

## MINIREVIEW

# How Flagellin and Toll-Like Receptor 5 Contribute to Enteric Infection<sup>∇</sup>

Theodore S. Steiner\*

*Division of Infectious Diseases, Vancouver Coastal Health Research Institute and  
University of British Columbia, Vancouver, BC, Canada*

Resistance to infection in vertebrates relies on a complex interaction between two separate but complementary arms of the immune system: adaptive immunity and innate immunity. Adaptive immunity refers to the recognition of specific molecular epitopes by either T or B lymphocytes and the resulting responses that powerfully target only a very narrow range of infectious agents. In contrast, innate immunity involves multiple cell types and tissues and relies on more general recognition of molecular patterns that are specific to classes of microorganisms. Severe defects in either arm of the immune system can be lethal.

In the last 10 years there has been a proliferation of interest in innate immunity, assisted by the discovery of the Toll-like receptors (TLRs). TLRs are single transmembrane proteins that recognize specific microbial molecules and trigger immune responses through direct interactions with intracellular signaling proteins. The target molecules of TLRs include nucleic acids, lipids, lipoproteins, proteins, and synthetic compounds. Of the 10 human TLRs, only TLR5 appears to be specific for a single protein moiety, bacterial flagellin. This specificity, along with the ease of protein mutagenesis techniques, has made the study of flagellin recognition unique in the TLR field.

### BACKGROUND: TLR5 AND FLAGELLIN

Many bacteria possess flagella, whiplike organelles which attach to a rotatory motor embedded in the bacterial cell wall, providing motility to the organism. The complex regulation and structure of the flagellum have been thoroughly reviewed elsewhere. The body of the flagellum consists of a mass of protofilaments, each of which is a long, end-to-end polymer of a single protein, flagellin. Because of the physical constraints imposed by its function, flagellin has a relatively conserved structure even among widely diverse bacterial species.

Many important human pathogens are flagellated, including agents of gastroenteritis (*Salmonella*, diarrheagenic *Escherichia coli*, *Campylobacter*), pneumonia (*Pseudomonas*, *Burkholderia*, *Legionella*), and invasive infections (*Proteus*, *E. coli*, *Clostridium*, etc.). For a long time expression of flagella has been examined as a virulence trait, but this has been largely in the context of motility rather than immune stimulation. Nev-

ertheless, flagellin was recognized decades ago to be highly immunogenic, although the tools with which to fully understand this property were not available at the time.

Starting in the late 1990s, flagellin was identified by several independent groups as the soluble mediator that causes intestinal epithelial or macrophage inflammation following infection with enteroaggregative *E. coli* (EAEC) or *Salmonella*. Wyant et al. and Ciacci-Woolwine et al. reported that *Salmonella enterica* serovar Typhi flagella induced cytokine release from human monocytes and impaired antigen presentation by human macrophages (7, 59, 60). McDermott et al. showed that this effect was highly potent and could be eliminated by trypsin treatment of monocytes, suggesting that there is a high-affinity interaction between flagellin and a cell surface receptor (33). Steiner et al. identified flagellin as the factor in culture supernatants of enteroaggregative *E. coli* that caused interleukin-8 (IL-8) release from intestinal epithelial cells in culture (50). Eaves-Pyles et al. reported that *Salmonella* flagellin caused cytokine release from intestinal epithelial cells in culture, as well as a shock-like syndrome when it was injected intraperitoneally into lipopolysaccharide (LPS)-resistant C3H/HeJ mice (14). The molecular basis of these effects was revealed by Hiyashi et al., who demonstrated that flagellin was the component of *Listeria* culture supernatants that activated TLR5, and subsequent work in many laboratories confirmed this finding for flagellins from various organisms (24). To date, flagellin is the only known activator of TLR5, and until recently flagellin-induced inflammation was believed to be fully dependent on TLR5 expression (see below).

