Salmonella typhimurium transcytoses flagellin via an SPI2-mediated vesicular transport pathway

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Summary

Apical colonization of polarized epithelia by *Salmonella typhimurium* results in translocation of flagellin to the basolateral membrane domain, thus enabling activation of toll-like receptor 5 (TLR5)-mediated pro-inflammatory gene expression. Such flagellin transcytosis occurred without a change in epithelial permeability to 40 kDa FITC dextran, did not require bacterial motility and was independent of transepithelial movement of intact bacteria. Flagellin transcytosis was blocked at 20°C, suggesting dependence on vesicular transport consistent with results from confocal microscopy that showed flagellin independent of bacteria inside epithelial cells. Furthermore, vesicles isolated from *S. typhimurium*-infected epithelia were highly enriched in flagellin. Flagellin transcytosis was dependent upon genes of *Salmonella* pathogenicity island (SPI)-2, which alter vesicular trafficking, but independent of SPI-1 that mediates bacterial invasion. Furthermore, such SPI-2 mutants were unable to mediate the localization of flagellin into intracellular vesicles consistent with flagellin transcytosis mediated by a *S. typhimurium* take-over of host vesicle trafficking pathways. As a result of their inability to transcytose flagellin, apical colonization by SPI-2 mutants induced substantially less epithelial IL-8 secretion than wild-type strains suggesting that such SPI-2 mediated transcytosis of flagellin plays a role in the pathogenesis of the mucosal inflammation characteristic of human Salmonellosis.

Key words: Toll-like receptor 5 (TLR5), Inflammation, IL-8, Cell polarity

Introduction

The clinical manifestations of human Salmonellosis result largely from the innate immune response elicited by this pathogen. Specifically, *Salmonella typhimurium* colonization of the intestinal epithelium induces the epithelium to secrete chemokines that recruit immune cells, especially polymorphonuclear leukocytes (McCormick et al., 1998a; McCormick et al., 1998b; McCormick et al., 1993). Such an innate immune inflammatory response generally results in clearance of the infection albeit resulting in substantial suffering in the host (Gewirtz et al., 2002a). A primary means by which this pathogen is recognized by intestinal epithelial cells is via the detection of flagellin monomers by toll-like receptor 5 (TLR5) (Gewirtz et al., 2001a). Although flagellin monomers with the potential to activate TLR5 are released by both commensal *Escherichia coli* strains and pathogens such as *S. typhimurium* (Gewirtz et al., 2001b; Moors et al., 2001), TLR5 expression appears to be limited to the basolateral surface of the intestinal epithelium; therefore only those bacteria with flagellin crossing the epithelium will activate this pro-inflammatory signaling pathway. We have observed that *S. typhimurium*, but not commensal *E. coli* can transcytose flagellin in this manner across the epithelium thus activating TLR5-mediated pro-inflammatory gene expression and thereby causing, at least in part, the gastroenteritis induced by this microbe (Gewirtz et al., 2001b). The goal of this study was to elucidate the mechanism by which *S. typhimurium* is able to transcytose flagellin across polarized epithelia. Using a variety of biochemical, cell biological and physiological techniques we show here that flagellin transcytosis occurs by a transcellular pathway mediated by vesicular trafficking and is dependent upon the function of the *Salmonella* pathogenicity island (SPI)-2.

Materials and Methods

Materials

The following *S. typhimurium* strains were used in this study: SL3201 wild-type parent and aflagellate *fliC/fljB* mutant (Schmitt et al., 1996); KK104 wild-type parent and *fliD* mutant were provided by K. Kutsukake (Hiroshima University, Japan) (Yokoseki et al., 1996); SL1344 wild-type parent and isogenic SPI-1 (invA) and SPI-2 (SSaR) were provided by B. Finlay (University of British Colombia, Vancouver, Canada); wild-type 12023 and SPI-2 mutants (ssaV, sseB) were provided by David W. Holden (London, UK) (Hindle et al., 2002). *E. coli* F-18 is a commensal clinical isolate that was previously described (Cohen et al., 1983). All reagents were from Sigma, unless otherwise stated.

Model epithelia

Polarized model epithelia were prepared by culturing T84 or MDCK cells on collagen-coated permeable supports as previously described (Gewirtz et al., 2002b).

Preparation of anti-flagellin antibody

Flagellin was purified as described (Gewirtz et al., 2001b) and purity was verified as previously described (McSorley et al., 2002). Rabbits were immunized twice (one month apart) with 100 μg flagellin per rabbit in complete Freund's adjuvant. One month later, serum was isolated and anti-flagellin Ig purified via affinity chromatography over a column of immobilized flagellin.

Flagellin transcytosis

Model epithelia of 5 cm^2 were apically colonized with 5×10^8 colony-forming units (CFU) *S. typhimurium* grown as microaerophilic overnight cultures as previously described (McCormick et al., 1993). Bacterial strains were applied for the indicated times, after which the apical and/or basolateral media was collected, centrifuged (10 minutes at 5000 ***g***) and supernatants were assayed for the presence of flagellin by SDS-PAGE and immunoblotting. Prior to such analysis, the flagellin present in the basolateral media was concentrated 20-fold via incubation with protein A sepharose beads, followed by centrifugation, aspiration and resuspension in SDS-PAGE loading buffer.

Measurement of TEER/macromolecular permeability

Model epithelia were apically colonized with 5×10^8 CFU *E. coli* or *S. typhimurium* as indicated in the presence of 10 mg/ml FITC-dextran (molecular weight 40 kDa) (ICN Biomedical) or 10 mg/ml FITC (Sigma Corp.). Transepithelial electrical resistance (TEER) was monitored as previously described (Gewirtz et al., 2002b). The basolateral reservoir was sampled at the indicated time points and FITC-dextran concentration quantified via spectrofluorimetry (λ_{ex}=492 nm, λ_{em}=525 nm).

IL-8 secretion

IL-8 secretion was measured using ELISA as previously described (Gewirtz et al., 1998).

Bacterial invasion transcytosis

Model epithelia were treated identically to those used for measuring flagellin transcytosis. At indicated times following addition of bacteria, basolateral reservoir was sampled and CFU quantified by standard microbiological techniques. Alternatively, epithelia were treated with gentamicin to allow quantification of intracellular bacteria as previously described (McCormick et al., 1993).

Cell fractionation

T84 model epithelia were colonized with 5×10^8 CFU *S. typhimurium* for 1 hour, at which time epithelia were washed in PBS, scraped with a rubber policeman and homogenized with a glass/Teflon homogenizer in ice-cold buffer containing 250 mM sucrose, 10 mM Tris pH 7.5, 1 mM PMSF, 1 μg/ml leupeptin and pepstatin (Gouraud et al., 2002). The disrupted cell suspension was then centrifuged at 800 ***g*** for 10 minutes at 4°C. The supernatant was then subjected to centrifugation at 17,000 ***g*** for 45 minutes and the supernatant was re-centrifuged at 200,000 ***g*** for 2 hours. Pellets were initially resuspended in PBS, analyzed for protein concentration (Pierce) and subsequently diluted to match the protein concentration of the light microsomal fraction. Fractions were subsequently diluted 1:2 in SDS-PAGE loading buffer and subjected to immunoblot analysis using anti-GroEl (Cal-Biochem) or anti-flagellin antibodies.

Confocal microscopy

Model epithelia were apically colonized with 5×10^8 CFU wild-type *S. typhimurium* or the indicated mutant. 90 minutes after the addition of bacteria, cells were fixed in paraformaldehyde and permeabilized with Triton-X-100 as previously described (Gewirtz et al., 2001a). Cells were then stained with Cy5-phalloidin and subsequently immunostained with rabbit polyclonal anti-flagellin (described above) and goat anti-*Salmonella* complete surface antigens (KPL Labs, Gaithersburg, MD). The former is specific for flagellin as no staining of aflagellate *S. typhimurium fliC/fljB* is observed (not shown) whereas the latter stains the bacterial surface in general as well as flagellin (shown here) consistent with existing knowledge that flagellin is a major *S. typhimurium* antigen. Microscopy was performed on a Zeiss LSM510 laser-scanning confocal microscope (Zeiss Microimaging Inc., Thornwood, NY) coupled to a Zeiss 100M axiovert and 63× or 100× Pan-Apochromat oil lens. Fluorescent dyes were imaged sequentially in frame-interlace mode to eliminate cross-talk between channels. Images shown are representative of at least three experiments, with multiple images taken per slide.

