Salmonella typhimurium translocates flagellin across intestinal epithelia, inducing a proinflammatory response

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This study investigated whether soluble paracrine factors mediated Salmonella-induced IL-8 expression in polarized model intestinal epithelia. We found that the basolateral media of model epithelia that had been apically infected with Salmonella typhimurium for a short period (10 minutes) could activate IL-8 secretion in virgin model epithelia, demonstrating that a proinflammatory factor (PIF) was indeed present. Initial characterization found that PIF was a heat-stable protein with a molecular mass of about 50 kDa that acts on the basolateral, but not apical, surface of model intestinal epithelia to elicit IL-8 secretion. PIF was not present in the media of model epithelia stimulated with other inducers of IL-8 secretion (TNF-α or carbachol) but was present in S. typhimurium supernatants, indicating PIF is of bacterial origin. PIF was purified from bacterial culture supernatants by anion/cation exchange chromatography and SDS-PAGE and found by using microsequencing to be the protein flagellin. In support of this finding, flagellin-deficient S. typhimurium mutants did not secrete detectable levels of PIF (i.e., a bioactivity that induced IL-8 secretion when placed basolaterally on model epithelia). Furthermore, viable flagellin-deficient mutant organisms (fliC/fljB and flhD) failed to elicit IL-8 secretion when added apically to model intestinal epithelia. These findings indicate that translocation of flagellin across epithelia, subsequent to apical epithelial–S. typhimurium interaction, is likely a major means of activating a mucosal inflammatory response.


Introduction

Infection of humans by the enteric pathogen Salmonella typhimurium generally results in severe abdominal cramping and diarrhea. These symptoms may largely result from the mucosal immune response elicited by this pathogen. Specifically, colonization of the human intestine by S. typhimurium leads to infiltration of polymorphonuclear leukocytes (PMNs) into the intestinal epithelium culminating in the formation of an intestinal crypt abscess (1). The action of these PMNs on the epithelium and the loss of barrier function that results are thought to be key events in mediating the clinical manifestations of Salmonellosis (for review see ref. 2). Interestingly, the pathobiology of the acute flares of inflammatory bowel disease appears to be very similar to that of Salmonellosis (2), though efforts to identify a responsible organism (or other provoking agonist) have not been successful.

We have shown previously that the first cells colonized by S. typhimurium, the intestinal epithelial cells, play an important role in directing the PMN movement that occurs in response to this pathogen. Specifically, in response to contact between S. typhimurium and their apical membranes, intestinal epithelial cells synthesize and secrete a panel of chemokines that participate in the recruitment of immune cells (3–5). One of the best characterized of these chemokines is the neutrophil chemoattractant IL-8. IL-8 is secreted basolaterally by polarized model intestinal epithelia such that it imprints a haptotactic gradient for PMNs on the subepithelial matrix, thus likely directing the movement of extravasated PMNs through the lamina propria (6).

We have shown that S. typhimurium induces IL-8 secretion via Ca++- mediated activation of the transcription factor NF-κB (7). However, the molecular interactions that activate these epithelial signaling pathways remain undefined. Morphologic studies of S. typhimurium adherence to intestinal epithelia indicate that this pathogen does not bind uniformly to epithelial cells, but rather a subset of epithelial cells bound (or internalized) many bacteria whereas most epithelial cells appeared free of attached (or internalized) organisms (8). However, the level of IL-8 secreted by S. typhimurium–infected model epithelia is similar to that observed in response to soluble agonists such as TNF-α that sought to uniformly activate these epithelial cells (7), suggesting that noncolonized epithelial cells might be involved in the IL-8 secretory response. Therefore, we...
speculated that *S. typhimurium* might induce the epithelial secretion of a proinflammatory factor (PIF) that would induce NF-κB activation and IL-8 secretion from cells without associated bacteria. The data herein show that, indeed, the basolaterally conditioned media of apically infected model intestinal epithelia possess the ability to elicit IL-8 secretion from unstimulated epithelia. However, to our surprise, we found the responsible factor was not of epithelial origin but rather related to translocation of the bacterial protein flagellin across epithelia where it subsequently acts on the basolateral surface of these cells to induce proinflammatory signaling events.