After TLR5-expressing cells are stimulated with flagellin, there is a signaling cascade that involves phosphorylation of interleukin-1 receptor-associated kinase 1, leading to activation of MEK kinases and I- $\kappa$ B kinases, which ultimately activates inflammatory protein production via NF- $\kappa$ B and p38 mitogen-activated protein kinase. The specifics of this signaling cascade are still being intensively studied and appear to be different in different cell types. It is clear, however, that TLR5 is responsible for flagellin-induced responses in epithelial cells, endothelial cells, macrophages, dendritic cells (DCs), and T cells. In the case of DCs and T cells, flagellin responses form an important bridge between the innate and adaptive arms of the immune system, which likely explains the unique properties of flagellin as both a pathogenicity factor and an immunogen. In addition, flagellin activates TLR5 on natural CD4<sup>+</sup>/CD25<sup>+</sup> T-regulatory cells, leading to increased suppressive activity, suggesting that flagellin has a complex role in bridging innate immunity and adaptive immunity (10).

\* Mailing address: Rm. D452, HP East, 2733 Heather St., Vancouver, BC V5Z 3J5, Canada. Phone: (604) 875-4588. Fax: (604) 875-4013. E-mail: tsteiner@interchange.ubc.ca.

<sup>∇</sup> Published ahead of print on 21 November 2006.

### STRUCTURAL FEATURES OF FLAGELLIN AND TLR5 RECOGNITION

The gene encoding flagellin has various names in different bacteria (*fliC*, *fljB*, *flaA*, etc.), but in each case its expression is tightly regulated as part of the complex chemotactic regulon. Under conditions that favor its expression (e.g., hypotonicity, nutrient-poor media, etc.), flagellin is expressed and transported to the membrane, where it is exported through a unique secretory mechanism that strongly resembles the type III secretion system (TTSS) that many virulent bacteria use to inject proteins into host cells. In contrast to the TTSS needle complex, however, flagellin is exported through the basal body of the flagellum, which consists of a rotatory motor and hook. Each flagellin monomer passes through this pore and spontaneously polymerizes with adjacent monomers to form an enlarging protofilament with a central pore through which additional subunits pass. This process is shown in Fig. 1A.

Because flagellin monomers spontaneously associate in aqueous solution, the crystal structure of the flagellin monomer has never been solved. However, limited proteolysis of flagellin removes the extreme N and C termini, leaving a central core that cannot polymerize; the structure of this fragment (called F41 in the case of *Salmonella*) has been solved (Fig. 1B) (42). The structure confirmed hypotheses generated from previous thermodynamic studies suggesting that the protein has a four-domain structure. The D0 and D1 domains are largely helical and are comprised of strongly conserved stretches of amino acids at the beginning and end of the primary sequence (referred to as C1 and C2, respectively). The D2 and D3 domains arise from the central portion of the primary sequence, which is highly variable. When flagellin monomers interact, the roughly convex surface of the conserved D0 and D1 domains from one monomer interacts with a corresponding concave surface on the adjacent monomer, leading to formation of a chain held together by the conserved domains (Fig. 1C). The variable D2 and D3 domains stick out from the chain, where they are largely surface exposed. Because of the cryptic location of the conserved domains (within the central pore of the molecule or buried in protein-protein interactions), they are relatively protected from immune surveillance.

There is strong evidence that the TLR5-activating region of flagellin is located within the conserved domains (Fig. 2). Deletional mutagenesis studies showed that loss of either the C1 or C2 domain resulted in a noninflammatory protein, whereas the variable domain could be replaced by an unrelated hinge domain without an effect on the inflammatory activity (15). Random transposon mutagenesis of the EAEC flagellin identified a short region in the C2 domain that was required for TLR5 activity, although deletion of the C1 domain also eliminated activity (13). Subsequently, it was determined by alanine scanning mutagenesis that specific amino acid residues within the C1 and C2 domains (L88 to Q97 in C1 and I411 in C2) were required to stimulate TLR5; mapping of flagellin based on the F41 crystal structure showed that these residues lie in adjacent alpha-helices in the folded protein (Fig. 1B) (45). Although these residues are largely hidden in the flagellin protofilament, they are predicted to be exposed in monomeric flagellin, and cross-linking studies demonstrated that flagellin monomers are much more potent than polymers for eliciting