Electron microscopy

Model epithelia were colonized for 1 hour with 5×10^8 CFU wild-type *S. typhimurium* SL1344 or isogenic SPI-2 mutant. Samples were then fixed and subjected to electron microscopic analysis as previously described (Gilmor et al., 1996).

Results

S. typhimurium, but not *E. coli* transcytoses flagellin across polarized epithelia

Both the pathogen *Salmonella typhimurium* and most commensal *Escherichia coli* strains are flagellated bacteria and thus, like most bacteria with unsheathed flagella, release substantial levels of flagellin into their milieu. The specific amount of flagellin released by *S. typhimurium* strains tends to be high, possibly because they generally have more flagella. Thus, apical colonization of model intestinal epithelia by either of these microbes leads to the substantial release of flagellin into the apical reservoir that can easily be observed by SDS-PAGE and immunoblotting even when samples are substantially diluted (Fig. 1). However, *S. typhimurium*, but not commensal *E. coli* is able to mediate the transcytosis of flagellin monomers across the epithelium (Gewirtz et al., 2001b). As shown in Fig. 1, western blotting of basolateral media following apical bacterial colonization of model intestinal epithelia revealed a detectable level of flagellin as early as 15 minutes following colonization. The concentration of basolateral flagellin following such apical *S. typhimurium* colonization increased throughout the 4-hour time course assayed. In contrast, following apical colonization by *E. coli*, in spite of its abundance in the apical reservoir, flagellin was not detected in the basolateral reservoir even after 4 hours. An identical trend was observed whether using polarized model epithelia made from the T84 intestinal epithelial cell line or from the well characterized polarized epithelial Madin-Darby kidney (MDCK) cell line, thus indicating that either model may be appropriate in studying this transcytotic process. Utilizing

purified flagellin as a standard allowed us to quantify approximately our immunoblotting analysis. Such measurement generally showed that by 1 hour after apical colonization by *S. typhimurium*, there was 5-20 ng/ml flagellin in the basolateral reservoir. Although this is a very small fraction of the flagellin released apically (1/2000-1/1000), it is well within the concentration range that we (and others) have shown to activate TLR5-mediated pro-inflammatory gene expression (Gewirtz et al., 2001a; Gewirtz et al., 2001b; Moors et al., 2001). Thus, *S. typhimurium*, but not commensal *E. coli* can mediate the transcytosis of flagellin across polarized model intestinal epithelium. As such flagellin transcytosis probably plays an important role in driving the intestinal inflammation associated with *S. typhimurium* infection we sought to elucidate this process mechanistically.

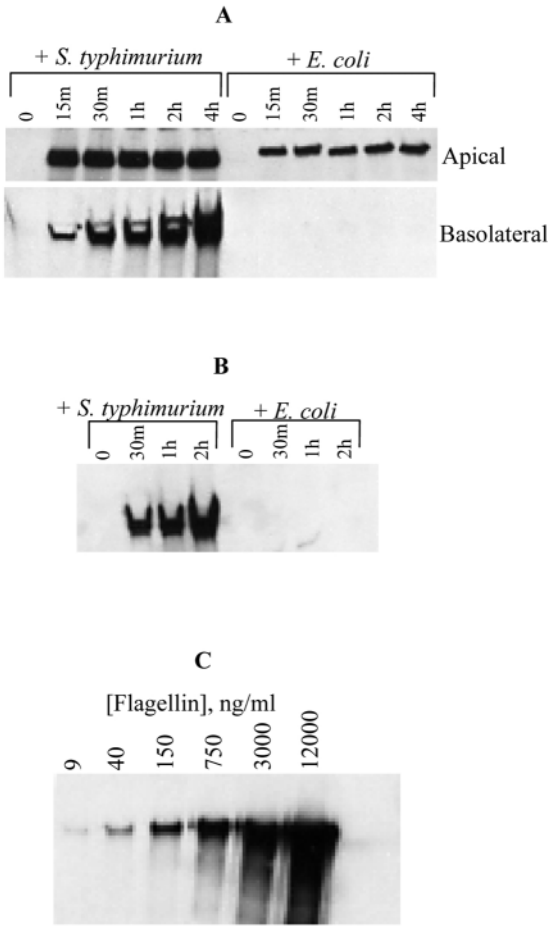

Fig. 1. *S. typhimurium*, but not *E. coli* transcytoses flagellin across polarized epithelia. (A) T84 model epithelia were apically colonized with 5×10^8 CFU *S. typhimurium (SL3201)* or a flagellated commensal *E. coli* strain as described in Materials and Methods. At the indicated times, the apical or basolateral media was isolated and assayed for flagellin by immunoblotting. Apical supernatants were diluted 100-fold and basolateral supernatants were concentrated 20-fold. (B) MDCK model epithelia were colonized as in panel A. Basolateral supernatants were assayed as above for flagellin at the indicated time points. (C) Known concentrations of flagellin were subjected to a similar analysis to provide quantitative data. All data are representative of several experiments.

Flagellin transcytosis occurs via a transcellular route uncoupled from bacterial movement

In order to understand how *S. typhimurium* transcytoses flagellin across the epithelium, we first sought to determine whether flagellin was traveling via a paracellular or transcellular pathway (i.e. between or across epithelial cells). Although the high degree of intestinal epithelial barrier function would greatly restrict the paracellular passage of a 50 kDa protein such as flagellin, some enteric pathogens are known to alter epithelial cell tight junctions resulting in increased paracellular permeability (Philpott et al., 1996; Spitz et al., 1995; Yuhan et al., 1997). Thus, we examined whether under our experimental conditions, *S. typhimurium* might alter epithelial permeability to facilitate paracellular passage of flagellin. Specifically, we monitored both transepithelial electrical resistance (TEER), which although technically a measure of resistance to charged particles is also thought to reflect permeability in general (Madara et al., 1992), as well as transepithelial flux of 40 kDa FITC-dextran (a molecule similar in size to flagellin). Under identical conditions to those when flagellin transcytosis was measured, *S. typhimurium*, but not commensal *E. coli* caused a significant drop in TEER, particularly after 2 hours (Fig. 2A). Consistent with the idea that such a drop in TEER may reflect an increased permeability at least to small molecules, we observed that *S. typhimurium* increased the transepithelial movement of unconjugated fluoroisothiocyanate (FITC) (with a molecular weight of 398 Da). However, such colonization of model epithelia did not have any effect on the permeability to FITC-dextran (Fig. 2C). Thus, although *S. typhimurium* may alter epithelial permeability to small and/or charged molecules like other enteric pathogens, it did not alter permeability to larger molecules suggesting that an increase in paracellular permeability was unlikely to be mediating the transcytosis of flagellin. The fact that we can measure movement of such FITC-dextran in the absence of bacteria indicates there is some non-bacterially mediated movement of large molecules. However, this is extremely inefficient, transferring less than 1/200,000 of the added FITC dextran in 4 hours, and is thus unable to account for movement of bioactive levels of flagellin, consistent with our inability to measure movement of added flagellin (Gewirtz et al., 2001b).

