moreover, the binding of type 1 fimbriated *E. coli* ORN103(pISF101) (Fig. 4) or *S. enterica* serotype Typhimurium AJB3 (data not shown) to DCs was impaired by α -methyl mannoside. Nearly the whole population of DCs (93 %) associated with *E. coli* ORN103(pISF101) in the absence of the inhibitor, whereas most DCs (76 %) were free of bacteria in the presence of α -methyl mannoside. This result demonstrated that α -methyl mannoside efficiently blocks fimbriae-mediated bacterial binding to DCs, and thus that the type 1 fimbriae of *S. enterica* serotype Typhimurium most probably recognize one or more mannosylated glycoprotein receptor(s) on the DCs. This result also confirmed that the mannose-specific adhesin FimH was needed for DC recognition by *Salmonella* type 1 fimbriae.

Fimbriae-mediated uptake by DCs

We investigated whether type 1 fimbriae-dependent binding of *Salmonella* to DCs influences intracellular uptake. Both strains AJB3 and AJB4(*fim*) express SPI-1 T3SS that mediates cellular uptake by mammalian cells. To determine

Fig. 2. Type 1 fimbriae-dependent binding of recombinant *E. coli* to DCs. (a) Type 1 fimbriated *E. coli* ORN103(pISF101) and non-fimbriated *E. coli* ORN103(pKT303) were incubated with DCs at 37 °C for 5, 30 or 60 min. The numbers of bound bacteria per DC were counted microscopically. The bars represent the mean $\pm$ SE values for ORN103(pISF101) (filled bars) and ORN103(pKT303) (shaded bars). There were statistically significant differences for the 30 and 60 min values ($P < 0.01$). (b) Bacterial binding to DCs analysed by flow cytometry. Only CD11c⁺-gated cells (DCs) are shown. DCs and bacteria were incubated for 30 min at an m.o.i. of 50 at 4 °C. The histograms represent control non-infected DCs (dotted line), DCs with type 1 fimbriae-deficient ORN103(pKT303, pKKGFP) (thin grey line) or type 1 fimbriae-expressing ORN103(pISF101, pKKGFP) (thick black line).

whether SPI-1 was involved in internalization by DCs, a *sipB* mutant that expressed type 1 fimbriae (DMS1508) was included in the study. Standard invasion assays (gentamicin

Fig. 3. FimH determines *S. enterica* serotype Typhimurium binding to DCs. DCs and bacteria were incubated at 4 °C for 30 min, at an m.o.i. of 50. Only CD11c⁺-gated cells (DCs) are shown. Top panel: complementation of *S. enterica* serotype Typhimurium LBH4(*fimH*) with plasmids carrying (pISF204) or lacking (pISF211) *fimH*. Bacteria were transformed with GFP-expressing plasmid pAZ8. The histograms represent control non-infected DCs (dotted line), or DCs with LBH4(pAZ8) (thin black line), LBH4(pISF211, pAZ8) (grey line) or LBH4(pISF204, pAZ8) (thick black line). The former three histograms overlapped. Bottom panel: recombinant *E. coli* AAEC189 expressing non-adhesive or adhesive *S. enterica* serotype Typhimurium type 1 fimbriae from plasmid pAZ36 or pISF101, respectively. The histograms represent control non-infected DCs (dotted line), DCs with *E. coli* AAEC189(pAZ36, pAZ8) (thin grey line) or DCs with AAEC189(pISF101, pAZ8) (thick black line).

Fig. 4. Inhibition of FimH-mediated bacterial adhesion to DCs with α -methyl mannoside. Bacteria were transformed with the GFP-expressing high-copy-number plasmid pKKGFP. DCs and bacteria were incubated as described in the legend to Fig. 3. The histograms represent control DCs stained with the appropriate isotype subclass (dotted line), or DCs incubated with recombinant *E. coli* ORN103 expressing adhesive *S. enterica* serotype Typhimurium type 1 fimbriae from plasmid pISF101, in the presence (grey thin line) or absence (black thick line) of 100 mM α -methyl mannoside.