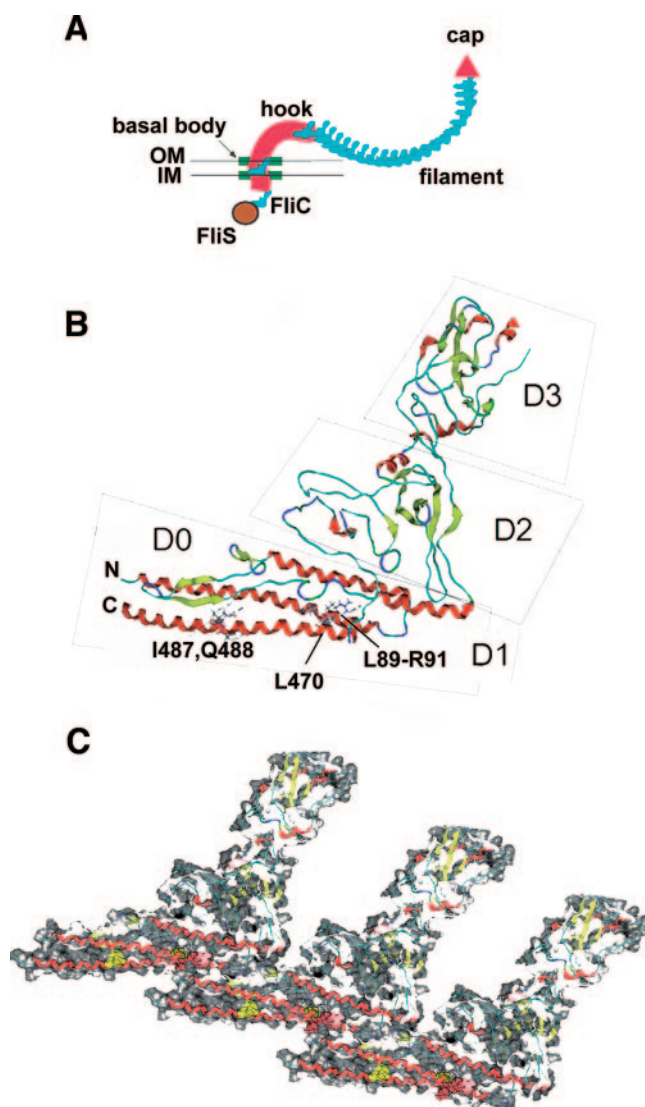


FIG. 1. Tertiary structure of flagellin and synthesis of flagella. (A) Schematic diagram of flagellin export and flagellum formation. Flagellin monomers (FliC) are synthesized and transported with a chaperone (FliS) to the inner membrane, where they are exported through the central pore of the flagellar basal body that straddles the inner membrane (IM) and outer membrane (OM) of the cell wall. FliC monomers continue through the pore of the flagellar filament, where they are added to the growing end beneath the cap protein (FliD). (B) Crystal structure of the F41 fragment of *Salmonella* FliC. The domains are labeled, and amino acids critical for TLR5 activation are indicated. (C) Space-filling model of flagellin, with sites of polymerization shown in the hypothetical flagellin trimer.

TLR5 activity (45). These results suggested that TLR5 evolved to recognize conserved domains of flagellin that are constrained by the need to provide motility. Similarly, the plant flagellin receptor, FLS2, recognizes a peptide fragment (Flg22) within the C1 domain of flagellin (although Flg22 itself has no activity with TLR5) (13, 16).