Whether flagellin was carried across the epithelium by bacteria or was trafficked independently of bacterial movement was investigated by measuring any temporal correlation between the appearance of live bacteria and flagellin in the basolateral reservoir (Fig. 3). We observed that flagellin appeared before the bacteria, suggesting that at least the initial burst of flagellin transcytosis was not dependent upon bacteria crossing the epithelium. At later time points we observed substantial numbers of bacteria in the basolateral reservoir and it seems likely that these significantly contribute to the level of basolateral flagellin seen at later time points. The role of bacteria crossing the epithelia in the transcytosis of flagellin was further investigated via a *S. typhimurium* mutant strain *fliD* that harbors a mutation in the flagellar hook protein that results in it being immobile and aflagellate but it still expresses and secretes flagellin monomers (Yokoseki et al., 1995). Following apical colonization, this non-motile strain was unable to migrate across the model epithelium itself. However, albeit with moderately slower kinetics, it was able to transcytose its

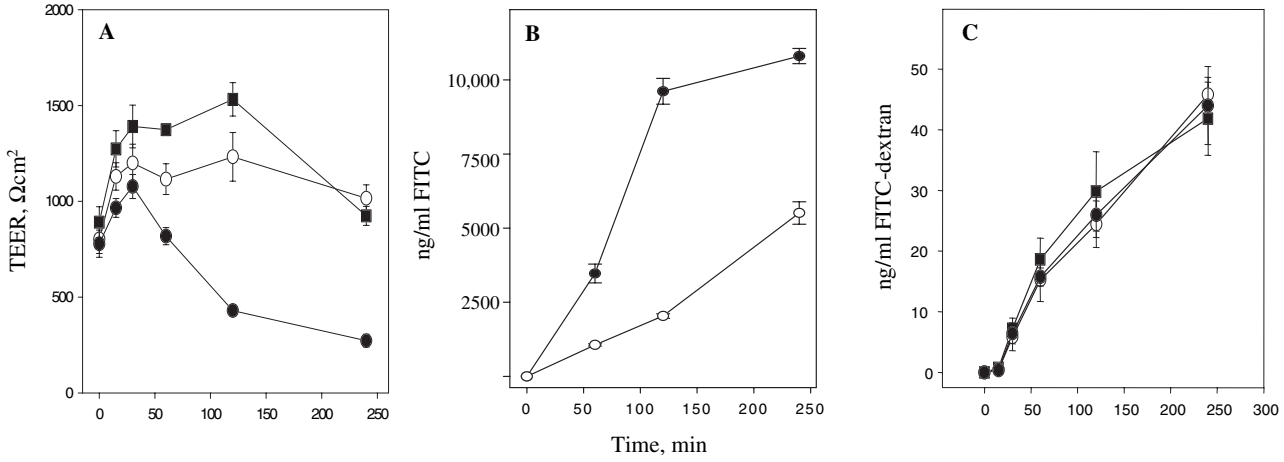

Fig. 2. *S. typhimurium* does not alter epithelial permeability to macromolecules. T84 model epithelia were colonized as in Fig. 1 with *S. typhimurium* and/or *E. coli* in the presence of 10 mg/ml FITC or 40 kDa FITC-dextran. (A) Transepithelial electrical resistance (TEER), (B) FITC or (C) FITC-dextran concentrations in the basolateral media were measured as described in Materials and Methods. ○, no bacteria; ●, *S. typhimurium*; ■, *E. coli*. Data are the means±s.e.m. of three parallel experiments.

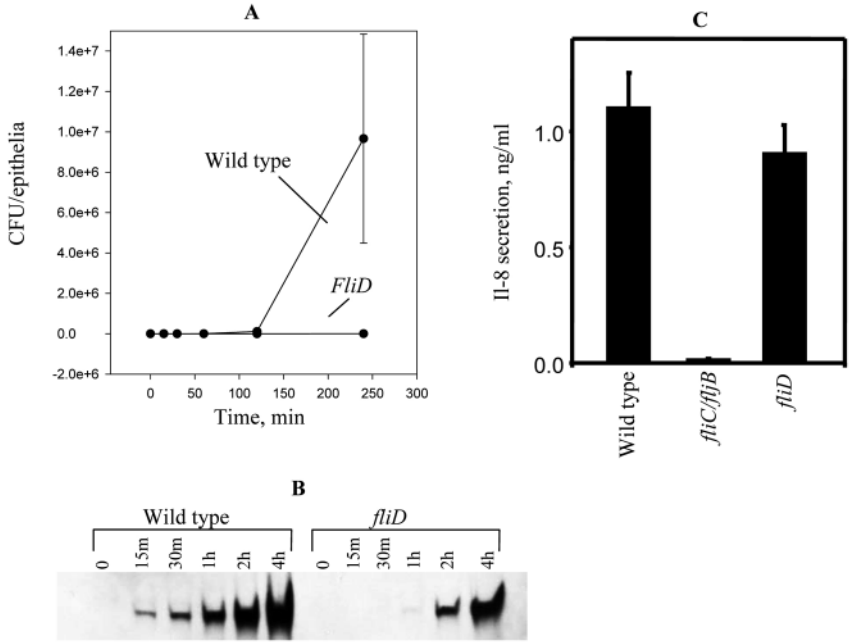

Fig. 3. Transcytosis of flagellin does not correlate with bacterial movement. (A,B) T84 model epithelia were colonized apically with 5×10^8 wild-type *S. typhimurium* or isogenic *fliD* mutant. At indicated time, basolateral media was assayed for CFU (A) and western blots were probed for flagellin (B). (C) IL-8 secretion was measured after 5 hours.

flagellin to the basolateral reservoir, indicating that flagellin crossing the epithelium need not be accompanied by live bacteria. Consistent with this notion, we also observed that induction of IL-8 secretion by apically applied bacteria (an event dependent upon flagellin transepithelial movement) could still be mediated by the *fliD* mutant but not by negative control strains of flagellin-deficient *S. typhimurium* or commensal flagellate *E. coli*. Thus, flagellin transcyotosis requires neither bacterial motility nor that bacteria traverse the epithelium, although it does seem likely that bacterial movement across the epithelium is one means of flagellin crossing the epithelium, particularly as the initial colonization progresses.

Flagellin is transcytosed via epithelial vesicular transport pathways

We next considered the possibility that, in response to *S. typhimurium* colonization, flagellin might be trafficked across the epithelium via host vesicular trafficking pathways. Vesicular trafficking in polarized epithelia is blocked at 20°C (Lencer et al., 1995), thus we examined the effect of this temperature on *S. typhimurium*-mediated transcytosis of flagellin across model epithelia. Although release of flagellin into the apical reservoir was the same at 37°C and 20°C, transcytosis of flagellin was nearly completely blocked at 20°C (Fig. 4A). In contrast, *S. typhimurium* invasion of model epithelia occurred nearly as efficiently at 20°C as it did at 37°C.