resistance) indicated that in comparison to the wild-type strain AJB3 (relative invasion of 100 %), significantly fewer AJB4(*fim*) cells were taken up by the DCs (5.5 ± 4.3 %, relative to AJB3, mean $\pm$ SE of three independent experiments). In contrast, the uptake of DMS1508(*sipB*) (67 ± 6 % of AJB3 invasion) was significantly less affected than the uptake of AJB4. These results indicated that SPI-1 does not affect the uptake of *Salmonella* by DCs as much as the type 1 fimbriae do. In contrast, DMS1508(*sipB*) was internalized significantly less efficiently by HeLa cells when compared to strain AJB3 (2.7 ± 0.6 % of AJB3 invasion), suggesting that *Salmonella* invasion of epithelial cells relied more on SPI-1 than *Salmonella* invasion of DCs. Strain AJB4(*fim*) was also significantly less invasive with HeLa cells (0.6 ± 0.3 % of AJB3 invasion). This result was anticipated, since AJB4(*fim*) had been previously shown to be significantly less adhesive and, as a consequence, less invasive than its parental strain AJB3 (Bäumler *et al.*, 1996c). The cellular uptake of *Salmonella* was further analysed by flow cytometry using O-antigen-specific antibody to label the bacteria and DC permeabilization to track the bacterial localization. Indeed, when DC membranes were permeabilized, both extra- and intracellular bacteria were detected by the antigen-specific antibody. The data are presented in Table 2 as the means of

Table 2. Apparent percentage of DCs with intra- and extracellular *Salmonella* (flow cytometry data)

Values are means $\pm$ SE of four independent experiments. All the data presented are based on apparent numbers, since DCs associated with one (or a few) bacteria might have escaped detection.

Strain	Total DCs counted*	Total DCs with associated <i>Salmonella</i> (%)†	DCs with extracellular <i>Salmonella</i> (%)	DCs with intracellular <i>Salmonella</i> (%)‡
AJB3	23 600	66.7 $\pm$ 10.4 ($P < 0.01$)§	22.5 $\pm$ 4.9 ($P < 0.05$)§	44.2 $\pm$ 8.7 ($P < 0.01$)§
DMS1508	26 945	71.7 $\pm$ 3.1 ($P < 0.0001$)§	30.4 $\pm$ 5.1 ($P < 0.05$)§	41.3 $\pm$ 3.4 ($P < 0.0001$)§
AJB4	28 228	10.4 $\pm$ 2.4	5.3 $\pm$ 3.0	5.1 $\pm$ 0.9

*DCs defined as cells positive for expression of CD11c.

†Permeabilized DCs with either internal, external, or both internal and external *Salmonella*.

‡DCs carrying only intracellular *Salmonella* (calculated as associated minus extracellular bacteria).

§*t*-Test comparisons with AJB4 data; there were no statistically significant differences between the AJB3 and DMS1508 data.

four experiments, one typical experiment being shown in Fig. 5. It should be noted that all the presented data are based on apparent numbers, since DCs associated with one (or a few) bacterium might have escaped detection. Among the DCs that were associated with the fimbriated *Salmonella* AJB3 (66.7 % of all DCs), a majority contained solely intracellular bacteria (Table 2). In contrast, only 10.4 % of DCs were associated with the non-fimbriated *Salmonella* AJB4. There were also fewer DCs with only intracellular

AJB4 bacteria (5.1 % of all DCs). Most DCs (71.7 %) were associated with the fimbriated *sipB* mutant DMS1508, and a majority of these DCs carried *Salmonella* exclusively intracellularly. DC association of the non-fimbriated *fim sipB* double mutant (DMS1509) was similar to that found for the non-fimbriated *fim* strain AJB4 (data not shown). Taken together, these data showed that type 1 fimbriae-mediated *Salmonella* association with DCs was the major determining factor for bacterial internalization.

Fig. 5. Type 1 fimbriae-dependent internalization of *S. enterica* serotype Typhimurium by DCs. Strain AJB3 (c, d), AJB4 (*fim*) (e, f) or DMS1508 (*sipB*) (g, h) were incubated with DCs for 30 min at an m.o.i. of 50 (37 °C). (a) and (b) represent mock-infected DCs. The DCs either were (b, d, f, h) or were not (a, c, e, g) permeabilized. Bacteria were labelled with anti-O antigen antibodies, followed by FITC-conjugated secondary antibodies. Only CD11c⁺-gated cells (DCs) are shown. The percentages represent the ratios of DCs carrying extracellular bacteria (a, c, e, g) or both extracellular and intracellular bacteria (b, d, f, h). The data are from one experiment and represent reproducible data from four experiments.