In a recent study Andersen-Nissen et al. found that many  $\alpha$ - and  $\epsilon$ -proteobacteria (including *Campylobacter*, *Helicobacter*, and *Bartonella*) have flagellin proteins that can avoid TLR5 recognition while maintaining motility (3). These flagellins dif-

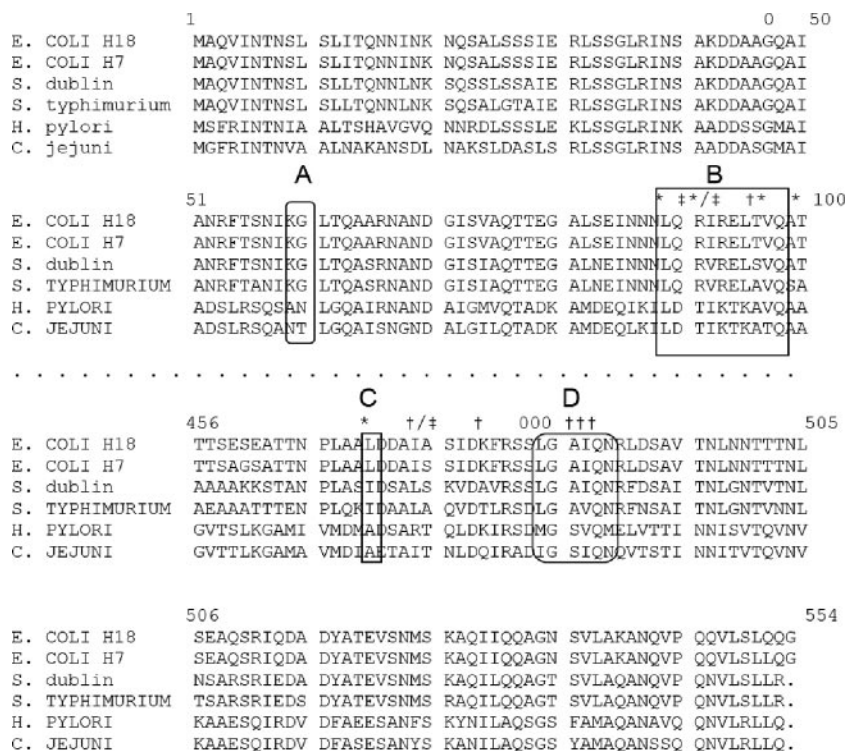


FIG. 2. Primary sequences of the critical regions of conserved domains of flagellins from selected  $\gamma$ - and  $\alpha$ - proteobacteria, numbered according to the *E. coli* H18 flagellin numbering system. The following regions are enclosed in boxes: the site of compensatory mutations that allow motility of  $\alpha$ -proteobacteria (region A); two regions where substitutions eliminate TLR5 activity (regions B and D); and the site of a point mutation that most strongly eliminates TLR5 activity (region C). Information for individual residues is indicated as follows: asterisk, point mutation that reduces or eliminates activity; dagger, site of random transposon insertion that reduces or eliminates activity; 0, point mutation that preserves activity; double dagger, point mutation that reduces inflammatory activity yet preserves motility.

fer from those of  $\gamma$ -proteobacteria in the critical region from amino acid 88 to amino acid 97, which prevents TLR5 activation. However, they also have an alanine at the position corresponding to I411 in *Salmonella*, which in *Salmonella* is required for both TLR5 recognition and motility. Motility of the  $\alpha$ - and  $\epsilon$ -proteobacteria is preserved by a third set of mutations in the C1 domain (residues 58 and 59). These three regions are predicted to be adjacent to one another in the folded flagellin protein within the region of polymerization, which likely explains how the various mutations complement each other to preserve motility.

Interestingly, the amino acids altered in these noninflammatory flagellins are the same amino acids predicted in other studies to be most critical for TLR5 recognition. Murthy et al. independently showed that amino acids 88 to 97 are required for inflammatory activity based on deletional mutagenesis and also showed that substitution or deletion of this motif eliminated activity (38). Verma et al. reported that single point mutations in this domain in the *Pseudomonas aeruginosa* flagellin reduced inflammatory activity but had a less pronounced effect on motility, indicating that there is not a strict correlation between TLR5 agonist activity and the ability to form functional flagella (55).

