The *Salmonella typhimurium* Flagellar Basal Body Protein FliE Is Required for Flagellin Production and to Induce a Proinflammatory Response in Epithelial Cells*

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During apical colonization by *Salmonella typhimurium*, intestinal epithelial cells orchestrate a proinflammatory response that involves secretion of chemotactants, predominantly interleukin-8, which coordinate neutrophil trans-epithelial migration at the site of infection. This host-pathogen interaction requires several *S. typhimurium* genes. To identify novel genes that participate in this pathogen-induced proinflammatory response, we created *S. typhimurium* Tn-10 transposon mutants and identified a single mutant with Tn-10 insertional inactivation within the fliE locus that was able to adhere to and invade intestinal epithelial cells normally but was unable to induce interleukin-8 secretion in host cells. The fliE-deficient mutant failed to secrete flagellin and lacked any surface assembly of flagellae. Unlike wild-type *S. typhimurium*, the fliE-deficient mutant did not activate the IκBα/NF-κB signaling pathway or induce the coordinated trans-epithelial migration of isolated human neutrophils. Transcomplementation of the fliE-deficient mutant with a wild-type fliE-harboring plasmid restored all defects and produced a wild-type *S. typhimurium* phenotype. Furthermore, functional down-regulation of basolateral TLR5 completely inhibited the monolayers’ ability to respond to both wild-type *S. typhimurium* and purified flagellin but had no effect on tumor necrosis factor α-induced responses. We therefore conclude that *S. typhimurium* fliE is essential for flagellin secretion, flagellar assembly, and *S. typhimurium*-induced proinflammatory responses through basolateral TLR5 and is consistent with the emerging model of *S. typhimurium* flagellin-induced inflammation.

The epithelial cells lining the gastrointestinal tract form a highly specialized barrier that separates two very distinct environments, thus maintaining the delicate balance between the gut lumen and the underlying tissue (1, 2). As part of its barrier function, the intestinal epithelium is able to detect surface attached enteric pathogens like *Salmonella enterica*, serovar *Typhimurium* (*S. typhimurium*), and orchestrate a proinflammatory response. This multifaceted response involves the rapid secretion of chemotactants, the development of a chemotactic gradient in the surrounding subepithelial matrix (3–5), and the migration of neutrophils to the site of infection, ultimately triggering secretory diarrhea (6–11). The concurrent apical release of a soluble factor, designated PEEC (for pathogen-glicited epithelial chemoattractant), aids in directing the final polymorphonuclear leukocyte (PMN) movement across the epithelial tight junctions into the gut lumen (12). In addition to IL-8, a variety of other chemokines are secreted in response to *S. typhimurium* attachment (13–15). *Salmonella*-induced secretion of IL-8 is known to require the activation of the nuclear transcription factor NF-κB (16, 17). In quiescent cells, the NF-κB heterodimer (p50/p65) is held in a stable, inactive, cytoplasmic complex with IκBα molecules until activated by an as yet undefined *Salmonella*-stimulated proinflammatory signaling cascade. The IκBα molecule is then phosphorylated and degraded, thus releasing the NF-κB molecule to translocate to the nucleus, bind the IL-8 promoter, and induce transcription of the IL-8 gene (16).

The flagellae of *S. typhimurium*, like many other motile bacteria, are comprised of the basal body, the hook, and the filament, which is composed mainly of the protein FljB or FljB (dependent on phase variation) (18). The assembly of flagellae in *Salmonella* is similar to the secretion of virulence factors and requires the complex regulation of export machinery across both inner and outer bacterial cell membranes. Thus, it is not surprising that certain components of the secretion system utilized in flagellar biosynthesis are structurally and functionally homologous to components of the type III secretion system used for the export of virulence factors in both *S. typhimurium* (19–21) and *Shigella* (22). Additionally, the significant structural and functional similarities between these two secretory systems may indicate that flagellar export machinery is an additional mechanism for secretion of virulence factors from *S. typhimurium* (23) and other pathogens (24).

Recently, interest has been focused on *Salmonella* flagellin and its role in the induction of host proinflammatory responses. Studies have demonstrated that purified flagellin can induce

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§ The abbreviations used are: IL, interleukin; MDCK, Madin-Darby canine kidney; TNF, tumor necrosis factor; Kan, kanamycin; ELISA, enzyme-linked immunosorbent assay; PMN, polymorphonuclear leukocyte(s); PBS, phosphate-buffered saline; WT, wild type.
proinflammatory mediators in epithelial cells (25) and must be translocated across the epithelial monolayer to the basolateral surface for these responses to occur (26). Additionally, flagellin has been shown to interact with the basolateral Toll-like receptor 5 (TLR5) on human intestinal epithelial cells (27) and can induce systemic responses in mice that require the cytosolic adaptor protein Myd88 (28).

