Yersinia pseudotuberculosis induces transcytosis of nanoparticles across human intestinal villus epithelium via invasin-dependent macropinocytosis

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Crohn's disease is characterized by a defect in intestinal barrier function, where bacteria are considered the most important inflammation-driving factor. Enteric bacteria, including *E. coli* and *Yersinia* spp, affect tight junctions in enterocytes, but little is known about bacterial effects on the transcellular pathway. Our objective was to study the short-term effects of *Y. pseudotuberculosis* on uptake of nanoparticles across human villus epithelium. Monolayers of human colon epithelium-derived Caco-2 cells and biopsies of normal human ileum were studied after 2 h exposure to *Y. pseudotuberculosis* expressing (inv+) or lacking (inv-) the bacterial adhesion molecule, invasin. Transepithelial transport of fluorescent nanoparticles (markers of transcytosis) was quantified by flow cytometry, and mechanisms explored by using inhibitors of endocytosis. Epithelial expressions of β 1-integrin and particle uptake pathways were studied by confocal microscopy. The paracellular pathway was assessed by electrical resistance (TER), mannitol flux, and expression of tight junction proteins occludin and claudin-4 by confocal microscopy. Inv+ *Y. pseudotuberculosis* adhered to the apical surface of epithelial cells and induced transcytosis of exogenous nanoparticles across Caco-2 monolayers (30-fold increase, $P < 0.01$) and ileal mucosa ($268 \pm 47\%$ of control; $P < 0.01$), whereas inv- bacteria had no effect on transcytosis. The transcytosis was concentration-, particle size- and temperature-dependent, and possibly mediated via macropinocytosis. *Y. pseudotuberculosis* also induced apical expression of β 1-integrin on epithelial cells. A slight drop in TER was seen after exposure to inv+ *Y. pseudotuberculosis*, whereas mannitol flux and tight junction protein expression was unchanged. In summary, *Y. pseudotuberculosis* induced apical expression of β 1-integrin and stimulated uptake of nanoparticles via invasin-dependent transcytosis in human intestinal epithelium. Our findings suggest that bacterial factors may initiate transcytosis of luminal exogenous particles across human ileal mucosa, thus presenting a novel mechanism of intestinal barrier dysfunction.

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Accumulating evidence demonstrates that genetic, environmental and immunological factors contribute to the pathogenesis of Crohn's disease (CD).¹ Enteric bacteria have been proposed as the most important inflammation-driving environmental factor in inflammatory bowel disease (IBD)^{2–4} including CD, and the link between bacterial components and CD has lent strong support from the discoveries of disease-promoting gene polymorphisms in components of innate immunity toward bacteria, eg, NOD2^{5,6} and

ATG16L1.^{7,8} Patients with CD demonstrate defect barrier function, with a combined increase in paracellular permeability,^{9,10} transcellular uptake of antigens^{11,12} and augmented mucosal immune responses to antigens and bacteria.^{13,14} It is therefore recognized that barrier function and immune responsiveness to environmental antigens are important in the disease process.^{1,15–17}

The mechanisms that link enteric bacteria and barrier function in the initiating steps of intestinal inflammation

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have in recent years been partly elucidated. For example, individuals with NOD2 alterations show increased intestinal permeability,¹⁸ and alterations in secretion of the antimicrobial peptides, α -defensins¹⁹ and DMBT1.²⁰ Moreover, CD-related *E. coli* strains can enhance the expression of CEACAM-6, which increases invasion of *E. coli* into ileal mucosa mediated by type-1 pili-dependent adhesion.²¹ Other enteric bacteria that have been implicated in CD pathogenesis include *Yersinia* spp. Two independent groups have shown increased frequency of DNA of *Y. enterocolitica* and *Y. pseudotuberculosis* in intestinal samples of CD patients,^{22,23} and several case reports have linked *Yersinia*-induced ileitis and lymphadenitis to the early phases of clinical CD.^{24,25} *Yersinia pseudotuberculosis* expresses two different virulence-associated adhesion proteins anchored to the outer membrane, ie, invasin and YadA. Together, these two proteins mediate initial adhesion, uptake and translocation of *Yersinia* across the intestinal epithelium.^{26,27} Invasin is known to exert its effect via a high-affinity interaction with β 1-integrin, which is expressed on the luminal surface of M-cells (epithelial cell type specialized in antigen and bacterial uptake),^{28,29} but only on the basolateral aspect of villus epithelial cells.

We have previously shown that *Y. pseudotuberculosis* affects the paracellular route in model epithelia via a β 1-integrin-dependent alteration of F-actin.³⁰ However, the effect of *Yersinia* spp. on the transcellular route across human intestine has not been previously addressed.