**Methods**

**Bacterial culture.** *S. typhimurium X1306* and SL3201 (wild-type strains), *S. typhimurium* PhoP+ and HilA (invasion-defective strains), and *Escherichia coli* F-18 (normal gut strain) were maintained as described previously (4, 6, 9). Flagellar mutants were prepared from the SL3201 strain (10). The *flbD* gene (flagella master operon) was mutated by P22 transduction of *flbD:Tn10* from strain KK2040 (kind gift of K. Kutsukake, Hiroshima University, Hiroshima, Japan) (11). The flagellar mutants SL3201 *fljB:Mu4*, SL3201 *flc:Tn10*, and SL3201 *fljB:Mu4* *flc:C:Tn10* were described previously (12). SL3201 *fljC* was transformed with the *fljC*-containing plasmid pKK1012 (13) and the *fljB*-containing plasmid pKK1025 (14), both of which were received from K. Kutsukake. *S. typhimurium* mutants *invA* and *invG*, and their parent strain SLR11, were received from Katherine Reed (Institute for Animal Health, Berkshire, United Kingdom). These studies also used seven strains of “normal” gut *E. coli.* *E. coli* F-18 (serotype rough:K1:H5) (15) was received from Beth McCormick (Harvard University Medical School, Boston, Massachusetts, USA). *E. coli* H5 was previously isolated from human feces (16). Additionally, five gut *E. coli* isolates obtained from the feces of healthy donors, as described previously (17), at the University of Illinois Medical Center (Chicago, Illinois, USA) were generously supplied by Gail Hecht (these five strains were assigned numbers 4–8 by Hecht). Cultures were prepared for experimental use under nonagitated, microaerophilic conditions by culturing 0.01 ml of stationary-phase culture in 10 ml of Luria broth (LB) overnight (15–18 hours) at 37°C, as outlined previously (4, 18). Bacterial overnight cultures were washed two times and concentrated 33-fold in HBSS. Samples were incubated overnight (15–18 hours) at 37°C. The bacterial cultures were reseeded and then incubated at 37°C for 55 minutes (unless otherwise specified). The model epithelia were then discarded and the basolateral media collected. The basolateral, PIF-containing media was subsequently filtered (0.22-μm pore; Millipore Corp., Bedford, Massachusetts, USA), boiled at 100°C for 20 minutes (except in initial experiments), then stored for later use at −20°C.

**Isolation of crude PIF (from bacterial culture supernatants).** Bacterial overnight cultures were washed two times and concentrated 33-fold in HBSS. Samples were incubated for 1 hour at 37°C. After the incubation, the bacteria were removed by centrifugation, and the PIF-containing supernatant was filtered (0.22-μm pore; Millipore Corp.) and boiled at 100°C for 20 minutes. The PIF-containing supernatant was then stored for later use at −20°C.

**Bacterial-induced IL-8 secretion.** IL-8 secretion in response to live apically added bacteria was assayed as described previously (7, 20).

**PIF-, TNF-α-, and carbachol-induced IL-8 secretion.** IL-8 was induced in the same manner except that PIF (various specified dilutions), recombinant TNF-α (R&D Systems Inc., Minneapolis, Minnesota, USA), and carbachol (100 μM), were added basolaterally. Epithelia were then incubated for 5 hours at 37°C. IL-8 levels were determined by ELISA, as described previously (9).

**Transepithelial electrical resistance measurements.** Apical and basolateral reservoirs of T-84 intestinal epithelia were connected to a voltage clamp using a pair of calomel and Ag-AgCl electrodes via agar bridges as described previously (19). The changes in potential differences generated by the passage of 25-μA current pulses were measured, and resistance was calculated as outlined previously (19). *Clostridium difficile* toxin A (a generous gift of Richard J. Obiso, Jr., East Tennessee State University, Johnson City, Tennessee, USA) (40 ng/ml) and PIF were added basolaterally.

**Purification of PIF: production of PIF-containing culture supernatants.** Three liters of LB was inoculated with 3 ml of a stationary-phase bacterial culture and incubated overnight (15–18 hours) at 37°C. The bacterial cultures were then concentrated 100-fold by centrifugation in HBSS and incubated for 1 hour at 37°C. After the incubation, the bacteria were removed by centrifugation, the PIF-containing supernatant was filtered (0.22-μm pore; Millipore Corp.) and boiled at 100°C for 20 minutes. The PIF-containing supernatant was then stored for later use at −20°C.