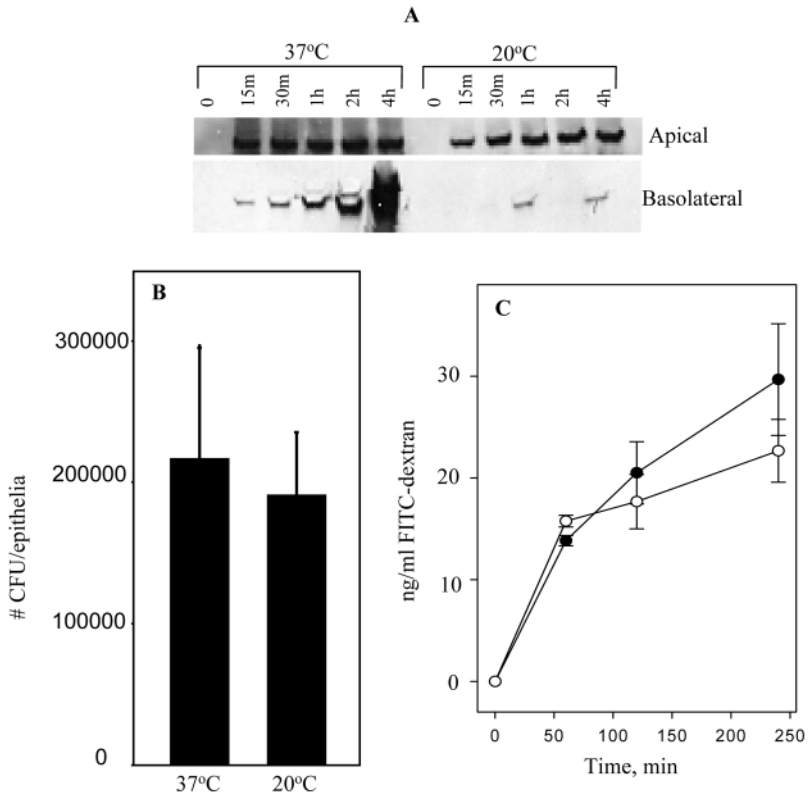

Fig. 4. Transcytosis of flagellin is blocked at 20°C. T84 model epithelia were treated in a 37°C incubator or at ambient room temperature (observed to vary between 18.7 and 20.4°C). (A,B) Epithelia were colonized by 5×10^8 CFU *S. typhimurium*. Flagellin transcytosis (A) and bacterial invasion (B) were measured as described in Fig. 1 and Materials and Methods. (C) 10 mg/ml FITC-dextran (40 kDa) was added to the apical surface and its appearance was measured in the basolateral reservoir. ○, 37°C; ●, room temperature.

Furthermore, movement of 40 kDa FITC-dextran across epithelia was not significantly altered by this reduced temperature, consistent with the idea that such FITC-dextran movement is occurring via the paracellular pathway. These results suggest that a substantial portion of flagellin transcytosis may be mediated by epithelial vesicular trafficking.

To investigate the notion that flagellin might be trafficked in epithelial vesicles further, we fractionated gently disrupted *S. typhimurium*-infected epithelia by centrifugation (Fig. 5). We observed that after a 30-minute 800 ***g*** centrifugation step (expected to pellet larger cellular components), the pellet contained most of the bacterial GroEL (a bacterioplasmic protein) indicating that this fraction contained most of the intracellular bacteria (a result seen whether fractions were normalized for protein content or volume, not shown). In contrast, this fraction did not contain much flagellin (although it could be detected if fractions were normalized for volume instead of protein content). Subjecting the supernatant to further centrifugation at 17,000 ***g*** to pellet endosomes and larger vesicles produced a pellet enriched in flagellin but containing very little GroEL indicating that, at 1 hour following colonization, a substantial portion of intracellular flagellin was no longer physically associated with bacteria. Furthermore, the flagellin was in a separate compartment to the bacteria. Lastly, we isolated small intracellular vesicles/microsomes by further centrifugation at 200,000 ***g***. These vesicles contained detectable levels of flagellin but did not contain GroEL. Together, these results indicate that, following *S. typhimurium* colonization, some flagellin is contained in intracellular compartments, distinct from the vacuoles known to contain the bacteria.

SPI-2 is required for transcytosis of flagellin across gut epithelia and subsequent maximal IL-8 expression

The bacterial determinants that mediate *S. typhimurium* transepithelial transcytosis of flagellin were investigated to enhance our understanding of the transcytotic mechanism. A substantial portion of the phenotypic differences between *S. typhimurium* and commensal *E. coli* strains are mediated by gene 'pathogenicity islands' (Marcus et al., 2000). *S. typhimurium* invasion of epithelial cells is mediated by genes on *Salmonella* pathogenicity island (SPI)-1 thus rendering SPI-1 mutants invasion defective (Eichelberg and Galan, 1999) (Fig. 6). In contrast, genes in SPI-2 are not required for invasion but rather allow this pathogen to survive in macrophages, primarily by altering vesicle trafficking, specifically phagosome-lysosome fusion (Hensel et al., 1997). SPI-2 is also known to play an essential role in generation/maintenance of the *Salmonella*-containing vacuole in epithelial cells although this vacuole is not necessary for *Salmonella* survival in epithelia (Steele-Mortimer et al., 2002). In light of these SPIs being major factors that distinguish *S. typhimurium* from *E. coli*, we investigated their role in transcytosis of flagellin across polarized epithelia. First, we examined release of flagellin into the apical compartment (Fig. 6A). Compared to their wild-type parents, both SPI-1 and SPI-2 mutants appeared to release, respectively, moderately to mildly higher levels of flagellin into the apical reservoir probably owing to the ability of these SPIs to suppress flagellar expression (Aldridge and Hughes, 2002). Next, we measured the amount of flagellin transcytosed across the model epithelium to the basolateral reservoir (Fig. 6A). In spite of invading about tenfold less efficiently than the wild type, SPI-1 mutants did not exhibit a reduced ability to transcytose flagellin across model epithelia indicating that invasion is not a limiting factor for this transcytotic process. In contrast, we observed a striking reduction in the ability of SPI-2 mutants to transcytose flagellin across model epithelia. This difference was observed in SPI-2 mutants derived from two different parental strains (Fig. 6B), indicating that a role for SPI-2 in flagellin transcytosis is not a strain-specific phenomenon. Although the apical supernatants of epithelia colonized with the SPI-2 mutant contained at least as much flagellin as those colonized by the parent strain, suggesting that the failure of the SPI-2 mutant was unlikely to be due to an inherent deficiency in flagellin expression and release by the mutant strain, we nonetheless examined this possibility directly in the absence of

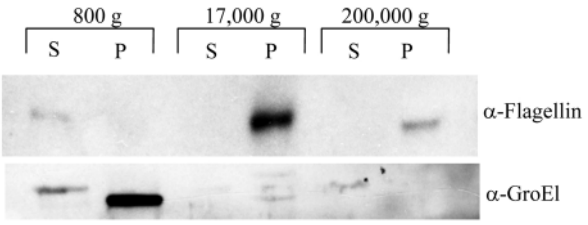

Fig. 5. Cellular fractionation reveals the presence of vesicular flagellin not associated with bacteria. T84 model epithelia were colonized with *S. typhimurium* for 1 hour after which epithelia were disrupted and fractionated by centrifugation as described in Materials and Methods. Fractions were normalized for protein content and then analyzed by SDS-PAGE and immunoblotting for bacterial GroEl and flagellin. This data is representative of several experiments.

host cells. As shown in Fig. 3C, when analyzed over a range of dilutions, supernatants of the wild type and SPI-2 mutant *S. typhimurium* contained similar levels of flagellin indicating the failure of the SPI-2 mutant to transcytose flagellin did not result from altered levels of flagellin expression by this mutant.

We next examined where in the transcytotic process SPI-2 mutants were deficient. First, we verified that, as has been shown in non-polarized epithelia, SPI-2 invaded polarized epithelia as efficiently as the parent strain (Fig. 6D) (Steele-Mortimer et al., 2002) whereas the SPI-1 mutant was invasion-defective as expected. Furthermore, there was also no difference between SPI-2 mutants and their parents in terms of the number of live bacteria that crossed the epithelium following colonization (data not shown). The relative level of invasiveness by these strains was similar to their relative ability to alter TEER, as this parameter was lowered by the wild-type parent and SPI-2 mutant but not the SPI-1 strain (Fig. 6E). This result indicates that such alteration of paracellular permeability is neither necessary nor sufficient for flagellin transcytosis consistent with our data indicating that such alterations in permeability do not permit the movement of large molecules. Next, we examined whether SPI-2 mutants might fail to mediate the movement of flagellin into intracellular vesicles. This was indeed the case as small vesicles prepared from SPI-2 infected epithelia contained strikingly less flagellin than those prepared from epithelia colonized by SPI-1 mutants or their isogenic parent (Fig. 6F). Furthermore, even crude cell lysates of SPI-2 infected model epithelia contained very little flagellin compared to those infected with wild-type strains (Fig. 6G). These results indicate that the incorporation of flagellin into vesicles is critical for its transcytosis across the epithelium and that such incorporation into vesicles does not appear to require the bacterial invasion of the epithelium.