The aim of the present study was therefore to investigate whether transcytosis across human absorptive intestinal epithelium is affected by interaction with *Y. pseudotuberculosis* strains. We used YadA-deficient *Y. pseudotuberculosis* strains with intact invasin or lacking invasin (inv+ or inv-), respectively.³⁰ Both of the bacterial strains upregulated the apical expression of β 1-integrins on Caco-2 cells and human ileal tissues, whereas only inv+ *Y. pseudotuberculosis* induced transcytosis of nanoparticles across the epithelia. Our results show for the first time that binding of invasin-expressing *Y. pseudotuberculosis* to apically localized β 1-integrins induces the uptake of nanoparticles via macropinocytosis and transcytosis across villus epithelium in human ileal tissue.

MATERIALS AND METHODS

Materials

Cell culture media and supplements were purchased from Sigma Chem. Co, unless otherwise stated. The human colon carcinoma cell line, Caco-2 (clone 1, passage $x + 7$ to $x + 11$), was kindly provided by Dr Maria Rescigno (University of Milano-Bicocca, Milano, Italy)³¹ and the *Y. pseudotuberculosis* strains by Professor Åke Forsberg (Umeå University and FOI, Umeå, Sweden). pEGFP was purchased from Clontech, Transwell polycarbonate inserts (12 mm diameter, 3 μ m pore size) from Costar-Invitrogen and Matrigel Basement Membrane Matrix from BD Biosciences. Yellow-green and red fluorescently labeled carboxylated polystyrene particles

(FluoSpheres), Alexa Fluor-647-phalloidin and Alexa-488-mouse-anti-IgG1 from Molecular Probes-Invitrogen, and the β 1-integrin antibody (clone J10) from Biogenex.

Bacterial Strains and Growth Conditions

YadA-deficient *Y. pseudotuberculosis* strains were used: (1) YpIII(pIB102) (inv+) and (2) Yp100(pIB103) (inv-). Strains were transformed with pEGFP, a plasmid carrying the gene for green fluorescent protein (GFP) and grown overnight on a rotary shaker at 26°C in brain heart infusion media supplemented with 5 mM EGTA, 20 mM MgCl₂, 100 μ g/ml ampicillin and 50 μ g/ml kanamycin. The overnight culture was diluted in fresh media and grown for 1 h at 26°C and 2 h at 37°C. Transcription of the pEGFP plasmid was induced by addition of 100 μ g/ml of isopropyl- β -D-1-thiogalacto-pyranoside.

Cell Culture

Caco-2 cells were grown at 37°C in a 10% CO₂ water-saturated atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 1% (v/v) nonessential amino acids and 1% (v/v) L-glutamine, supplemented with antibiotics (100 U/ml penicillin, 100 μ g/ml streptomycin). Cells were seeded in Transwell inserts coated with Matrigel (12 μ g protein/cm²) and grown for 18–22 days. The last two days before incubation with bacteria, cell culture medium without antibiotics was used.

The cell monolayers were equilibrated in Hank's buffered salt solution (HBSS) for 20 min and transepithelial electrical resistance (TER) was measured at 37°C with a volt-ohm meter (EvoHm; World Precision Instruments, Sarasota, FL, USA), before experiments, after bacterial infection and after transport studies. Monolayers with TER < 175 Ω cm² before experiments were excluded. Integrity of monolayers was measured using [¹⁴C]mannitol, a hydrophilic paracellular marker molecule.³²

Bacterial Incubations

The bacterial strains were suspended in HBSS to a final concentration of 1×10^2 , 1×10^4 or 1×10^6 bacteria/ml. The Caco-2 cells were first exposed to bacteria for 2 h at 37°C. To determine the number of intracellular bacteria, a gentamicin protection assay was performed as described earlier.²⁸ Briefly, cultures were washed with PBS and incubated for 1 h in the presence of gentamicin (100 μ g/ml) to kill remaining extracellular bacteria. Cells were lysed with 1% Triton X-100 in PBS, and intracellular viable bacteria assessed by plating serial 10-fold dilutions on plates with kanamycin (50 μ g/ml) and ampicillin (100 μ g/ml).

Bacterial translocation across Caco-2 cells monolayers was determined by plating basolateral samples as above. Plates were incubated for 48 h at 26°C before counting of colony forming units (CFU).