**Purification of PIF: gel filtration chromatography.** PIF was purified using a Biologic HR chromatography system (Bio-Rad Laboratories, Hercules, California, USA). This system was connected to a flow adapter (Bio-Rad Laboratories) and glass Econo-column (75 cm, 124 ml; Bio-Rad Laboratories) packed with 125 ml of Superose-6.
S. typhimurium could cause the epithelial exocytosis of a proinflammatory mediator that could then activate IL-8 expression by a mechanism similar (or identical) to that used by TNF-α. Thus, we collected the conditioned media of S. typhimurium–infected model epithelia 1 hour after application of the bacteria and examined the ability of these samples to activate IL-8 secretion in virgin (i.e., noninfected) epithelia. After filtration through a 0.2-μm pore filter to remove bacteria, the apically conditioned media was transferred to the apical surface of virgin epithelia while the basolaterally conditioned media was transferred to the basolateral aspect of an additional virgin epithelia. Five hours later, we measured the IL-8 concentration in the basolateral reservoir because IL-8 secretion is known to occur in a basolaterally polarized manner regardless of the type or location of stimulus (23). As shown in Figure 1a, transfer of basolaterally conditioned media, but not apically conditioned media, significantly induced IL-8 secretion, indicating the existence of a PIF. Nonphysiological transfer of apically conditioned media to the basolateral surface of virgin epithelia, but not transfer of basolaterally conditioned media to the apical surface, also induced IL-8 secretion (data not shown), suggesting the response to PIF, rather than the release of PIF, was polarized (addressed later in this article). PIF bioactivity (i.e., ability to induce IL-8 secretion) became detectable in the basolateral media 15 minutes after apical addition of the

![Figure 1](http://www.jci.org/)

Figure 1

Basolaterally conditioned media of S. typhimurium–infected epithelia induce IL-8 secretion in virgin epithelia. Model intestinal epithelia were apically exposed to S. typhimurium. The epithelial media was then transferred to untreated model epithelia. IL-8 was assayed in basolaterally conditioned media of S. typhimurium–infected epithelia 1 hour after addition of bacteria. Live S. typhimurium (added apically for 5 hours) served as a positive control. A) Basolateral media was transferred at the indicated time after addition of bacteria.
PIF is a heat-stable protein. Samples from a and b were applied basolaterally to model epithelia, and their ability to induce IL-8 secretion over a 5-hour period was measured. (a) PIF was centrifuged through indicated Amicon concentrators followed by dilution of retentate to original volume. (b) PIF, TNF-α (20 ng/ml), and carbachol (100 μM) were subjected to boiling (20 minutes) or proteinase K treatment (1 mg/ml for 1 hour followed by 10 minutes of boiling to inactivate proteinase K).

PIF bioactivity can be isolated from bacterial supernatants. Having failed to obtain PIF secretion in a bacteria-free assay, we next sought to identify a PIF bioactivity in supernatants of bacteria that had not interacted with epithelial cells. The same S. typhimurium inoculum that we typically use to induce epithelial IL-8 secretion was harvested by centrifugation, washed in HBSS, and incubated for 1 hour at 37°C. The culture was then centrifuged and the supernatant filtered through a 10-kDa MW cut-off filter. The retentate exhibited no more ability to induce IL-8 secretion than a similarly treated retentate (carbachol-containing) that had not been preconditioned by epithelia, indicating that carbachol, like TNF-α, does not induce PIF secretion from model epithelia.

PIF bioactivity can be isolated from bacterial supernatants.
off filters (Figure 4b). These results indicated that PIF is likely of bacterial origin. We next investigated whether bacterial LPS was involved in this response by using polymyxin B–coated agarose to remove this compound from PIF (24). Removal of endotoxin in this manner did not result in any loss of PIF activity (control and polymyxin B bead–treated PIF induced 1.54 ± 0.23 and 1.64 ± 0.27 ng/ml IL-8 secretion, respectively). To be sure that the polymyxin B agarose did actually remove endotoxin, we verified that our treatment abolished (99.98 ± 3.2 % inhibition) the ability of a solution of LPS to activate IL-8 secretion in HUVECs (a well-characterized LPS responsive cell type). Thus, LPS is not a component of PIF.