Here we demonstrate that a Salmonella mutant in which the fliE gene has been disrupted by a Tn-10 transposon insertion (29) retains the ability to interact with intestinal epithelial cell apical membranes, but such interactions are nonproductive in terms of signaling epithelia to initiate proinflammatory signaling cascades. Disruption of fliE results in Salmonella mutants that are unable to secrete flagellin or assemble flagellae. Complementation of the fliE mutant strain with a plasmid containing wild-type fliE results in recovery of a wild-type Salmonella phenotype. We have also established for the first time that TLR5 is expressed exclusively on the basolateral membrane of Madin-Darby canine kidney cells and is functionally down-regulated by preincubation with purified flagellin. This TLR5 functional down-regulation results in monolayers of T84 cells that do not secrete TNFα-stimulated IL-8 secretion.

T84 cells were grown on 96-well polycarbonate plates (MDCK) (Costar Corp., Cambridge, MA). Transcomplementation of KAR729 was carried out using plasmid pMM1001 containing wild-type fliE cDNA (a gift from R. M. Macnab). Competent KAR729 cells were transformed with plasmid DNA, and amp'/kan' colonies were selected for further investigation; this new strain was designated KAR729(pMM1001).

Induction of fliE—Confluent epithelial monolayers were placed on a 0.4-μm pore-size filter in an inverted 24-well plate. MDCK cells were grown as stated above and equilibrated with 1.0 ml of Hank's balanced salt solution plus Ca2+, Mg2+; 14 mM NaHCO3, 100 units/ml penicillin, 8 mg of ampicillin, and 90 mg of streptomycin per ml; and 5% F-12 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 14 mM NaHCO3. Monolayers were incubated at 37°C for 4 h. The motility of each strain and incubated for 1 h at 37°C. Monolayers were then carefully washed of excess bacteria and plated in 300 μl of HBSS++ in the lower (basolateral) well and incubated at 37°C. The basolateral medium was collected 4 h later, and the concentration of IL-8 was analyzed by ELISA. For cells in the 96-well format, the final 30 min was modified; 50 μl of a 1% Triton X-100 solution in 10 mM EDTA was added to the upper surface of the cells. Negative controls with HBSS++ alone and positive controls with TNFα (100 ng/ml) added to the basolateral medium were employed in each assay.

IL-8 ELISA—IL-8 was measured by a sandwich enzyme-linked immunosorbent assay (ELISA). For T84 cells, the assay was performed as previously described (3), with some modifications. For MDCK cells, a canine ELISA was developed. For both assays, 96-well plates were coated with 100 μl of 0.1% bovine serum albumin (Costar) and utilized 3–5 and 6–14 days postseeding, respectively. T84 monolayers received weekly and MDCK biweekly feedings postseeding. Cultured inverted monolayers of T84 cells, for PMN transmigration studies, utilized 5.0-μm pore size and were constructed as previously described (31, 32). All culture medium supplies were purchased from Invitrogen.

Bacterial Strains, Plasmids, and Growth Conditions—All S. typhimurium and Escherichia coli strains were grown in LB medium at 37°C. T84 monolayers were maintained in a 1:1 mixture of Dulbecco-Vogt modified Eagle's medium and Ham's F-12 medium supplemented with 15 mM HEPES buffer (pH 7.5) (Sigma); 14 mM NaHCO3, 40 mg of penicillin, 8 mg of ampicillin, and 90 mg of streptomycin per ml; and 5% newborn calf serum. MDCK monolayers were maintained in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 14 mM NaHCO3, 100 units/ml penicillin, and 100 μg/ml streptomycin. Monolayers of T84 and MDCK cells were grown on 96-well polycarbonate plates (MDCK) (Costar Corp., Cambridge, MA) or 0.33-cm2 ring-supported polycarbonate filters (T84 and MDCK) (Corning). The total culture area (basal) was used to evaluate the motility of the following S. typhimurium strains: wild type (y3306), Phop' (CS022), fliE knock-out (KAR729), and transcomplemented fliE (KAR729(pMM1001)). Small wells were made in semisolid agar plates, loaded with 5 μl from a maximally invasive culture (described above) of each strain and incubated at 37°C for 4 h. The motility of each Salmonella strain in the agar was evaluated by the diameter of the "halo" of colony growth produced.