Confocal Microscopy Studies

After exposure to bacteria, cell monolayers and tissues, respectively, were incubated with red-fluorescent 200-nm particles for 45 min, washed in cold HBSS and fixed for 20 min with 2% formaldehyde in PBS, pH 7.4. F-actin was stained by AlexaFluor-647-phalloidin. For studies in monolayers of β 1-integrin, claudin 4 and occludin expression, filters were fixed after the exposure to bacteria. Samples were boiled for 10 min in 10 mM citrate, pH 6 (β 1-integrin, claudin 4) to unmask antigenic epitopes, cooled and rinsed in distilled water. All monolayers were then permeabilized with 0.2% Triton-X-100 for 5 min. After blocking with 1% bovine serum albumin (BSA), samples were stained with mouse anti- β 1-integrin (500 μ g/ml), anti-claudin 4 (3 μ g/ml) or anti-occludin (10 μ g/ml) for 2 h at room temperature and incubated with goat-anti-mouse or goat-anti-rabbit IgG1 antibodies conjugated to Alexa 488 for 1 h at room temperature. Samples incubated with isotype- and concentration-matched primary antibody were used as negative controls. The cell monolayers were then mounted in Fluoromount-G (SouthernBiotech). For studies of β 1-integrin, claudin 4 and occludin expression in ileal tissue, samples were fixed in 4% formalin and mounted in Cryomount. Cryosections (10 μ m) were cut using a microtome (Leica, Wetzlar, Germany) and dried at room temperature on glass slides overnight. Cryosections were then immersed in boiling citrate buffer (10 mM, pH 6) for 40 min (β 1-integrin, claudin-4) or incubated with protease solution for 10 min at 37°C (occludin), as recommended by the manufacturers of the antibodies. After cooling to room temperature, the sections were rinsed several times in PBS. Cryosections were then blocked in PBS containing 5% BSA and stained with anti- β 1-integrin (500 μ g/ml) for 2 h at room temperature, anti-claudin 4 (3 μ g/ml) or anti-occludin (10 μ g/ml) for 1 h at room temperature. The primary antibodies were detected with secondary goat-anti-mouse or goat-anti-rabbit IgG1 antibodies conjugated to Alexa 488. Monolayers and cryosections were then examined using confocal laser scanning microscopy (BioRad Radiance 2000 MP with LaserSharp 2000 software). The β 1-integrin staining was quantified by measuring the mean intensity in a defined area in the apical part of the epithelial cells (apical to the nucleus) across multiple sections of the specimens.

Particle Transport Across Caco-2 Cell Monolayers

Cell monolayers were exposed to *Y. pseudotuberculosis* inv+ and inv- strains in a concentration of 1×10^6 CFU/ml, for 2 h before addition of nanoparticles. The particle transport was studied at 37, 20 and 4°C, respectively, in HBSS supplemented with 1% FCS. A solution of yellow-green fluorescent particles (200 or 500 nm) was added apically at a final concentration of 2.5×10^9 particles/ml, and samples drawn basolaterally at regular intervals to monitor particle transport. The amount of transported particles was quantified by flow cytometry (FACScan, BD and Cytomics FC500 MPL, BeckmanCoulter). To determine the pathway involved in

the nanoparticle, transport cells were incubated with the following substances after exposure to bacteria. 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA; 50 μ g/ml) was used as an inhibitor of macropinocytosis. Chlorpromazine (60 μ mol/ml) and methyl- β -cyclodextrin (2.5 mmol/ml) were used as inhibitors of clathrin-mediated endocytosis and caveolar endocytosis, respectively. Nystatin (50 μ g/ml) was used as an inhibitor of lipid-rafts mediated endocytosis, including caveolae and macropinocytosis. Cell monolayers were preincubated for 20 min with each specific inhibitor before particles were added to the monolayers. Inhibitors were added to the apical and basolateral side and were present during the whole experiment.

Particle Transport across Human Ileum after Exposure to Bacteria

Human ileum biopsies were obtained during routine colonoscopy follow-up after local treatment for colonic polyps in six otherwise healthy patients (four men, two women; age range 45–66 years), as approved by the Regional Committee of Human Ethics in Linköping. The human tissues were immediately transported to the laboratory in ice-cold oxygenated Krebs buffer, pH 7.4, examined under dissection microscope to assure that only tissue without areas of follicle-associated epithelia (FAE) were studied,³³ and mounted in modified Ussing chambers³⁴ (Harvard Apparatus Inc.). The mucosal compartments were filled with 10 mM mannitol in Krebs buffer, whereas 10 mM glucose was used in the serosal compartment. Both sides were continuously oxygenated with 95% O₂/5% CO₂ and circulated by gas flow. Transmucosal potential difference (PD), short circuit current and electrical resistance were assessed using two pairs of electrodes (Ag/AgCl with agar-salt bridges and platinum electrodes, respectively) for assessment of tissue viability as previously described.^{33,35} Before transport experiments chambers were equilibrated for 20 min to achieve steady-state conditions in PD. Live *Y. pseudotuberculosis* (inv+ or inv-) were added to the mucosal side at a final concentration of 1×10^8 CFU/ml. After two hours, the bacterial suspension was removed and replaced with red fluorescent 200 nm particles at a final