PIF represents flagellin. We first sought to obtain PIF’s MW by using gel-filtration chromatography. Bacterial supernatant was filtered (0.2-μm pore size), applied to a Superose column (13-μm particle size), and fractions assayed for PIF activity by measuring their ability to induce IL-8 secretion from model epithelia. PIF bioactivity eluted as a single peak with a molecular mass of about 50 kDa. We purified PIF by an ion exchange chromatography, using as a starting material bacterial supernatants that had been boiled, filtered, and concentrated tenfold with a 30-kDa MW cut-off filter. By performing test bindings, we observed that PIF bioactivity readily bound to anion exchange, but not cation exchange resins. Thus, we used cation exchange beads to remove proteins not related to PIF bioactivity and then applied the resulting sample to a Q-Sepharose anion-exchange column. PIF bioactivity eluted from the anion-exchange column as a single peak that upon SDS-PAGE analysis contained a prominent 50-kDa band (Figure 5). This band was digested with trypsin, extracted from the gel, analyzed using amino acid sequencing (Edman degradation), and the sequences FNSAITNLG and TTSYT obtained. A search of the Salmonella database indicated that the first sequence was from FliC and the second from FljB. Thus, the band contained both of the two known S. typhimurium isoforms of the protein flagellin, the primary structural component of flagella.

We next investigated whether we had indeed purified the protein responsible for PIF bioactivity. S. typhimurium has two highly homologous genes for flagellin, and growth conditions may dictate which isoform is expressed. Functional flagella can be made from either protein. We thus generated S. typhimurium strains deficient in flagellin (lacking the genes for both fliC and/or fljB). We isolated supernatant from these strains and their wild-type parent and measured the ability of these supernatants to induce IL-8 secretion when applied to the basolateral surface of model intestinal epithelia. In
striking contrast to the wild-type strain, the flagellin-deficient mutant (fliC/fljB) supernatant did not display any IL-8–inducing bioactivity, even when added at a 1,000-fold greater concentration (Figure 6). We also analyzed whether S. typhimurium deficient in either, but not both, fliC or fljB secreted the IL-8–inducing factor. The supernatants of either of these two strains contained significant, albeit somewhat reduced, levels of IL-8–inducing activity when compared with the wild-type strain. Thus, although our wild-type cultures (originating from a single colony) contain a mix of FliC and FljB (perhaps the result of a growth-phase change during overnight incubation), either flagellin isoform can function as a PIF. The relatively reduced potencies of supernatants lacking FliC or FljB probably resulted from a lower concentration of flagellin rather than synergy between the two flagellin isoforms present in the wild-type supernatant (see below). Lastly, we verified that we could rescue the inability of flagellin-deficient S. typhimurium supernatants to induce IL-8 secretion by transforming them with plasmids encoding fliC or fljB. As shown in Figure 7b, transformation with either flagellin gene restored the ability of the organism’s supernatants to induce IL-8 secretion, further confirming our conclusion that FliC and FljB are proinflammatory factors.