DISCUSSION

Following oral ingestion, *S. enterica* serotype Typhimurium reaches the intestines, where it first makes contact with M cells, enterocytes and DC. There is evidence that DCs sending luminal dendrites or DCs within Peyer's patches interact closely with *Salmonella* (Niess *et al.*, 2005; Rescigno *et al.*, 2001; Salazar-Gonzalez *et al.*, 2006). Although studies have identified that specific fimbriae preferentially target the *Salmonella* towards M cells or intestinal epithelial cells (Bäumler *et al.*, 1996a, b), the bacterial ligand initiating the interaction with DCs is not known. This study showed that the type 1 fimbriae are extremely efficient at mediating the binding and internalization of *S. enterica* serotype Typhimurium into murine DCs. Whether binding directs bacterial uptake by creating the necessary conditions for other interactions to occur or by triggering bacterial internalization itself remains to be determined.

Microscopic analysis and flow cytometry showed that type 1 fimbriated *S. enterica* serotype Typhimurium, in contrast to an isogenic *fim* mutant, binds to bone-marrow-derived DCs, suggesting specificity of the fimbriae for one or more cell receptor(s). The binding specificity was confirmed with recombinant *E. coli* expressing *S. enterica* serotype Typhimurium type 1 fimbriae and with isolated fimbriae, which bound to DCs in a dose-dependent manner. The fimbrial structure consists of different types of subunits, all being encoded on the *fim* gene cluster, together with the chaperone-usheer system that coordinates the export and assembly of the subunits (Clegg & Swenson, 1994). The helically arranged structural subunit FimA forms most of the polymerized fimbrial shaft, whereas the binding determinant for mannosylated proteins is a minor structural subunit designated FimH (Hancox *et al.*, 1998). Here, type 1 fimbriae-mediated *Salmonella* binding to DCs was shown to be mediated by the FimH adhesin. Moreover, this interaction was dependent on mannosylated residues. Strikingly, FimH-mediated binding was also required for efficient bacterial internalization. Under our experimental conditions, the SPI-1-encoded mammalian cell invasion machinery, did not significantly affect *S. enterica* serotype Typhimurium binding to DCs and was dispensable for DC invasion.

Type 1 fimbriae have previously been shown to confer *S. enterica* serotype Typhimurium adhesion to a variety of mammalian epithelial cells such as human Hep-2, HeLa, and small intestinal and bladder epithelial cell lines (Bäumler *et al.*, 1996c; Boddicker *et al.*, 2002; Hancox *et al.*, 1998; Thankavel *et al.*, 1999), and to contribute to the bacterial invasion of some of these cells (Bäumler *et al.*, 1997; Ernst *et al.*, 1990; Horiuchi *et al.*, 1992; van der Velden *et al.*, 1998). More recently, *S. enterica* serotype Typhimurium colonization of the murine intestine by biofilm formation was shown to require type 1 fimbriae carrying an adhesive allelic FimH (Boddicker *et al.*, 2002). However, unlike epithelial cell internalization, which requires an active bacterial invasion machinery, the observed uptake of *S.*

enterica serotype Typhimurium by DC was SPI-1-independent, as described recently by others (Kiama *et al.*, 2006; Petrovska *et al.*, 2004; van der Velden *et al.*, 2003). A recent *in vitro* study with a macrophage cell line showed that the route of entry of SPI-1-induced, non-opsonized and complement- or IgG-opsonized *Salmonella* influenced their vacuolar surroundings and intracellular gene expression (Drecktrah *et al.*, 2006). *Salmonella* uptake by murine DCs is greatly enhanced by serum complement (Eriksson *et al.*, 2003). What mediated *Salmonella* binding and uptake remained unknown. The novel finding of this study is that the type 1 fimbriae-mediated binding of *S. enterica* serotype Typhimurium to DCs concomitantly targets the bacteria to the intracellular compartment of these professional migratory cells. The potential effect of fimbrial lectins on the phagocytic process of DCs was not evaluated in other studies (Drecktrah *et al.*, 2006; Eriksson *et al.*, 2003; Petrovska *et al.*, 2004; van der Velden *et al.*, 2003). DCs are known to recognize and take up bacteria and bacterial molecules via different groups of receptors, including C-type lectin and pattern recognition receptors (Geijtenbeek *et al.*, 2004). One or more unidentified mannosylated DC glycoprotein(s) might represent a new class of phagocytic DC receptors that interact with bacterial lectins, such as the type 1 fimbriae of *Salmonella*. Whether this lectinophagocytic interaction (Ofek & Sharon, 1988) directs uptake by a distinct mechanism of bacterial entry and triggers the formation of a new vacuolar compartment, as described for type 1 fimbriated *E. coli* with mast cells or macrophages (Baorto *et al.*, 1997; Shin *et al.*, 2000), is a question that requires further studies.