The localization of flagellin in model epithelia colonized with *S. typhimurium* or isogenic SPI mutants was investigated

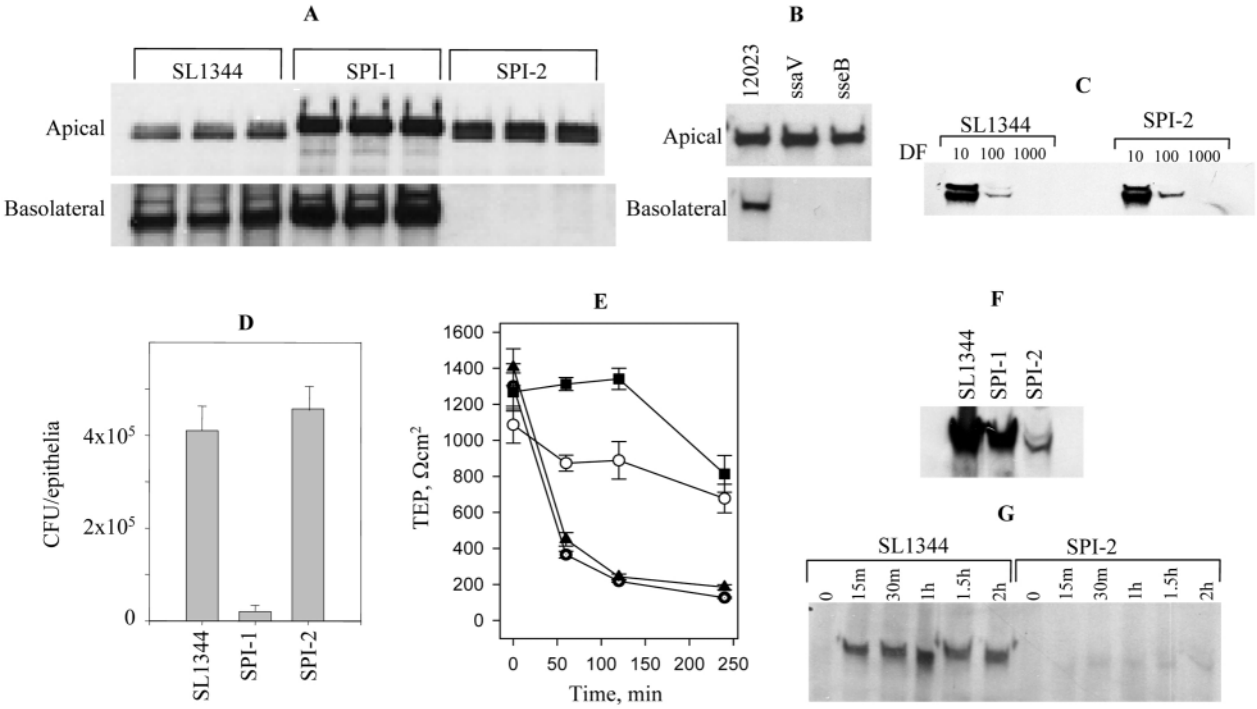

Fig. 6. *S. typhimurium* transcytosis of flagellin is dependent on SPI-2, but not SPI-1. T84 model epithelia were apically colonized with 5×10^8 CFU wild-type *S. typhimurium* strains (SL1344 or 12023) or corresponding isogenic mutants (SPI-1 and SPI-2 mutants of SL1344 are invA and ssaR, SPI-2 mutants of 12023 are ssaV and sseB). (A,B) 2 hours after addition of bacteria, flagellin in the apical and basolateral supernatants was measured by SDS-PAGE and immunoblotting as described in Materials and Methods. Results in A are from three parallel experiments whereas those in B are representative of several experiments. (C) Supernatants, in the absence of host cells, of the wild type and SPI-2 mutants were diluted by indicated dilution factor (DF) and subjected to SDS-PAGE immunoblot analysis for flagellin. (D) Internalized bacteria were quantified 1 hour after colonization as described in Materials and Methods. (E) TEER was measured at indicated times in model epithelia colonized by the wild type or SPI mutants. ○, no bacteria; ●, SL1344; ■, SPI-1 mutant; ▲, SPI-2 mutant. Results in D and E are the means±s.e.m. of three parallel experiments. (F,G) At 1 hour or indicated time point, epithelial cells were fractionated as in Fig. 5 and flagellin was assayed in small vesicle isolation (F) or initial lysate (G). Results are representative of several experiments.

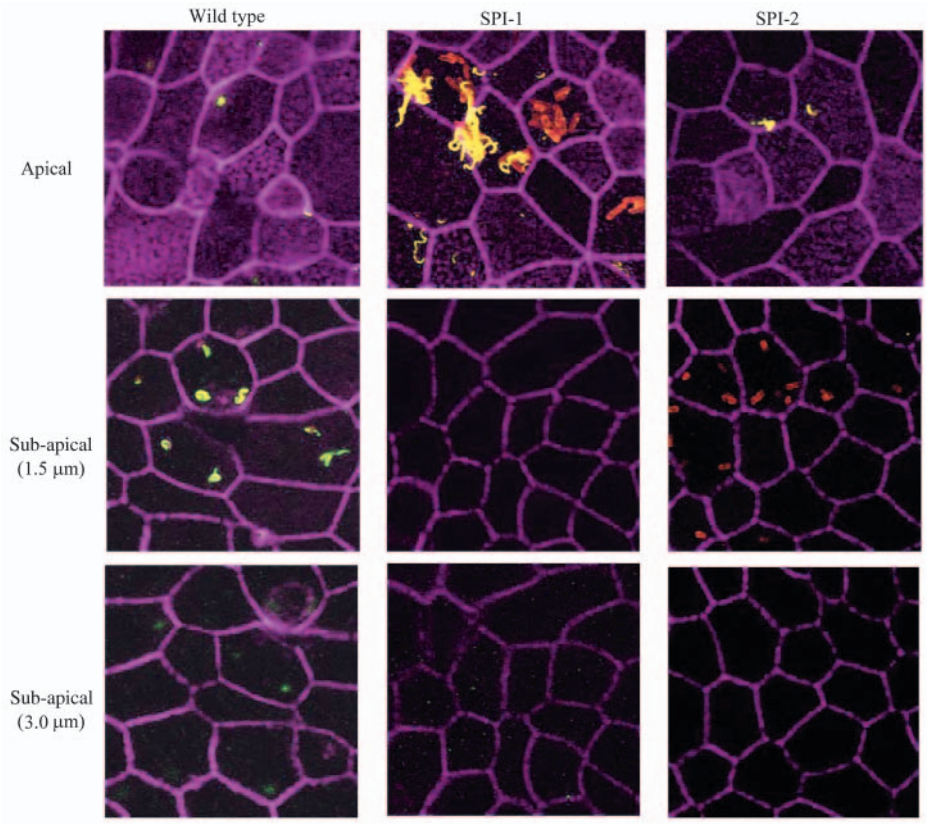

Fig. 7. Altered levels/localization of flagellin in epithelia infected with SPI-2 deficient *S. typhimurium*. MDCK model epithelia were colonized with wild-type *S. typhimurium* (SL1344) or isogenic mutants for 1 hour, after which epithelia were paraformaldehyde-fixed, immunostained and analyzed by confocal microscopy. Actin is shown in purple. *Salmonella* complete surface antigens (CSA) are shown in red. Flagellin is shown in green. Flagellae stain with both CSA and flagellin antibodies and thus appear yellow. The sub-apical planes 1.5 μm and 3 μm below the apical surface are shown. Magnification ×1000.