**Bacterial-induced PIF responses require epithelial translocation of flagellin.** Many flagellated bacteria will release flagellin into their media, and thus supernatants of most Salmonella strains would be expected to activate IL-8 secretion when applied (basolaterally, see below) to epithelia. Indeed, as shown in Figure 7, supernatants from every Salmonella strain we tested (except flagellar mutants), and five out of seven strains of non-pathogenic gut E. coli, contained flagellin as indicated by Western blotting (Figure 7a) and induced epithelial IL-8 secretion (Figure 7b) when applied basolaterally. Diluting some of these bacterial supernatants and then measuring their ability to induce IL-8 secretion indicated that the supernatants of these flagellated bacterial strains contained similar levels of PIF/flagellin bioactivity (Figure 7c). Furthermore, the PIFs isolated from these strains had the same biochemical properties as PIF/flagellin isolated from wild-type S. typhimurium (resistance to boiling, sensitivity to proteinase K; data not shown), indicating that flagellin was likely responsible for this IL-8 induction. However, in contrast to their supernatants, the PhoP c mutant and the E. coli strains themselves elicited very little IL-8 secretion when added apically (i.e., physiologically) to model epithelia (refs. 9, 17 and Figure 7d). We reasoned that this apparent contradiction could perhaps be explained by epithelia exhibiting a polarized response to flagellin; i.e., perhaps epithelia could respond only to flagellin that reaches its basolateral surface. This was indeed the case because flagellin added apically to model epithelia failed to elicit IL-8 secretion even when added at 100-fold greater concentration than was necessary to elicit a significant response when added basolaterally (Figure 8). Therefore, as we normally add bacteria only to the apical (lumenal) surface (consistent with the physiology of the intestine), there would only be a response to flagellin if and when it is
translocated across the epithelia. However, the fact that a flagellin-mediated bioactivity was isolated from the basolateral supernatants (Figure 1) indicates that indeed such translocation does occur. Additionally, Western blotting of such basolateral-conditioned media indicate that these samples do indeed contain detectable levels of flagellin within 30 minutes after apical addition of the bacteria (Figure 9a).

We next sought to gain some insight into how flagellin translocation might occur. Flagellin itself, added apically in the absence of bacteria, was unable to translocate across model epithelia (Figure 9b). Nor could flagellin translocation be mediated by a flagellin-secreting strain of normal gut E. coli. Furthermore, using our more sensitive and quantitative indicator of flagellin translocation (i.e., detecting flagellin by its IL-8–inducing bioactivity), we found that flagellin-deficient S. typhimurium could not translocate added soluble flagellin (IL-8 induction by basolateral media of epithelia apically col-

Figure 7
Various bacteria release PIF/flagellin. An overnight culture of the indicated bacterial strain was pelleted, washed two times by centrifugation, and resuspended in HBSS (10^10 CFU/ml). One hour later, bacterial supernatant was isolated. Flagellin-deficient strain (flkC/flkB) transformed with plasmids encoding flkC and flkB is indicated by flkC/flkB/flkC' and flkC/flkB/flkB', respectively. (a) Supernatants were analyzed by SDS-page/immunoblotting using a mAb to flagellin. (b-d) Model epithelia were placed in HBSS. (b) Supernatants were applied basolaterally as a 1:100 dilution to model epithelia. IL-8 secretion was assayed 5 hours later. (c) Bacterial supernatants were applied basolaterally, at indicated dilution, to model epithelia. IL-8 secretion was assayed 5 hours later. (d) Live bacteria were applied to apical reservoir (10^8 bacteria/epithelia). IL-8 secretion was assayed 5 hours later.

Figure 8
Basolateral flagellin, but not apical flagellin, induces IL-8 secretion. Flagellin was isolated from S. typhimurium supernatant as described in Methods, diluted as indicated, and added to the indicated reservoir of model epithelia, and IL-8 secretion was measured 5 hours later.
Flagellin rapidly appears in the basolateral supernatants of apically infected model epithelia. Model intestinal epithelia were apically exposed to indicated bacteria. Basolateral epithelial supernatants were isolated and Western blotted for flagellin. (a) Supernatants were isolated at the indicated time after addition of S. typhimurium. Isolated surface flagellin from S. typhimurium expressing FliC or FljB and PIF were directly Western blotted (i.e., not exposed to epithelia) to serve as positive controls. (b) Supernatants were isolated 1 hour after exposure to indicated organism or purified flagellin (100 ng/ml).

**Figure 9**

Flagellin rapidly appears in the basolateral supernatants of apically infected model epithelia. Model intestinal epithelia were apically exposed to indicated bacteria. Basolateral epithelial supernatants were isolated and Western blotted for flagellin. (a) Supernatants were isolated at the indicated time after addition of S. typhimurium. Isolated surface flagellin from S. typhimurium expressing FliC or FljB and PIF were directly Western blotted (i.e., not exposed to epithelia) to serve as positive controls. (b) Supernatants were isolated 1 hour after exposure to indicated organism or purified flagellin (100 ng/ml).


tiva, and invG was 30.7 ± 6.5, 6.0 ± 1, and 9.3 ± 4 × 10⁴ bacteria per 0.33 cm² epithelia). Together, these results indicate that the type III secretory-mediated interactions between S. typhimurium and epithelia may modulate, but are not absolutely required for, flagellin translocation. Moreover, whereas many flagellated Gram-negative organisms secrete flagellin, this flagellin will only activate an inflammatory response when an organism can translocate this protein across the epithelia. Thus, the ability to translocate flagellin may be an important determinant in whether a bacteria will elicit an inflammatory response.