Table 1 Transepithelial resistance during transport experiment

Time (h) ^a	0	2	4
Control (n = 136)	276 $\pm$ 42	290 $\pm$ 52	257 $\pm$ 46
Inv+ (n = 137)	273 $\pm$ 38	263 $\pm$ 42	191 $\pm$ 40*
Inv- (n = 13)	244 $\pm$ 27	246 $\pm$ 36	228 $\pm$ 38

^aThe integrity of the cells was controlled three times during the experiments: at the beginning (t = 0 h), after bacterial exposure (t = 2 h) and after particle exposure at the end of the experiment (t = 4 h). Values are expressed in Ω cm² (average $\pm$ s.d.).

*P < 0.05 as compared to controls.

Figure 1 Expression of tight junction proteins, occludin and claudin 4 in cell monolayers and ileal tissue after 2 h exposure to *Y. pseudotuberculosis* inv+ and inv-, respectively. Monolayers and tissue not exposed to bacteria were used as controls. (a) Digitally acquired XZ stack sections (upper panel) and optical XY cross sections (lower panel) of Caco-2 monolayers, showing expression of occludin in controls (left panel) and after exposure to inv- (middle panel) and inv+ (right panel). (b) Digitally acquired XZ stack sections (upper panel) and optical XY cross sections (lower panel) of Caco-2 monolayers, showing expression of claudin 4 in controls (left panel) and after exposure to inv- (middle panel) and inv+ (right panel). (c) Expression of occludin in optical cross sections of ileal tissue, controls (left panel), after exposure of inv- (middle panel) and inv+ (right panel). (d) Expression of claudin 4 in optical cross sections of ileal tissue, controls (left panel), after exposure of inv- (middle panel) and inv+ (right panel). (× 63 objective, × 2 zoom.)

concentration of 2.5×10^9 particles/ml. Background particle transport was determined in tissues that were not exposed to bacteria. Translocation of particles was measured after additional 2 h, by analyzing the serosal buffer in a Varian Eclipse™ fluorescence reader at 580/620 nm. In a second set of experiments, EIPA ($100 \mu\text{M}$) was added to the serosal side, after exposure to inv+ *Y. pseudotuberculosis*. After 20 min incubation, particles were added, samples withdrawn after 2 h, and translocation of particles analyzed as described above.

Statistics

Particle transport is expressed as mean $\pm$ s.e.m. The differences between treated groups were analyzed using nonparametric one-tailed Mann–Whitney tests. Bacterial internalization was evaluated with one-way analysis of variance in combination with Bonferroni's multiple comparison tests.

RESULTS

Cell Monolayer and Tissue Integrity

Previous studies in our laboratory have shown that TER values above $175 \Omega\text{cm}^2$ represents an intact barrier function of the cell monolayer.^{36,37} In agreement with this the cell monolayers expressed TER values within the given range during the experiment. Similarly, the excised ileal human tissue maintained viability during the experiment as confirmed by a starting PD of at least -0.2 mV and a PD that remained at a level of $>75\%$ of initial value.^{35,37} Thus,

neither the bacteria nor the nanoparticles affected the integrity of the cell monolayers or the tissue.

Paracellular Pathway

Following 2 h of incubation with the *Y. pseudotuberculosis* (inv– or inv+) at concentrations of 1×10^6 bacteria/ml, followed by 2 h of exposure to nanoparticles, a small decrease in TER was observed for the inv+ exposed cells compared to unexposed controls (Table 1). The transport of mannitol, a marker commonly used for paracellular transport, however did not change significantly during the bacterial incubation ($6.7 \times 10^{-7} \pm 1.4 \times 10^{-7} \text{ cm/s}^7$ as compared to controls ($6.0 \times 10^{-7} \pm 0.65 \times 10^{-7} \text{ cm/s}^7$).

To further elucidate the effects of inv– or inv+ *Y. pseudotuberculosis* on the paracellular pathway, the expression of tight junction proteins occludin and claudin 4 were studied. (Figure 1a–d). As shown by confocal microscopy occludin remained at the junctional complex after infection with the two *Y. pseudotuberculosis* strains in human ileal tissue as well as in monolayers. Similarly, distribution of claudin 4 at the lateral cell margins remained equal to the control after infection with bacteria, showing that the staining pattern of these tight junction proteins did not change during the 2 h incubation with bacteria.