by confocal microscopy (Fig. 7). In these assays, actin was visualized with Cy5-phalloidin (purple) whereas double immunostaining was performed using anti-flagellin (green) and anti-*Salmonella* complete surface antigens (CSA) (red). As flagella are major surface antigens of Salmonellae, they are recognized by both antibodies and thus appear in yellow. However, whereas our purified anti-flagellin antibody recognizes both flagella and flagellin monomers, the *Salmonella* CSA antibody has little ability to recognize flagellin monomers (as assessed by western blotting, data not shown) and thus the green channel fluorescence that is not seen using the red channel, probably reflects localization of flagellin monomers. One hour following apical colonization by a wild-type strain, there were relatively few bacteria on the apical surface (at least that adhered sufficiently to survive the washing/staining process), but bacteria could be easily seen inside the cells just beneath the apical surface. Although these bacteria did not appear to be highly flagellated, a substantial level of flagellin monomer was present both in the immediate and general vicinity of the bacteria. Furthermore, flagellin staining was observed a few micrometers below the apical surface, which did not appear to be in the immediate vicinity of bacteria. For SPI-1 mutants, as expected, hardly any intracellular bacteria could be found, however groups of bacteria were clearly seen adhering to the apical surface. These bacteria appeared to have produced large clusters of flagella but had seemingly shed them; the fact that the bacteria had survived the washing/staining suggested that these flagella had specifically adhered to the apical surface. Despite the fact that no intracellular bacteria were observed, punctate intracellular staining of flagellin could be observed consistent with the notion that transcytosis of flagellin need not necessarily be directed by internalized bacteria but rather can also be directed by *S. typhimurium* adherent to the apical surface. In contrast to the SPI-1 mutant, intracellular SPI-2 *S. typhimurium* were at least as abundant as their wild-type parent but in striking contrast to the wild-type strain, intracellular bacteria did not have flagellin in their immediate or general vicinity. Furthermore, no punctate intracellular staining of flagellin was observed consistent with our biochemical approach that showed lysates of SPI-2 infected epithelia contained no flagellin. As an additional approach to understand the inability of SPI-2 mutants to transcytose flagellin, the localization of intracellular bacteria was examined by electron microscopy (Fig. 8). Whereas, 2 hours following colonization wild-type bacteria were found only in vacuoles (commonly referred to as *Salmonella*-containing vacuoles or SCV), the SPI-2 mutant was only observed free in the cytoplasm: a localization consistent with that recently observed for SPI-2 mutants in non-polarized epithelial cells (Brumell et al., 2002). Together, these results indicate that the SPI-2 plays a role in mediating and/or maintaining the localization of flagellin into intracellular vesicles that play an essential role in transcytosing *S. typhimurium* across intestinal epithelia.

In light of the role of flagellin transcytosis in mediating *S. typhimurium*-induced pro-inflammatory gene expression, we measured the ability of SPI-2 mutants to induce IL-8 secretion from polarized model epithelia (Fig. 9). In this assay, the aflagellate mutant serves as a negative control. However, it should be noted that its inability to induce IL-8 expression may be caused not only by its inability to activate TLR5, but also in part by an inability to stimulate intracellular pattern recognition receptors, as we and others have shown that such mutants have substantially reduced invasion capacities (Gewirtz et al., 2001b; Van Asten et al., 2000). When bacteria are placed in the basolateral reservoir, an experimental system that obviates the transcytotic process, SPI-2 mutants induced at least as much IL-8 secretion as their parent strains. However, when colonizing the apical physiological surface of such epithelia, SPI-2 mutant strains were substantially reduced in their IL-8 inducing capacity compared to wild-type strains,

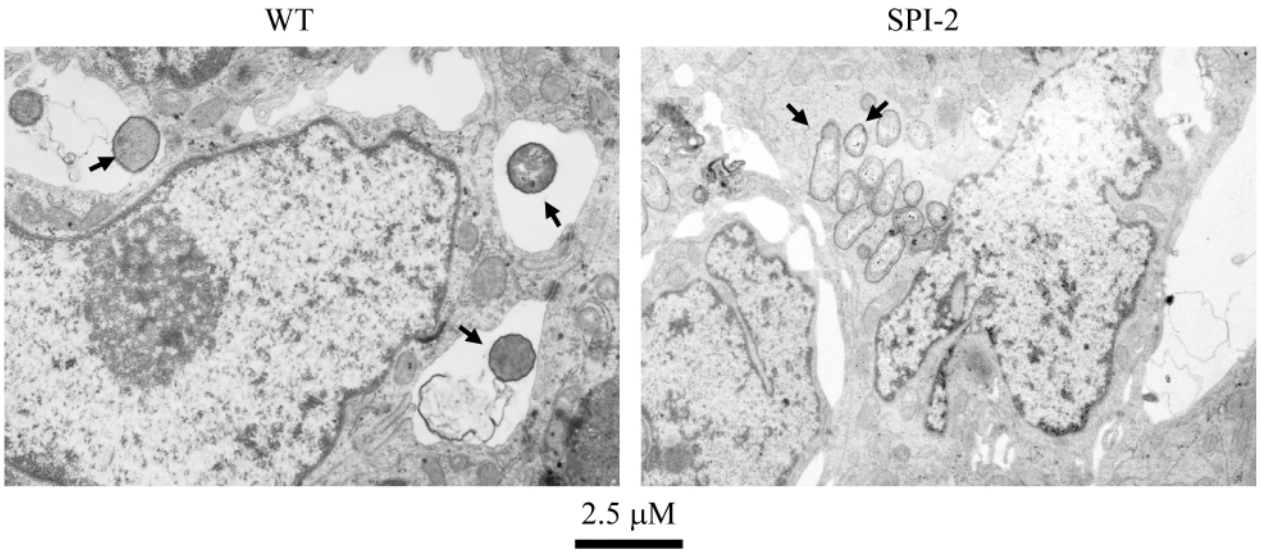

Fig. 8. *S. typhimurium* SPI-2 mutant localizes to the cytoplasm in polarized epithelia. T84 Model epithelia were colonized with wild-type *S. typhimurium* or isogenic SPI-2 mutant. Two hours following colonization, cells were fixed and analyzed by electron microscopy as described in Methods. Arrows indicate bacteria. Bar, 2.5 μm.

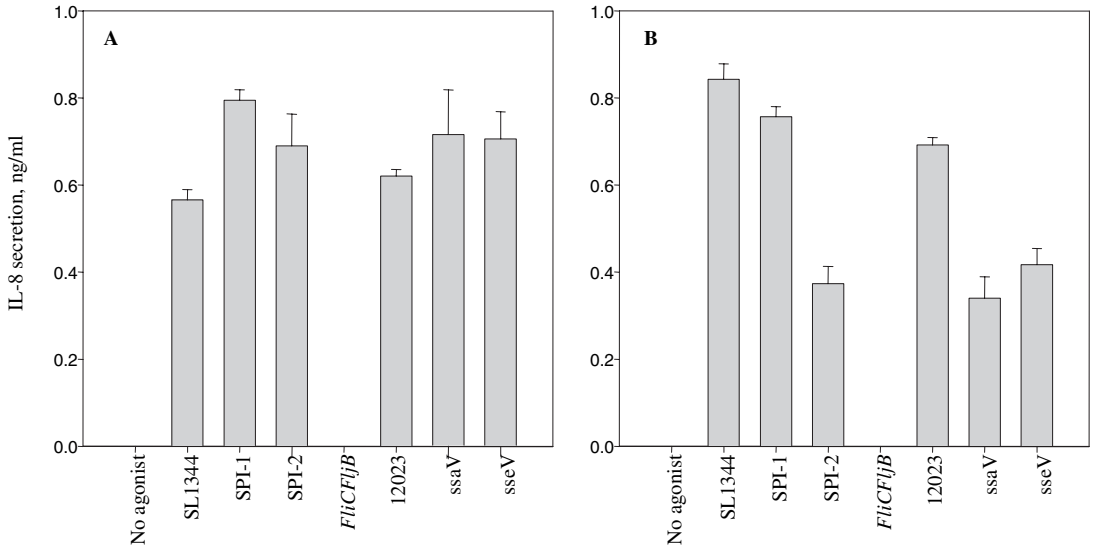

Fig. 9. *S. typhimurium* SPI-2 mutant exhibits reduced ability to activate IL-8 expression when colonizing the physiologically relevant apical surface of model intestinal epithelia. The indicated bacterial strains were applied to polarized model epithelia and IL-8 secretion measured as described. (A) Bacteria were applied directly to the basolateral reservoir. (B) Bacteria were applied to the apical reservoir; i.e. the physiological site of colonization. IL-8 in the basolateral compartment was assayed 5 hours later by ELISA. Data are the means±s.e.m. of three parallel experiments.

suggesting that the failure of SPI-2 *S. typhimurium* to transcytose flagellin across epithelia may result in these strains inducing less intestinal inflammation than wild-type strains when colonizing the human intestine.