S. typhimurium Adherence to and Invasion of Epithelial Monolayers—Adherence of T84 or MDCK epithelial monolayers was performed by the method described previously (3). Monolayers were placed in a 24-well tissue culture plate with 300 μl of HBSS++ added to the lower (basolateral) well and 100 μl of HBSS++ added to the upper (apical) well. After a 30-min equilibration, 10 μl of bacterial solution (~20 bacteria/epithelial cell) was added apically, and bacterial adherence and invasion were assessed after 1 h. Cell-associated bacteria represent bacteria adhered to and/or internalized into the monolayers and were released by incubation with 100 μl of 1% Triton X-100 (Sigma). Internalized bacteria were those obtained after lysis of the monolayer with 1% Triton X-100 followed by a 45-min incubation with gentamicin (50 μg/ml). For both assays, bacterial density was carried out as described above.
Salmonella-induced Inflammation Requires FliE Expression

Transmission Electron Microscopy—Bacterial pellets were fixed in 3% glutaraldehyde and 1% paraformaldehyde and processed for embedding in Epon. Semithin (0.1 μm) and ultrathin (0.05 μm) sections were stained with uranyl acetate and lead citrate and observed in a Hitachi 7100 transmission electron microscope.

RESULTS

Tn-10 Insertional Inactivation of Salmonella fliE Prevents Induction of IL-8 Secretion—Approximately 1000 Salmonella Tn-10 mutants were screened for their ability to induce the secretion of IL-8 using a canine IL-8-specific ELISA and MDCK cells in a 96-well format. KAR729 was identified. B, WT (y3306), PhoPc (CS022), and fliE mutant (KAR729) S. typhimurium strains were assessed for their ability to induce IL-8 secretion from filter-grown MDCK cell monolayers. Basolateral supernatants collected from apically infected filter-grown MDCK cell monolayers were analyzed by ELISA. Control MDCK monolayers were incubated with buffer alone, whereas positive controls were incubated with TNFα added to the basolateral buffer. Data are presented as the mean ± S.D. of assays performed in triplicate. C, schematic diagram of Tn-10 transposon disruption of the fliE gene of S. typhimurium KAR729. S. typhimurium genomic DNA EcoRI fragments were cloned into pBluescript and sequenced using primers to T7 and M13 as well as primers to regions of the KanR cassette adjacent to the insertion sequences (IS10) of the Tn-10 transposon. Allocation of the genes upstream and downstream of fliE is based on sequence analysis of the 5′ and 3′ DNA regions.
Salmonella-induced Inflammation Requires FliE Expression

Salmonella-induced inflammation requires FliE expression. FliE is a flagellar protein that is normally found in abundance in the cell. It is involved in the assembly of the bacterial flagellum, which is essential for bacterial motility.

To determine whether the biochemical and morphological lack of flagellin observed in KAR729 had an effect on bacterial motility, we performed a motility assay. Semisolid agar provides sufficient physical resistance to nonflagellated bacterial strains to prevent their movement and spread through the medium but allows free movement of flagellated strains. As demonstrated in Fig. 2C, wild-type \( \chi_{3306} \) was able to easily move through the semisolid agar and form a large colony; however, the less flagellated \( E. coli \) and PhoP\(^\text{a} \) formed only small colonies. Not surprisingly, we observed a complete lack of movement by KAR729 (Fig. 2C). Taken together, these data support the idea that the Tn-10 disruption of \( \text{fliE} \) in KAR729 produces a nonflagellated Salmonella strain.

Given the complete lack of flagellae in KAR729, we next assessed whether the inability to induce IL-8 secretion from host epithelial cells was a result of an inability to interact with the apical cell surface of host epithelium. We have previously shown that adherence, but not invasion, is required for productive interactions between Salmonella and host epithelium (40). Surprisingly, the ability of KAR729 to adhere to and invade both MDCK and T84 cell monolayers was virtually identical to wild-type \( \chi_{3306} \) (Fig. 3, A and B). As expected, the noninvasive mutant PhoP\(^\text{a} \) showed both reduced adherence and very low levels of invasion (Fig. 3, A and B). These data demonstrate that the failure of KAR729 to induce IL-8 secretion from epithelial monolayers is not a result of an inability to properly interact with the apical membrane.