Primary S. typhimurium induction of IL-8 occurs by a flagellin-mediated mechanism. We next sought to better assess the biological relevance of flagellin induction of epithelial IL-8 secretion. First, we measured the concentration dependence of this response. Flagellin purified from wild-type S. typhimurium (likely a mix of FliC and FljB) induced detectable IL-8 secretion at concentrations below 1 ng/ml and had an ID₅₀ of about 2 ng/ml (Figure 11a). FljB (flagellin purified from fliC S. typhimurium) had an ID₅₀ of 1.5 ng/ml, whereas FliC (purified from the fljB strain) had an ID₅₀ of 2.4 ng/ml. This is more potent than IL-8 induction elicited by an equal concentration of the potent proinflammatory agonist TNF-α. In contrast, these cells do not secrete detectable levels of IL-8 in response to basolaterally added S. typhimurium LPS (no response was observed at any concentration we tested, the highest being 500 µg/ml), analogous to the results of nonpolarized epithelia (28, 29) or polarized epithelia treated apically (4, 17). Similarly, we did not observe detectable IL-8 secretion between 1 ng/ml and 500 ng/ml (Figure 11a).

**Figure 10**

Flagellin translocation by S. typhimurium mutants. Model epithelia were apically exposed to wild-type (WT) S. typhimurium or indicated mutant or nonpathogenic E. coli. For 45 minutes. After exposure to bacteria, basolateral supernatants were collected, boiled, filtered (through 2-µM pore size filter), and transferred to basolateral surface of virgin epithelia. IL-8 secretion was measured 5 hours later using ELISA. Wild-type represents PhoP+ parent (14028s). An indistinguishable result was obtained using invA parent SR-11 (not shown).
secretion in response to two commercially available *E. coli* LPS preparations or LPS that we isolated from *E. coli* F-18. Thus, whereas LPS is thought to be important for activating subepithelial (i.e., lamina propria) macrophages, flagellin may play an important role in activating epithelial orchestration of an inflammatory response when the epithelial barrier has been breached. We next assessed whether flagellin would stimulate IL-8 production from model epithelia made from cell lines other than T84. HT29cl.19A model epithelia also secreted IL-8 in response to flagellin (unstimulated and 100 ng/ml flagellin-treated HT29cl19A epithelia secreted 0.045 ±.25 and 1.53 ± 0.45 ng/ml IL-8, respectively), indicating this response was not unique to a single cell line. Like TNF-α, and unlike bacterial toxins such as *C. difficile* toxin A, flagellin does not itself cause significant changes in epithelial barrier function within the time it induces IL-8 induction (Figure 11b), indicating it is likely activating an inflammatory response rather than simply being cytotoxic.

Lastly, we investigated what portion of the IL-8 secretory response induced by *S. typhimurium*, applied in a physiologically polarized manner (i.e., apically), was accounted for by flagellin. Specifically, we examined the ability of the *fliC/fljB* mutant, as well as an *flhD* mutant (flagellar master operon), to induce IL-8 secretion when applied apically to model epithelia. Similar to their supernatants (Figure 6 and 7), neither of these live organisms elicited significant levels of IL-8 secretion (Figure 11c) from model epithelia. In contrast, strains lacking only FliC or FljB elicited IL-8 secretion to an extent that was only marginally reduced from the wild-type parent strain. Furthermore, transformation of the flagellin-deficient strain with plasmids that encode either *fliC* or *fljB* fully restored the bacteria’s ability to induce this response. Thus, flagellin is not merely an amplifier of IL-8 expression. Rather, flagellin appears to play a major role in activating epithelial orchestration of the immune inflammatory response.