Discussion

The histopathologic hallmark of human Salmonellosis, caused by non-typhoidal strains of *Salmonella* enterica of which *S. typhimurium* is one of the most common serovars, is the recruitment of neutrophils to the intestinal mucosa resulting in the formation of an intestinal crypt abscess (Takeuchi, 1967). Such neutrophils are not merely a marker of such infections but play a key role in both clearing the infection and in causing the clinical manifestations (i.e. cramping, diarrhea) characteristic of the infection (Gewirtz et al., 2002a). Recent inquiries into the mechanisms that drive such neutrophil recruitment revealed that a key event in triggering this inflammatory response was the detection of flagellin by epithelial basolateral TLR5 resulting in epithelial cell secretion of chemokines, thus driving the influx of innate immune cells (Gewirtz et al., 2001a). Epithelial polarity is an absolutely critical aspect of this response as flagellin secreted by *Salmonella* strains or commensal *E. coli* strains are all

potentially potent TLR5 ligands (Gewirtz et al., 2001b; Moors et al., 2001). However, apical colonization by *S. typhimurium* but not commensal *E. coli*, results in flagellin attaining a basolateral localization thus allowing ligation of TLR5 (whose expression is strongly polarized to the basolateral surface), thus explaining in part, why *S. typhimurium* but not commensal *E. coli*, is proinflammatory. Here, we addressed the mechanism by which such flagellin transcytosis occurs and showed that flagellin travels via a transcellular pathway mediated by host vesicular transport. Such *S. typhimurium* transcytosis of flagellin was not dependent on motility, or on SPI-1, which mediates invasion of epithelial cells, but was strictly dependent upon SP1-2, which is thought to permit *S. typhimurium* to alter vesicle trafficking (Linehan and Holden, 2003).

Consistent with the role of flagellin transcytosis in mediating *S. typhimurium* activation of epithelial pro-inflammatory gene expression, SPI-2 deletion resulted in a substantial reduction in the ability of *S. typhimurium* to induce IL-8 secretion when colonizing the physiologically relevant apical surface of model gut epithelia. However, whereas aflagellate *S. typhimurium* does not activate IL-8 secretion, SPI-2 mutants retained significant, albeit reduced, activation of such a pro-inflammatory pathway despite a complete inability to transcytose flagellin to the site of TLR5 localization. Considering that aflagellate *S. typhimurium* are unable to invade epithelial cells (Gewirtz et al., 2001b; Van Asten et al., 2000), whereas the SPI-2 mutant invades efficiently but resides in the cytoplasm, the IL-8 activation by the SPI-2 mutant may result from activation of the cytosolic pattern recognition receptor Nod1, which is known to mediate pro-inflammatory gene expression in response to invasive pathogens that reside in the cytoplasm such as enteroinvasive *E. coli* (Girardin et al., 2001).

SP1-2 was first characterized based on its ability to promote *Salmonella* survival in macrophages. However, SPI-2 has also been shown to play a role in *S. typhimurium* infection of epithelial cells by generating/maintaining the SCV (Steele-Mortimer et al., 2002; Waterman, 2003). In both of these environments, the primary general function of SPI-2 is to alter normal vesicle/endocytic trafficking. Thus, considering all our results together, we speculate that genes in SPI-2 permit flagellin transcytosis by diverting endocytic flow away from the lysosomes, which would otherwise result in the destruction of the internalized flagellin and loss of the SCV. Under this scenario we envisage that, upon colonization by wild-type *S. typhimurium*, such SPI-2 mediated flagellin transport would be mediated by internalized bacteria. However, our observation that flagellin transcytosis occurred in response to the SPI-1 mutant that invaded tenfold less efficiently suggests that such SPI-2 mediated alteration of vesicular transport need not absolutely occur from internalized bacteria. SPI-2 effectors, at least under some circumstances, might be translocated into the epithelial cells from bacteria that adhered intimately to the apical surface. Alternatively, it is possible that the residual level of internalized SPI-1 is sufficient to alter vesicle trafficking to permit flagellin transcytosis. In either of these scenarios, the fact that none of the tested bacteria upregulated transepithelial movement of FITC dextran suggests that the transcytosed flagellin is not occurring via fluid-phase transport but rather that flagellin is binding a molecule on the apical (or apical endosomal) surface that is internalized and reaches the basolateral surface only when vesicular trafficking is altered by genes in SPI-2. A number of glycolipids including asialoglycoprotein 1 and muc-1 have been shown to bind flagellin (Lillehoj et al., 2002; McNamara et al., 2001) and thus we speculate that these would be reasonable candidates to serve as transepithelial carriers of flagellin.

Although SPI-2 mediated survival in macrophages has clearly been shown to be important in murine infections where *S. typhimurium* causes a systemic typhoid-like illness, the role of SPI-2 in human Salmonellosis where the infection is generally limited to the intestinal mucosa is less clear, although the consistent presence of SPI-2 in clinical isolates suggests an important role. As epithelial cells lack the microbial killing power of macrophages, the role of maintenance of the SCV in epithelia has been particularly unclear as *Salmonella* can grow as well or better in the cytosol than in the SCV (Brumell et al., 2002). However, our observation that SPI-2 mutants can not transcytose flagellin across the epithelia and subsequently activate less epithelial chemokine secretion suggest the possibility that *S. typhimurium* SPI-2 mediated maintenance of the SCV may also be a part of a pathogenic strategy to induce the secretory diarrhea that aids in dissemination to new hosts. Although altering vesicular trafficking is a fairly widely used strategy for intracellular pathogens, our observations that, at least in vitro, apically adherent bacteria with functional SPI-2 can alter vesicular trafficking suggests the possible physiological existence of non-invasive apically adhering bacteria that can also alter host vesicular trafficking with one consequence being the transfer of microbial components across the epithelium. Such bacteria, if they exist in nature, may be currently unrecognized pathogens that might play a role in idiopathic intestinal inflammation such as occurs in inflammatory bowel disease.

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References

Aldridge, P. and Hughes, K. T. (2002). Regulation of flagellar assembly. *Curr. Opin. Microbiol.* **5**, 160-165.

Brumell, J. H., Tang, P., Zaharik, M. L. and Finlay, B. B. (2002). Disruption of the Salmonella-containing vacuole leads to increased replication of Salmonella enterica serovar typhimurium in the cytosol of epithelial cells. *Infect. Immun.* **70**, 3264-3270.

Cohen, P. S., Rossoll, R., Cabelli, V. J., Yang, S. L. and Laux, D. C. (1983). Relationship between the mouse colonizing ability of a human fecal Escherichia coli strain and its ability to bind a specific mouse colonic mucous gel protein. *Infect. Immun.* **40**, 62-69.

Eichelberg, K. and Galan, J. E. (1999). Differential regulation of Salmonella typhimurium type III secreted proteins by pathogenicity island 1 (SPI-1)-encoded transcriptional activators InvF and hilA. *Infect. Immun.* **67**, 4099-4105.

Gewirtz, A. T., McCormick, B., Neish, A. S., Petasis, N. A., Gronert, K., Serhan, C. N. and Madara, J. L. (1998). Pathogen-induced chemokine secretion from model intestinal epithelium is inhibited by lipoxin A4 analogs. *J. Clin. Invest.* **101**, 1860-1869.