KAR729 Induces Rapid Host Cell Cytoskeletal Remodeling but Fails to Induce Early Events Required for Nuclear Signalization—During the initial stages of bacterial-host interactions, Salmonella uses a type III secretory apparatus to inject bacterial effector proteins into the host cytoplasm (for a review, see Ref. 21), many of which have been shown to reorganize actin filaments just beneath the sight of bacterial attachment (41, 42). Since KAR729 physically interacts with the apical surface of host cells but fails to initiate pro-inflammatory responses, we sought to determine whether KAR729 could induce the rapid actin cytoskeletal remodeling. In monolayers labeled with both rhodamine phalloidin and an anti-Salmonella antibody, confocal microscopy of infected monolayers revealed that the KAR729 mutant not only adhered to the apical surface of epithelial cells but also induced localized alterations in the host cell cytoskeleton similar to the wild-type \( \chi_{3306} \) (Fig. 3C).

Following the rapid cytoskeletal remodeling, early signaling events result in activation of the IxB-kinase complex, followed by nuclear translocation of the transcription factor NF-xB and nuclear signaling. This process involves phosphorylation of the inhibitory molecule IxBa by IxB-kinase, followed by ubiquitination and subsequent degradation in a proteasome. Degradation of IxBa provides an excellent indicator of activation of the NF-xB signaling pathway. As shown in Fig. 4, incubation of monolayers with basolateral TNFa or apical wild-type \( \chi_{3306} \) led to an almost complete degradation of IxBa, as detected by immunoblot. However, monolayers that were incubated with either PhoP\(^\text{a} \), KAR729, or the nonflagellated \( \text{fliC}^-/\text{fliB}^- \) had levels of IxBa comparable with that seen in untreated control monolayers (Fig. 4). Taken together, these results demonstrate that while KAR729 has retained the ability to induce rapid localized cytoskeletal remodeling in the host cell, it has lost the ability to stimulate signaling pathways that lead to IxBa degradation and the subsequent NF-xB-mediated cytokine expression.
then determined for each bacterial strain (see procedures). A noninvasive S. typhimurium strain PhoP- was used as a negative control. Data are presented as the mean ± S.D. of triplicate samples. C. filter-grown monolayers were infected apically for 30 min at 37 °C with WT (χ3306) or KAR729. Cells were fixed, permeabilized, and stained with rhodamine phalloidin and anti-Salmonella mAb followed by a fluorescein isothiocyanate-conjugated secondary Ab (see “Experimental Procedures”). Intact cell monolayers were optically sectioned every 0.5 μm. Horizontal (x-y) sections were taken in the plane of the apical (Ap) membrane (lower panels). Digitally compiled vertical (x-z) sections perpendicular to the plane of the apical membrane are shown directly above x-y sections. The arrows indicate sites of bacterial invasion and actin remodeling.

KAR729 Does Not Induce Spontaneous Transepithelial Migration of PMN—The proinflammatory program in epithelia, which is triggered by apical membrane attachment of wild-type S. typhimurium, orchestrates PMN movement toward and across the epithelium (9, 40, 43). We next sought to establish whether PMN transepithelial migration is induced by apical colonization of T84 monolayers with KAR729. Inverted confluent monolayers were used to analyze directed neutrophil migration in the physiologically relevant basolateral to apical direction. In this assay, the apical surface of T84 cell monolayers was incubated for 1 h in the presence of bacteria followed by washing to remove nonadherent bacteria. We then assessed the ability of the infected epithelial monolayers to orchestrate the PMN trans-epithelial migration by adding human PMN to the basolateral compartment. As shown in Fig. 5, we found that not only KAR729, but also fliC–/fliB- (the control nonflagellated strain) had a dramatically reduced (~90%) ability to direct the movement of PMN to the apical aspect of the monolayers when compared with wild-type χ3306.