Localization of *Y. pseudotuberculosis* and Nanoparticles in Caco-2 Cell Monolayers and Intestinal Villus Tissue

The localization of the GFP-expressing inv+ and inv– *Y. pseudotuberculosis* and the red-fluorescent nanoparticles in the cell monolayers (Figure 2) and in the ileal tissues (Figure 3)

Figure 2 Visualization of *Y. pseudotuberculosis* and particles in Caco-2 cell monolayers. Cells were exposed to bacteria during 2 h. Particles were added and after 45 min the cells were rinsed and fixed. The Caco-2 cells were stained for F-actin (blue), the bacteria were identified by their GFP-expressing properties (green) and the particles by their fluorescent label (red). (a) Apical images and (b) optical cross sections of the Caco-2 cell monolayers. In the unexposed (Control) monolayers and inv– exposed cells no attached bacteria and only small numbers of particles were seen on the apical surface of the cells, whereas in the inv+ -exposed cell monolayers both attached bacteria (large arrows) and surface-bound and internalized particles (small arrows) were found ($\times 63$ objective, $\times 3$ zoom).

Figure 3 Visualization of *Y. pseudotuberculosis* and particles in human intestinal villus epithelium. Ileal mucosal tissues were mounted in Ussing chambers and exposed to bacteria during 2 h. Particles were added and after 45 min tissues were rinsed and fixed. White = F-actin, green = *Y. pseudotuberculosis*, red = nanoparticles ($\times 63$ objective, $\times 4$ zoom). Internalized particles (small arrows) were found only in tissues exposed to inv+ *Y. pseudotuberculosis*.

were investigated by confocal microscopy. Only inv+ *Y. pseudotuberculosis* remained adherent to the cell monolayers after washing, suggesting that invasin was needed for binding to the cell surface (Figure 2a, right panel). Optical cross-sections revealed a very small number of bacteria inside or on the basolateral side of the cells. The nanoparticles were found inside the cell monolayers exposed to inv+ *Y. pseudotuberculosis* (Figure 2b, right panel) and could not be observed between the cells. However, no particle uptake or transport could be identified in untreated monolayers or in monolayers exposed to inv- *Y. pseudotuberculosis* (Figure 2a and b, middle panels). The results from the corresponding experiments in excised human ileal tissues (Figure 3) were similar to those obtained with the monolayers. Ileal mucosa exposed to inv+ *Y. pseudotuberculosis* displayed significant binding of

the bacteria and nanoparticles to the apical surface of epithelial cells. Nanoparticles transported across the epithelia to lamina propria were also identified, indicative of trans-epithelial transport (Figure 3, lower panel). In tissue exposed to inv- *Y. pseudotuberculosis* only a few bacteria and nanoparticles could be identified on the apical cell surface (Figure 3, middle panel), whereas no attached or transcytosed nanoparticles were found in the untreated tissues (Figure 3, upper panel).

Quantification of Nanoparticle Transport across Caco-2 Monolayers and Human Ileum

The number of fluorescent nanoparticles transported by the Caco-2 monolayers gradually increased with the number of inv+ *Y. pseudotuberculosis* (10^2 , 10^4 or 10^6 bacteria/ml;

Figure 4a). The largest amount of transported nanoparticles was obtained after exposure to the 10^6 bacteria/ml concentration, which was used in the subsequent experiments. After exposure to inv- *Y. pseudotuberculosis*, only a very small transepithelial transport of nanoparticles was observed, supporting the notion that the induction of nanoparticle transport required invasin (Figure 4b). The results obtained in Caco-2 cells were further confirmed in ileal tissues. Exposure to inv+ *Y. pseudotuberculosis* resulted in a significant increase in particle translocation (to $268 \pm 47\%$ of control), whereas inv- did not (Figure 4c).

Invasin-Induced Particle Transport is Size and Temperature Dependent

The effect of inv+ *Y. pseudotuberculosis* exposure on nanoparticle transport was dependent on the size of the particles. Although the increase in the number of transported particles was 77-fold for the 200 nm particles, the number of transported nanoparticles having a diameter of 500 nm increased only 12-fold (Figure 4d). However, when recalculated to the transported volume, the total volume of the transported 500 nm particles was fourfold higher than the volume transported by the 200 nm particles.

In order to elucidate whether the particle transport induced by inv+ *Y. pseudotuberculosis* was mediated by an energy-dependent process we investigated the transport at 4 and 20°C. At 4°C, the transport was only 7% of the transport occurring at 37°C at 120 min and similar to the unexposed controls. The same pattern could also be observed at 20°C, where transport decreased by 50% suggesting that the nanoparticle transport required an energy-dependent process (data not shown).