So far, there has been no formal demonstration of direct binding between TLR5 and flagellin, but there is strong evidence of association between the two proteins in whole-cell systems. Flagellin coimmunoprecipitates with murine TLR5

when the latter is overexpressed (with an epitope tag) in CHO cells (46). Likewise, flagellin can precipitate human TLR5 from lysates of transiently transfected COS cells (36). Similar results have been obtained for flagellin from two *Vibrio* species and both human TLR5 and fish soluble TLR5 (29, 53). Fluorescent colocalization of flagellin and TLR5 on whole cells as determined by microscopy has also been reported. Adamo et al. showed that flagellin colocalized with the ganglioside GM1 on the apical surface of polarized airway epithelial cells initially but at later times induced apical migration of TLR5, which then localized to the same clusters (1). West et al. showed that flagellin colocalized with fluorescently tagged TLR5 on the surface of transfected RAW macrophages (57). Interestingly, these authors also reported that increasing expression of TLR5 correlated with greater binding of flagellin, which is the first indication that TLR5 could act as a high-affinity receptor.

In general, the techniques described above are useful for demonstrating interactions between flagellin and TLR5, but only in the context of the complex cellular environment. Hence, they cannot rule out involvement of other proteins or nonprotein factors. In fact, flagellin from *P. aeruginosa* binds to several gangliosides on the surface of respiratory epithelial cells, and extrinsic addition of gangliosides inhibits both binding of flagellin to whole cells and inflammatory signaling (57). Thus, it is possible that flagellin encounters TLR5 primarily in the context of ganglioside-rich membrane regions, with other

proteins playing a necessary role. This could explain observations that flagellin binds nonspecifically (with low affinity) to cell membranes (35).

Despite these concerns, an *in silico* modeling study by Jacchieri et al. raised the possibility that there is direct interaction between flagellin and TLR5 at specific sites on the two proteins (27). Based on hydrophobicity profiles, these authors determined that there is a high likelihood of interaction between predicted  $\alpha$ -helices on TLR5 (residues 552 to 561) and flagellin (the LQRIRELAVQ motif in the C1 domain). They also found a smaller complementary region between TLR5 amino acids 323 and 327 and a short region in the C2 domain of flagellin (LGAIQN).

However, comparative analysis of these studies revealed several problems with this TLR5/flagellin binding model. First, the region of TLR5 predicted by Jacchieri et al. to bind flagellin is different from the region identified by Mizel et al. (using deletion constructs) as the region required to trigger flagellin responses (35). Second, certain TLR5 constructs (such as a construct containing amino acids 386 to 857) fail to signal despite coprecipitating with flagellin. Finally, Verma et al. demonstrated that glycosylation of *Pseudomonas* flagellin is required for full inflammatory activity, whereas *E. coli* does not glycosylate its flagellin (55). This raises the likelihood that flagellin activation of TLR5 requires a complicated interaction that could involve additional protein-protein or protein-lipid interactions that are not yet understood.

#### ROLE OF FLAGELLIN AND TLR5 IN ENTERIC INFECTIONS

Inflammatory responses to flagellin were first observed in enteric infection models, and much subsequent work has focused on these models. The most intensively studied model is *Salmonella* gastroenteritis (Fig. 3). The unique pathophysiology of *Salmonella* infection has been extensively reviewed elsewhere, but a brief summary here is warranted. Upon encountering the intestinal epithelium, *Salmonella* invades epithelial cells via bacterium-mediated endocytosis that requires expression of genes in *Salmonella* pathogenicity island 1 (SPI-1), one of two TTSS that the organism possesses. This invasion process induces an inflammatory response in epithelial cells, but in susceptible hosts enough organisms survive to pass through the basolateral surface of the epithelial cells into the lamina propria, where they are taken up by macrophages and/or DCs. *Salmonella* survives within vesicles through a complex and incompletely understood mechanism that requires the second TTSS, SPI-2. Once within antigen-presenting cells, the organisms disseminate to mesenteric lymph nodes and secondary lymphoid organs, where their fate (clearance or fatal infection) depends on host resistance. Most patients clear the infection spontaneously, but people at the extremes of age, people who are immunocompromised, and people without a functional spleen are at risk of disseminated or fatal infection.