Trans-complementation of KAR729 with Wild-type fliE Restores a Wild-type S. typhimurium Phenotype—To determine whether the observed inability of KAR729 to induce signal transduction events necessary to promote IL-8 secretion is directly a result of fliE inactivation, we have complemented the defective fliE in KAR729 with wild-type fliE on an expression plasmid. The inducible expression plasmid pTrc99A carrying the wild-type fliE gene (pM1001) was introduced into KAR729 S. typhimurium cells by transformation. All of the resulting colonies were resistant to ampicillin and had normal colony morphology indistinguishable from wild-type χ3306. The addition of wild-type fliE into the KAR729 mutant resulted in the inducible expression of flagellin as detected by immunoblot of bacterial protein extracts and Transmission Electron Microscopy (Fig. 6, A and B). The expression of wild-type fliE in KAR729 restored the ability of this strain to move through semisolid agar in a manner identical to wild-type χ3306 (Fig. 6C). More importantly, expression of wild-type fliE reestablished the ability of the KAR729 mutant to induce IκBα degradation (Fig. 6D), provoke IL-8 secretion (Fig. 6E), and stimulate PMN transepithelial migration from basolateral to apical surface across T84 cell monolayers (Fig. 6F). Taken together, these data demonstrate that the only defect in KAR729 was the lack of FliE, and restoration of a wild-type S. typhimurium phenotype was achieved by reintroduction of the wild-type fliE gene.

Functional Down-regulation of Basolaterally Expressed Toll-like Receptor 5 (TLR5) Inhibits Salmonella- and Flagellin-induced Epithelial Secretion of IL-8—It is becoming increasingly clear that Salmonella-induced epithelial proinflammatory responses are mediated by soluble flagellin through interaction with basolateral TLR5 (25–28). Since our model epithelia are unresponsive to Salmonella mutants lacking flagellin secretion (KAR729, fliC–/fliB-), we sought to determine whether MDCK cells express TLR5 in a polarized manner and, second, whether functional down-regulation of TLR5 would inhibit the monolayers ability to respond to Salmonella.
Using cell surface-specific biotinylation, MDCK cell monolayers were incubated with sulfo-N-hydroxysuccinimide-biotin (Pierce) applied to either the apical or basolateral chambers, quenched, lysed, and affinity-purified with streptavidin-agarose beads (Pierce). The isolated proteins were separated by SDS-PAGE and immunoblotted with anti-TLR5 antibody (Santa Cruz Biotechnology). MDCK monolayers expressed TLR5 predominantly (~95%) at the basolateral membrane as a ~100-kDa doublet that is a result of differential glycosylation (Fig. 7A). Thus, it is clear that MDCK monolayers have the same polarized TLR5 expression as has been previously reported for human T84 cell monolayers (27). We next sought to determine whether the epithelial proinflammatory response to Salmonella or purified flagellin could be inhibited by functional down-regulation of basolateral TLR5. This was achieved by preincubation of monolayers for 24 h with medium containing purified flagellin in the basolateral compartment. The monolayers were washed extensively and then incubated for 5 h with either wild-type Salmonella (γ3306) applied apically or purified flagellin in the basolateral chamber. The basolateral supernatants were collected, and secreted IL-8 was quantified by a
canine-specific ELISA. Control monolayers that were not pre-incubated with basolateral flagellin responded normally to wild-type Salmonella, basolateral flagellin, and TNFα (a positive control) (Fig. 7B). However, monolayers that had been pre-incubated with basolateral flagellin did not secrete IL-8 in response to either wild-type Salmonella or basolateral flagellin, while the response of the monolayers to TNFα was unaffected (Fig. 7B). Taken together, these results dramatically demonstrate three things: first, MDCK monolayers are equipped with basolateral TLR5 exquisitely poised to respond to Salmonella colonization in a polarized manner; second, that functional down-regulation of TLR5 completely blocks the ability of the epithelial monolayer to mount a proinflammatory response to Salmonella; and, finally, that the flagellin/TLR5 interaction is the sole mediator of the epithelial proinflammatory response to Salmonella.

DISCUSSION

The goal of this study was to identify novel S. typhimurium genes involved in the pathogen-induced proinflammatory response. This was achieved by creating random Tn-10 transposon insertion mutants, which were then screened for a reduction in their ability to induce epithelial IL-8 secretion. A single mutant was identified containing an insertional inactivation of the Salmonella fliE gene that not only completely lacked the ability to induce epithelial IL-8 secretion but also lacked flagellae and flagellin secretion. Using genetic, biochemical, and cell biological methods, we have demonstrated the role of fliE in the secretion and assembly of flagellin into flagellar structures and verified its importance in the inflammatory process.