SPI-1 gene products are involved in the invasion of intestinal epithelial cells (Galan, 2001) and participate in M cell uptake (Penheiter *et al.*, 1997). However, studies have suggested that SPI-1 is not essential for crossing the intestinal barrier *in vivo* (Clark *et al.*, 1998; Jepson & Clark, 2001; Murray & Lee, 2000), and variable use of the SPI-1 proteins has been observed between host species and *Salmonella* serotypes (Morgan *et al.*, 2004). Here, *Salmonella* uptake by murine DCs was mainly determined by FimH-mediated processes and not by its SPI-1-mediated invasive attributes. Although SPI-1 gene expression might not be optimal under the growth conditions used, recent microarray data showed that SipB and other SPI-1 genes are transcribed in stationary phase (Adkins *et al.*, 2006). Moreover, since the SPI-1 apparatus and plasma membrane ruffling are activated within 2 min of macrophage exposure to *S. enterica* serotype Typhimurium grown to stationary phase (Alpuche-Aranda *et al.*, 1994), it is unlikely that our interpretation of the data was significantly affected by the experimental conditions used for the adhesion assays presented. Nevertheless, it remains possible that SPI-1 plays some role in *Salmonella* uptake *in vivo*.

That *Salmonella enterica* serotype Typhimurium express their type 1 fimbriae *in vivo* was clearly shown in an earlier study which compared the bacterial colonization of murine

organs two days after oral administration of a wild-type strain together with its isogenic *fim* mutant (Lockman & Curtiss, III, 1992a). Higher numbers of the former strain were isolated from reticulo-endothelial tissues such as the spleen and liver. In contrast, more *fim* mutants were found in the blood. This observation is consistent with the more efficient uptake of *fim*⁺ *Salmonella* by DCs, together with the previously described extraintestinal systemic distribution of *Salmonella* by CD18-expressing phagocytes (Vazquez-Torres *et al.*, 1999) that were later postulated to be mainly DCs (Niess *et al.*, 2005; Rescigno *et al.*, 2001). In spite of the increased uptake of type 1 fimbriated *S. enterica* serotype Typhimurium by DCs, such strains were previously found to be less virulent than isogenic *fim* mutants in mice, as determined by their LD₅₀ (Lockman & Curtiss, III, 1992b; van der Velden *et al.*, 1998). How the type 1 fimbriae attenuate *S. enterica* serotype Typhimurium in mice is not clear. Whether the targeting of *Salmonella* towards DCs amplifies an antibacterial inflammatory response *in vivo*, as expected from *in vitro* studies (Marriott *et al.*, 1999; Yrlid *et al.*, 2000), or whether *S. enterica* serotype Typhimurium-activated resident DCs induce a non-inflammatory response with IL-10 secretion in mice, as observed with epithelial-cell-conditioned human DCs *in vitro* remains to be determined (Rimoldi *et al.*, 2005). Moreover, DC targeting is expected to affect the adaptive immune response (Bueno *et al.*, 2005). In any case, the amplified interaction of type 1 fimbriated *S. enterica* serotype Typhimurium with DCs might play an important role in attenuating *Salmonella*, as observed with isogenic *fim*⁺ and *fim*⁻ strains (Lockman & Curtiss, III, 1992b; van der Velden *et al.*, 1998). By acting as 'anti-virulence' factors (Foreman-Wykert & Miller, 2003), type 1 fimbriae might be beneficial to the intestinal establishment of *S. enterica* serotype Typhimurium as a persistent inhabitant of immunocompetent adult mice or carriers.

In this study, we showed that the expression of type 1 fimbriae optimized *Salmonella* uptake by DCs. Efficient bacterial internalization depended on the mannose-specific adhesin FimH and did not require SipB, an essential gene of the T3SS of *Salmonella* involved in epithelial cell invasion. The role of type 1 fimbriae-mediated binding and uptake of *Salmonella* by DCs and the importance of these interactions for infection remain to be investigated in the mammalian host.

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