Induction of $\beta 1$ -expression in Caco-2 Cells and Human Ileum

In control Caco-2 cell monolayers, $\beta 1$ -integrin expression was mainly localized to the cell borders below the tight junctions (Figure 5a), which is in agreement with the reported staining pattern in the human villus epithelium³⁸ and MDCK cells.³⁰ After exposure to inv+ *Y. pseudotuberculosis*, the expression of $\beta 1$ -integrin increased and was redistributed to the apical cell membrane (Figure 5a and b). It has previously been shown that an increased expression of $\beta 1$ -integrin in the apical membrane is due to increased maturation of existing precursors and hence, redistribution of the protein, rather than an elevated production of $\beta 1$ -integrin.^{38,39} An equivalent pattern was observed after exposure to inv- *Y. pseudotuberculosis* (Figure 5a and b), suggesting that other factors than invasin contributed to the altered $\beta 1$ -integrin expression and distribution. To assure that the interaction between *Y. pseudotuberculosis* and $\beta 1$ -integrins was in agreement with previous reports,⁴⁰ we investigated bacterial uptake into the Caco-2 cells, using a gentamycin protection assay.³⁴ The results showed that inv+ but not inv- bacteria were taken up to a low, but significant

Figure 4 The transport of nanoparticles across Caco-2 monolayers and human intestinal villus epithelium was studied after 2 h exposure to *Y. pseudotuberculosis* strains expressing (inv+) or lacking (inv-) invasin. (a) Effects of increasing concentrations inv+ on 200 nm particle transport across cell monolayers ($n=4-5$). (b) Effects of invasin on particle transport across Caco-2 cells ($n=4-5$). (c) Particle transport across human intestinal villus epithelium after exposure to inv+ and inv- *Y. pseudotuberculosis* strains, respectively ($n=3-4$). (d) Cell monolayers were exposed to inv+ and the transport of 200 nm and 500 nm particles was studied across Caco-2 cells ($n=3-4$). Statistically significant differences are indicated by * $P<0.05$ and ** $P<0.01$.

extent, comparable to that reported previously (Table 2). These results confirm that there is a functional invasin-dependent internalization of the bacteria in Caco-2 cells. No transport of

bacteria across the Caco-2 cell monolayers was however observed during the 2-h incubation period (data not shown).

In human ileal tissue a more pronounced increase in expression and apical redistribution of β 1-integrin could also be observed after exposure to inv+ *Y. pseudotuberculosis* (Figure 5c and d). A qualitative difference between the redistribution induced by the inv+ and inv- bacteria was also detected. Although staining of tissue exposed to inv- *Y. pseudotuberculosis* showed a more even distribution of β 1-integrin in the epithelial cells in a pattern similar to the control tissue (Figure 5c), exposure to inv+ *Y. pseudotuberculosis* generated a strong uniform staining of β 1-integrin at the apical aspect of the epithelial cells.

Invasin-dependent Particle Transport Reduced by Endocytosis Inhibitors

The transport of nanoparticles in monolayers exposed to inv+ *Y. pseudotuberculosis* was investigated, as previously described, after incubation with chlorpromazine, methyl- β -cyclodextrin, nystatin and EIPA. Chlorpromazine, known to function as a calmodulin-antagonist and most commonly used as an inhibitor of clathrin-mediated endocytosis,⁴⁰ reduced the transport of particles significantly and the inhibition was nearly 100% compared to the transport occurring at 37°C after exposure of inv+ *Y. pseudotuberculosis*. Methyl- β -cyclodextrin and Nystatin deplete cholesterol from the plasma membrane and are used as inhibitors of caveolae and macropinocytosis,^{42,43} and had an almost equal inhibitory effect on the particle transport in Caco-2 cells (Table 3). Similarly, EIPA used as a selective inhibitor of macropinocytosis^{41–43} decreased the transport to the same extent and inhibition was 80% of the inv+ values (Figure 6). Together these data support that the inv+ *Y. pseudotuberculosis* induced transport of particles are primarily mediated by macropinocytosis. In agreement with this, the nanoparticle transport was reduced in ileal tissue to 32% of inv+ in the presence of EIPA (Figure 6).

DISCUSSION

Previous studies using other models and longer times of exposure have shown that *Y. pseudotuberculosis* affects the paracellular route in model epithelia. To our knowledge, we are here the first to show that short-term exposure to the enteroinvasive bacteria *Y. pseudotuberculosis* can induce a substantial increase in transcytosis of nanoparticles in

intestinal villus epithelium, whereas the paracellular pathway was only modestly affected during these experimental conditions. Earlier *in vitro* studies have demonstrated a transformation of epithelial phenotype to M cell-like cells in Caco-2 cells co-cultured with lymphocytes isolated from Peyer's patches or with Raji B cells.^{37,45} We have also shown that human FAE has a very high capacity for uptake of nanoparticles and bacteria compared with villus epithelium.^{33,36} Moreover, previous *in vivo* studies in rodents demonstrated an increased transcytosis across FAE after microbial challenge, explained either by induction of the M cell phenotype and/or an increased transport rate in already present M cells.^{46,47} In the present study we showed that inv+ *Y. pseudotuberculosis* rapidly altered the transcytosis pattern of Caco-2 monolayers in complete absence of lymphocytes or lymphocyte-derived products. Moreover, we verified this