Disruption of the S. typhimurium fliE gene prevents the secretion of flagellin and flagellar assembly. The product of the fliE gene, a protein of ~11 kDa, has been previously localized to the basal body of the flagellum (23, 44). FliE has also recently been shown to interact with the flagellar hook protein FlgB, a component of the basal body, and is thought to be required for the export of flagellar hook and rod components (39, 40, 44, 45). Although the precise role of FliE in flagellar assembly is not well understood, our results are in agreement with data from Komoriya et al. (23), who previously observed that any defect in a flagellar rod protein resulted in the absence of flagellae. We have also found that while this lack of flagellar assembly prevents bacterial locomotion, it does not affect bacterial adherence or invasion of epithelial cells. Presumably, the Salmonella type III effector proteins necessary for invasion are secreted normally, since rapid, localized actin remodeling and bacterial invasion were observed with the fliE-deficient mutant. This is consistent with recent observations that nonflagellated S. typhimurium mutants secrete higher levels of type III virulence factors and suggests a putative relationship between the two secretion systems (23).

Orchestration of PMN migration across an epithelial monolayer in response to S. typhimurium colonization requires recognition of the bacterial invader in a milieu of prokaryotic flora before secretion of basolateral IL-8 and apical PEEC can occur. Previously accepted models of Salmonella-induced proinflammatory responses developed from studies in nonpolarized cells relied on Salmonella secretion of type III effector proteins to induce nuclear events necessary for proinflammatory responses (46, 47). However, two recent studies have demonstrated a definite link between flagellin secretion by Salmonella and induction of proinflammatory responses in epithelial cells (25, 26). Of late, the role of flagellin (FlgC) in stimulating the proinflammatory response through TLR5 receptors has been reported (27, 28). We have shown that Salmonella foci infect epithelial cells in polarized monolayers, yet even those epithelial cells that are not surface-colonized uniformly signal through nuclear NF-κB (48). This result suggested (and it was subsequently shown) that epithelial transcytosis of a proinflammatory factor that diffuses laterally has an affect on all cells in the monolayer, thus explaining the discrepancy between focal infection and the uniform epithelial response. Additionally, flagellin by itself is able to induce epithelial proinflammatory activation when added basolaterally but not apically (26).

Not surprisingly, we have found that fliE-dependent secretion of flagellin is necessary to induce proinflammatory activation of the IκBα/NF-κB signaling pathway, IL-8 secretion, and PMN trans-epithelial migration. This fliE-deficient phenotype can be completely rescued by trans-complementation with the wild-type fliE gene. This is the first report of the regulatory role of FliE expression on the secretion of flagellin. Our results complement previous work demonstrating the role of flagellin in the innate immune response and would seem to be in contrast to the previous notion that Salmonella type III effector proteins translocated into the cytoplasm directly activate proinflammatory nuclear signaling events (46). However, the two ideas may not be mutually exclusive; secreted flagellin can activate proinflammatory signaling through the basolateral TLR5, and type III effectors may provide the stimulus by which flagellin is transcytosed across the epithelium. This idea is in complete agreement with a recent study that demonstrates that translocation of flagellin is critical for eliciting proinflammatory responses (26). However, flagellin is also synthesized and secreted by many nonpathogenic bacteria that are capable of colonizing the apical surface of epithelial monolayers, yet these bacteria do not induce a proinflammatory response. In aggregate, these findings paired with other current findings suggest that a critical difference between pathogenic and non-pathogenic bacteria is the ability to induce transcytosis of flagellin. In turn, this suggests that bacterial virulence factors, probably provided by injection via the type III secretory apparatus, may play an important role in usurping the transcytotic pathway as a means of delivering a critical proinflammatory factor (flagellin) to the responsive basolateral domain.

In summary, we have established that expression of the S. typhimurium fliE gene is necessary for bacterial secretion of flagellin and the assembly of functional flagellae. Additionally, using physiologically relevant epithelial model systems, we have demonstrated that S. typhimurium fliE-regulated secretion of flagellin is necessary to stimulate epithelial proinflammatory pathways that lead to IL-8 secretion and ultimately direct PMN trans-epithelial migration. While FliE is not a direct mediator of this immune inflammatory response, it does play a pivotal role in the secretion of flagellin. We have also demonstrated that TLR5 signaling can be functionally down-regulated by preincubation with purified flagellin. Further characterization of the mechanisms by which flagellin is translocated across the intestinal epithelium and how it interacts with TLR5 to induce proinflammatory signals may provide a better understanding of the aberrant signaling that occurs in chronic inflammatory diseases of the intestine.

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