**Discussion**

This report shows that the protein flagellin, the primary structural component of *E. coli* and *Salmonella* flagella (30), is a potent inducer of IL-8 expression in model intestinal epithelia when this protein obtains access to the basolateral (serosal) aspect of such epithelia. While it is not entirely clear why bacteria release soluble flagellin (it is unlikely to be shedding from flagella that depolymerize because gentamicin-killed bacteria do not release significant amounts of soluble flagellin; data not shown), it appears to occur among a variety of bacterial strains. Expression of flagellin by the pathogen *S. typhimurium* was found to be essential for this organism to induce the epithelial secretion of IL-8. As IL-8 expression is both a marker and mediator of mucosal inflammation in the intestine (31–33), this finding has profound implications on the understanding of the mechanisms by which enteric organisms can activate mucosal inflammation.

The mucosal surface of the human colon is very heavily colonized with Gram-negative organisms, many of which (e.g., nonpathogenic *E. coli*) likely release flagellin. Thus, flagellin is normally likely to be ubiquitous at the lumenal epithelial membrane domain. In contrast, since the intestinal epithelium is normally impenetrable to most bacteria, the basolateral membrane environment (i.e., lamina propria) likely is normally a flagellin-free environment. In this context, our
observation that flagellin exposed to the basolateral, but not apical (lumenal), surface of model epithelia induces IL-8 secretion has significance for two reasons. First, the pathogen *S. typhimurium* was able to mediate the translocation of flagellin across the epithelium, and such translocated flagellin was able to activate proinflammatory gene expression in uninfected epithelial cells. That flagellin-deficient *S. typhimurium* did not elicit detectable IL-8 secretion suggests that this mechanism of epithelial activation may be essential for epithelial orchestration of a mucosal inflammatory response. While flagellin-deficient *S. typhimurium* also exhibit considerably reduced ability to invade epithelial cells (34) (in our model system invasion of *flgC/fsB* was 4.3 ± 1.1 x 10^3 bacteria/epithelia compared to 26.0 ± 3.1 x 10^3 bacteria for the parent strain), this fact is insufficient to explain their inability to induce IL-8 secretion, because we have shown these events can be uncoupled (9). Second, in intestinal diseases associated with epithelial dysfunction such as inflammatory bowel disease (IBD), there is diminished barrier function that may allow the luminal contents access to the serosal surface of the epithelium. This could result in flagellin inducing epithelial cells to orchestrate an inflammatory response in the absence of any normally pathogenic organisms. This proposed role for flagellin in IBD is similar to that proposed for LPS acting on lamina propia macrophages. While monocytic cells have been shown to secrete cytokines in response to flagellin (35), the epithelial response to flagellin may be of particular importance since epithelial cells are, by far, the most numerous cells in the intestinal mucosa, and, in contrast to macrophages, are relatively unresponsive to LPS (17, 28, 29), even when added basolaterally (our results herein).

Currently, the mechanism by which flagellin translocation occurs is unknown, but some insights can be drawn. Neither flagellin isolated from *S. typhimurium* or *E. coli* were, by themselves, able to cross intact model epithelium. Rather, only live *S. typhimurium* were able to mediate the translocation of this protein, indicating direct bacterial-epithelial interaction is involved. It seems unlikely that *S. typhimurium* causes this rather large molecule to pass through epithelial cell tight junctions because the transepithelial electrical resistance (a direct indicator of passive permeability) drops only modestly in response to this invasive pathogen (4). It is possible that *S. typhimurium* that invade epithelia may simply basolaterally exit the epithelium and secrete flagellin at such a location. However, analogous to the case for IL-8 induction, invasion may not be required for flagellin translocation because we have found that the invasion-defective *hilA* mutant was able to induce PIF/flagellin translocation (refs. 9 and 29 and herein). Thus, it is possible that *S. typhimurium* attached to the apical membrane can translocate flagellin into epithelial cells and that this protein could be trafficked to exit the basolateral membrane. *S. typhimurium* is well-known to secrete effector molecules into epithelial cells, particu-
expression as a mucosal inflammatory response eventuating in diarrhea and may offer a disseminating advantage to this organism. Regardless, normal gut microflora flagellin may, through its actions on the intestinal epithelium, play a role in inflammation associated with IBD and hence may serve as a strategic target for treating this disorder.

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