*Salmonella* expresses two different but related flagellin proteins, encoded by *fliC* and *fliB*, which are controlled through phase variation. Expression of flagella has long been recognized to play a role in *Salmonella* infection. In 1984, Weinstein et al. demonstrated that flagellated *Salmonella* is more virulent than nonflagellated strains, with enhanced survival within mac-

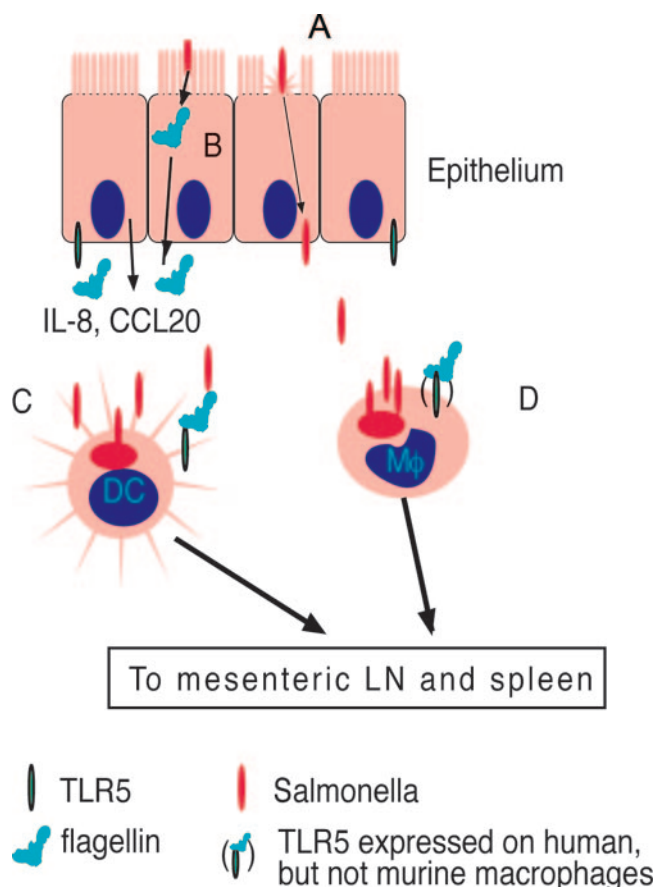


FIG. 3. Schematic diagram of the various roles of flagellin in *Salmonella* gastroenteritis. A, internalization of organisms by epithelial cells, with membrane ruffling (SPI-1 dependent); B, transport of flagellin monomers through the epithelium to encounter basolateral TLR5 (SPI-1 dependent); C, encounter with lamina propria DC (flagellin activation of TLR5 may facilitate uptake and/or survival of organisms, which is also SPI-2 dependent); D, encounter with lamina propria macrophage (M $\phi$ ) (organisms are internalized). In resistant hosts, flagellin-dependent Ipaf recognition activates caspase-1, leading to IL-1 secretion and cell death. TLR5 is expressed on human macrophages but not on murine macrophages; the role of TLR5 in human salmonella gastroenteritis remains uncertain. LN, lymph nodes.

rophages and increased bacterial counts in spleens of infected animals (56). More recently, however, Schmitt et al. reported that increased virulence of several *Salmonella* strains could be fully attributed to decreases in flagellin expression and that aflagellar *Salmonella* was less inflammatory *in vitro* but more pathogenic *in vivo* (43, 44).

Specific mechanisms of *Salmonella* flagellin expression and presentation to host cells have been described over the past 5 years. In a study of model epithelia, Gewirtz et al. showed that *Salmonella* infection causes transcytosis of flagellin to the basolateral surface of intestinal epithelial monolayers, where it encounters basolaterally expressed TLR5 to induce an inflammatory response (18, 19). This was later shown to be an SPI-2-dependent process (31). The situation *in vivo* may be more complicated, since polarized expression of TLR5 on human intestinal explants or biopsies has been difficult to demonstrate due to low-level expression and a lack of high-affinity antibod-

ies; apical expression of TLR5 in mice has been demonstrated (4). Functionally, however, flagellin responses in intact human intestinal explants appear to be limited to the serosal surface, as shown by Rhee et al. (41). Whether this is primarily due to engagement of TLR5 on epithelial cells or other cell types has not been determined.