Gewirtz, A. T., Navas, T. A., Lyons, S., Godowski, P. J. and Madara, J. L. (2001a). Cutting edge: bacterial flagellin activates basolaterally expressed tlr5 to induce epithelial proinflammatory gene expression. *J. Immunol.* **167**, 1882-1885.

Gewirtz, A. T., Simon, P. O., Jr, Schmitt, C. K., Taylor, L. J., Hagedorn,

C. H., O'Brien, A. D., Neish, A. S. and Madara, J. L. (2001b). Salmonella typhimurium translocates flagellin across intestinal epithelia, inducing a proinflammatory response. *J. Clin. Invest.* **107**, 99-109.

Gewirtz, A. T., Liu, Y., Sitaraman, S. V. and Madara, J. L. (2002a). Intestinal epithelial pathobiology: past, present and future. *Best Pract. Res. Clin. Gastroenterol.* **16**, 851-867.

Gewirtz, A. T., Reed, K. A., Merlin, D., Neish, A. S. and Madara, J. L. (2002b). Modeling Microbial-Epithelial Interactions in the Intestine. *Methods Microbiol.* **31**, 377-396.

Gilmor, M. L., Nash, N. R., Roghani, A., Edwards, R. H., Yi, H., Hersch, S. M. and Levey, A. I. (1996). Expression of the putative vesicular acetylcholine transporter in rat brain and localization in cholinergic synaptic vesicles. *J. Neurosci.* **16**, 2179-2190.

Girardin, S. E., Tournebize, R., Mavris, M., Page, A. L., Li, X., Stark, G. R., Bertin, J., DiStefano, P. S., Yaniv, M., Sansonetti, P. J. et al. (2001). CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive Shigella flexneri. *EMBO Rep.* **2**, 736-742.

Gouraud, S., Laera, A., Calamita, G., Carmosino, M., Procino, G., Rossetto, O., Mannucci, R., Rosenthal, W., Svelto, M. and Valenti, G. (2002). Functional involvement of VAMP/synaptobrevin-2 in cAMP-stimulated aquaporin 2 translocation in renal collecting duct cells. *J. Cell Sci.* **115**, 3667-3674.

Hensel, M., Shea, J. E., Raupach, B., Monack, D., Falkow, S., Gleeson, C., Kubo, T. and Holden, D. W. (1997). Functional analysis of ssaJ and the ssaK/U operon, 13 genes encoding components of the type III secretion apparatus of Salmonella Pathogenicity Island 2. *Mol. Microbiol.* **24**, 155-167.

Hindle, Z., Chatfield, S. N., Phillimore, J., Bentley, M., Johnson, J., Cosgrove, C. A., Ghaem-Maghami, M., Sexton, A., Khan, M., Brennan, F. R. et al. (2002). Characterization of Salmonella enterica derivatives harboring defined aroC and Salmonella pathogenicity island 2 type III secretion system (ssaV) mutations by immunization of healthy volunteers. *Infect. Immun.* **70**, 3457-3467.

Lencer, W. I., Moe, S., Rufo, P. A. and Madara, J. L. (1995). Transcytosis of cholera toxin subunits across model human intestinal epithelia. *Proc. Natl. Acad. Sci. USA* **92**, 10094-10098.

Lillehoj, E. P., Kim, B. T. and Kim, K. C. (2002). Identification of Pseudomonas aeruginosa flagellin as an adhesin for Muc1 mucin. *Am. J. Physiol. Lung Cell Mol. Physiol.* **282**, L751-L756.

Linehan, S. A. and Holden, D. W. (2003). The interplay between Salmonella typhimurium and its macrophage host–what can it teach us about innate immunity? *Immunol. Lett.* **85**, 183-192.

Madara, J. L., Colgan, S. P., Nusrat, A., Delp, C. and Parkos, C. A. (1992). A simple approach to measurement of electrical parameters of cultured epithelial monolayers: use in assessing neutrophil epithelial interactions. *J. Tissue Cult. Methods* **15**, 209-216.

Marcus, S. L., Brumell, J. H., Pfeifer, C. G. and Finlay, B. B. (2000). Salmonella pathogenicity islands: big virulence in small packages. *Microbes Infect.* **2**, 145-156.

McCormick, B. A., Colgan, S. P., Archer, C. D., Miller, S. I. and Madara, J. L. (1993). Salmonella typhimurium attachment to human intestinal epithelial monolayers: transcellular signalling to subepithelial neutrophils. *J. Cell Biol.* **123**, 895-907.

McCormick, B., Gewirtz, A. and Madara, J. L. (1998a). Epithelial crosstalk with bacteria and immune cells. *Curr. Opin. Gastro.* **14**, 492-497.

McCormick, B. A., Parkos, C. A., Colgan, S. P., Carnes, D. K. and Madara, J. L. (1998b). Apical secretion of a pathogen-elicited epithelial chemoattractant (PEEC) activity in response to surface colonization of intestinal epithelia by Salmonella typhimurium. *J. Immunol.* **160**, 455-456.

McNamara, N., Khong, A., McKemy, D., Caterina, M., Boyer, J., Julius, D. and Basbaum, C. (2001). ATP transduces signals from ASGM1, a glycolipid that functions as a bacterial receptor. *Proc. Natl. Acad. Sci. USA* **98**, 9086-9091.

McSorley, S. J., Ehst, B. D., Yu, Y. and Gewirtz, A. T. (2002). Bacterial flagellin is an effective adjuvant for CD4(+) T cells in vivo. *J. Immunol.* **169**, 3914-3919.

Moors, M. A., Li, L. and Mizel, S. B. (2001). Activation of interleukin-1 receptor-associated kinase by gram-negative flagellin. *Infect. Immun.* **69**, 4424-4429.

Philpott, D. J., McKay, D. M., Sherman, P. M. and Perdue, M. H. (1996). Infection of T84 cells with enteropathogenic Escherichia coli alters barrier and transport functions. *Am. J. Physiol.* **270**, G634-G645.

Schmitt, C. K., Darnell, S. C. and O'Brien, A. D. (1996). The attenuated phenotype of a Salmonella typhimurium flgM mutant is related to expression of FliC flagellin. *J. Bacteriol.* **178**, 2911-2915.

Spitz, J., Yuhan, R., Koutsouris, A., Blatt, C., Alverdy, J. and Hecht, G. (1995). Enteropathogenic Escherichia coli adherence to intestinal epithelial monolayers diminishes barrier function. *Am. J. Physiol.* **268**, G374-G379.

Steele-Mortimer, O., Brumell, J. H., Knodler, L. A., Meresse, S., Lopez, A. and Finlay, B. B. (2002). The invasion-associated type III secretion system of Salmonella enterica serovar Typhimurium is necessary for intracellular proliferation and vacuole biogenesis in epithelial cells. *Cell Microbiol.* **4**, 43-54.

Takeuchi, A. (1967). Electron microscope studies of experimental Salmonella infection. *Am. J. Pathol.* **50**, 109-119.

Van Asten, F. J., Hendriks, H. G., Koninkx, J. F., van der Zeijst, B. A. and Gaastra, W. (2000). Inactivation of the flagellin gene of Salmonella enterica serotype Enteritidis strongly reduces invasion into differentiated Caco-2 cells. *FEMS Microbiol. Lett.* **185**, 175-179.

Yokoseki, T., Kutsukake, K., Ohnishi, K. and Iino, T. (1995). Functional analysis of the flagellar genes in the fliD operon of Salmonella typhimurium. *Microbiology* **141**, 1715-1722.

Yokoseki, T., Iino, T. and Kutsukake, K. (1996). Negative regulation by fliD, fliS, and fliT of the export of the flagellum-specific anti-sigma factor, FlgM, in Salmonella typhimurium. *J. Bacteriol.* **178**, 899-901.

Yuhan, R., Koutsouris, A., Savkovic, S. D. and Hecht, G. (1997). Enteropathogenic Escherichia coli-induced myosin light chain phosphorylation alters intestinal epithelial permeability. *Gastroenterology* **113**, 1873-1882.