Table 2 Internalization of viable *Y. pseudotuberculosis* by Caco-2 cells

Strain	Incubation time (h)	No of internalized bacteria ^a	Internalization (%)
Inv+	1	7 ± 6	0.09
Inv–	1	0 ± 0	0.00
Inv+	2	19 ± 3 ^b	0.24
Inv–	2	2 ± 2 ^c	0.02
Control	4	0 ± 0	0.00

^aCFU ± s.e.m., n = 4.

^bSignificantly changed compared to control ($P < 0.001$).

^cSignificantly changed compared to inv+ 2 h ($P < 0.01$).

Table 3 Particle transcytosis during 60 min in *Yersinia* inv+ exposed Caco-2 cells in the presence of endocytosis inhibitors

Inhibitor (concentration)	% Inhibition ^a
Nystatin (50 µg/ml)	76 (***)
Chlorpromazine (60 µM)	99 (**)
Methyl- β -cyclodextrin (2.5 mM)	80 (*)
EIPA (50 µM)	77 (***)

^aValues are expressed as % inhibition compared to inv+ exposed cells.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Figure 5 β 1-Integrin expression and quantification in Caco-2 cells and human intestinal villus tissue. Monolayers and tissues were exposed to inv+ and inv- *Y. pseudotuberculosis* during 2 h followed by immunofluorescence staining for β 1-integrin, the adhesion molecule for invasins. (a) Digitally acquired XZ stack sections of Caco-2 cell monolayers (upper panel) and optical XY cross sections (lower panel) in control (left panel), cells exposed to inv- (middle panel) and inv+ (right panel). Insets in lower panels show high-power XY cross sections at the level of microvilli, corroborating the increased intensity of apical β 1-integrin expression in the XZ sections. (b) Quantification of fluorescence intensity on the Caco-2 cells; (c) Optical cross sections of human ileal tissue, showing the expression of β 1-integrin in control (left panel), and after exposure to inv- (middle panel) and inv+. (d) Quantification of fluorescence intensity of β 1-integrin expression in the apical part of the villus epithelium. Green = β 1-integrin (Alexa-488). Statistically significant differences are indicated by * $P < 0.05$ and ** $P < 0.01$.

Figure 6 Particle transport across Caco-2 cells (a) and human intestinal villus epithelium (b) after 2 h exposure to *Y. pseudotuberculosis* inv+ in the presence of the pharmacological macropinocytosis inhibitor 5-(N-ethyl-N-isopropyl) amiloride (EIPA; 50 μ M). In (a) a control group was included at each time-point to monitor batch-to-batch variations in background particle transport and in (b) tissue not exposed to bacteria were included to monitor background particle transport (control). Bars show mean values $\pm$ s.e.m., ($n = 4-6$). Statistically significant differences are indicated by * $P < 0.05$ and ** $P < 0.01$.

rapidly inducible invasin-dependent transport in villus epithelium of human ileum, which implicated bacterial binding to $\beta 1$ -integrins as an important step in the induction of transcytosis. *Y. pseudotuberculosis* induced increased expression or redistribution of $\beta 1$ -integrin on Caco-2 cells and ileal villus tissues mounted in Ussing chambers. This is intriguing, as $\beta 1$ -integrins are normally not expressed on the surface of absorptive epithelial cells.³⁸ The increase was detectable already after 2 h of exposure to the bacteria, which is compatible with earlier reports in rodents, where an increased number of M cells has been detected in ileal loops already 1 h after inoculation with *Streptococcus pneumoniae* R36a.⁴⁷ Our investigation was focused on the cellular responses in non-phagocytic cells after exposure to *Y. pseudotuberculosis*, but it is possible that an increased transcytosis also can be seen after exposure to other enteroinvasive bacteria. In support of this notion, Lyons *et al* recently showed that *Salmonella typhimurium* transcytoses flagellin via a SPI2-mediated vesicular transport across polarized epithelial cells. Transcytosed flagellin then triggers the production of proinflammatory cytokines via the activation of Toll-like receptor 5, thereby contributing to the intestinal inflammation known to be caused by *S. typhimurium*.⁴⁸

In agreement with what we report here, Lyons *et al* show that flagellin is incorporated in vesicles and transcytosed across the epithelium without being dependent on bacterial invasion. In addition, several bacterial species are known to interact with $\beta 1$ -integrin⁴⁹ and some species, such as *Listeria*, share some common strategies for cellular uptake.⁵⁰ However, it remains to be elucidated by future studies whether these have the possibility to induce transcytosis of microparticles.

The nanoparticle transport was temperature-dependent, indicating that the process required an active ATP metabolism in the cells. To further elucidate the transport mechanisms, cells were treated with different pharmacological endocytosis inhibitors. Chlorpromazine, methyl- β cyclodextrin and nystatin all decreased the transport of nanoparticles across monolayers. Chlorpromazine is known to function as a calmodulin antagonist and has mainly been used as an inhibitor of clathrin-mediated endocytosis.⁴⁰ However, calmodulin is also known to interact with several small GTPases including Rac-1,⁵¹ which is known to stimulate actin filament reorganization and membrane ruffling^{52,53} processes essential for macropinocytosis. Methyl- β -cyclodextrin is known to extract cholesterol from the plasma membrane and has mostly been used as an inhibitor of caveolae-mediated endocytosis.⁵⁴ However, depletion of cholesterol from the plasma membrane has also been shown to inhibit the localization of activated Rac-1 to the plasma membrane, and hence the stimulation of actin filaments and membrane ruffling.⁵⁵ In agreement with this treatment with methyl- β -cyclodextrin resulted in a decreased particle transport. In addition, nystatin, also known to remove cholesterol from the plasma membrane,^{42,43} significantly reduced particle transport across cell monolayers. Together these data suggested that the transport of particles was mediated via macropinocytosis.

Macropinocytosis is a challenging mechanism to study because no specific markers or drugs are available that interfere with this process. It is, however known that this particular pathway acquire proteins common to other endocytotic pathways and it is therefore not surprising that the above-mentioned inhibitors affects macropinocytosis as well.⁵⁶ Lately, amiloride analogues have been used as selective inhibitors for this pathway. It has been suggested that amiloride, as a potent inhibitor of the plasma membrane Na^+/H^+ exchange protein⁵⁷ changes the intracellular pH and thereby induces alterations in the cytoskeleton⁵⁸ or other processes related to macropinocytosis.⁵⁹ To further elucidate if the *Y. pseudotuberculosis* inv+ induced transport of particles is mediated via macropinocytosis we used EIPA as a selective inhibitor. EIPA significantly reduced the particle transport across Caco-2 cells as well as in human villus tissue.

In other words, these results propose that the inv+ bacteria induced an active energy-dependent transport process, possibly via macropinocytosis, across the epithelium. Future studies will need to be focused on further elucidating the signaling pathways involved. One possibility is that

Yersinia via injection of various Yop proteins can affect the Rho proteins, RhoA, Rac1 and Cdc42,^{52,60,61} that have been implicated as important regulators in the trafficking of vesicles during phagocytosis and macropinocytosis.^{52,53} Alternatively, *inv*⁺ *Yersinia* (but not *inv*[−]) activate MAP-kinases,⁶² among them p38 MAPK, which in turn can promote endocytosis.⁶³

The dysregulation of the mucosal immune response in the pathogenesis of IBD involves microbial factors,¹ but may also include nanoparticles. In Western diets a high number (>10¹² per day) of microparticles (100–700 nm in diameter) are ingested daily. These have previously been considered immunologically inert, but accumulating evidence suggests that ingested microparticles are taken up by the intestinal mucosa and induce an immunological response.⁶⁴ It was shown that conjugates of titanium oxide microparticles and lipopolysaccharide increase secretion of IL-1 β in intestinal mucosal organ cultures.⁶⁵ Moreover, a higher microparticle-induced release of IL-1 β was seen in IBD explants.⁶⁶ The microparticles seem to act as adjuvants,⁶⁷ and may thereby aggravate inflammatory responses toward bacterial antigens in the intestinal lumen.

It has generally been believed that nanoparticles as well as *Yersinia* are only taken up at FAE, and recently a dysfunction of FAE leading to increased antigen exposure and a local immune response has been implicated in CD.^{16,68,69} The present study shows that *inv*⁺ *Y. pseudotuberculosis* can indeed induce a rapid increase in transport of nanoparticles in Caco-2 monolayers and human ileal tissues devoid of FAE. These results give further support to the notion that absorptive epithelial cells under the influence of external stimuli like enterobacteria can attain FAE-like properties, ie, start to sample antigens or exogenous, potentially proinflammatory particles from the intestinal lumen. This 'uncontrolled' sampling in villus epithelium represents a novel mechanism of barrier dysfunction that in the predisposed host may be involved in mucosal pathophysiology.

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CONFLICT OF INTEREST

None to declare.

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