There are potential explanations for the effect of flagellin on early *Salmonella* infection of epithelia that do not involve epithelial TLR5. For example, anti-flagellin antibodies inhibit binding of organisms to intestinal epithelial cells (26), and flagellin appears to assist with *Salmonella* invasion (51). One possible mechanism for this is a mechanism in which flagellin also facilitates translocation of SopE2, a *Salmonella* SPI-1 effector that acts as a guanine exchange factor (25). In addition, infection of intestinal epithelial cells induces expression of ICAM-1 in cocultured microvascular endothelial cells, which express high levels of TLR5 constitutively (32). This suggests a mechanism by which *Salmonella* flagellin could potentiate inflammation, by inducing infected epithelial cells to "prime" endothelial cells for inflammation, which would then be further induced by direct exposure to flagellin either transcytosed through the epithelium or expressed on the surface of bacteria that have successfully penetrated the epithelial barrier.

The relative roles of SPI-1, SPI-2, and flagellin in *Salmonella* gastroenteritis in vivo have only recently been understood, with the help of a murine gastroenteritis model that requires pretreatment of animals with high doses of streptomycin (5). Early induction of colitis requires expression of flagella in the context of an intact chemotactic system, suggesting that motility rather than TLR5 activation is critical (48). SPI-1 is clearly required for colitis, but more recent reports have shown that SPI-2 is also required for the complete inflammatory phenotype (8, 9). Interestingly, the inflammatory responses to SPI-1- and SPI-2-deficient *Salmonella* strains were quite different; bacteria expressing SPI-1 alone induced diffuse cecal inflammation that was independent of MyD88 (i.e., preserved in MyD88<sup>-/-</sup> mice), whereas SPI-2-expressing bacteria induced focal subepithelial inflammation that was attenuated in MyD88<sup>-/-</sup> mice (22). These findings indicate that TLRs are involved more in the later, intracellular stages of *Salmonella* infection. Together, these studies suggest that full manifestation of *Salmonella* gastroenteritis involves coordinated expression of SPI-1, SPI-2, and flagellin, with subsequent activation of innate immune responses in different cell compartments. The specific mechanisms by which *Salmonella* introduces flagellin monomers into cells and the potential evolutionary advantages are areas in which there is active exploration; one explanation may involve TLR5-independent flagellin recognition by intracellular pattern recognition receptors (see below).

There is strong evidence that acquired immunity plays an important role in *Salmonella* infection, as demonstrated by severe and/or recurrent disease in patients with AIDS. Lamina propria DCs are probably the key interface between innate immunity and adaptive immunity in *Salmonella* gastroenteritis. Uematsu et al. recently demonstrated that in mice high levels of TLR5 are expressed on lamina propria DCs but not on mesenteric lymph nodes or splenic DCs (54). Moreover, while DCs from TLR5<sup>-/-</sup> mice were hyporesponsive to flagellin in terms of cytokine production, TLR5<sup>-/-</sup> mice actually survived oral *Salmonella* infection better than wild-type mice survived

such an infection (but there was no difference following intraperitoneal infection). These results suggest that the flagellin-induced, TLR5-dependent inflammatory response in lamina propria DCs helps facilitate *Salmonella* uptake, survival, and/or dissemination and could partially explain the attenuated virulence of aflagellar *Salmonella*.

Other interesting effects of flagellin on adaptive immune responses to *Salmonella* have been described. Immunization with purified flagellin alone produces a very potent T<sub>H</sub>2-type immune response. However, mucosal exposure to whole, flagellated *Salmonella* instead produces a strong T<sub>H</sub>1 anti-flagellin response (11). Flagellin-independent effects of *Salmonella* on DCs likely account for this effect. For example, while live *Salmonella*, purified *Salmonella* LPS, and purified flagellin all induce cytokine responses in macrophages, maximal cytokine production from DCs requires live organisms (39). In fact, intracellular-phase *Salmonella* appears to downregulate flagellin expression to the point where infected splenocytes are unable to induce proliferation of flagellin-specific T cells (2). This may explain the observation that oral infection with a large *Salmonella* inoculum led to specific anti-flagellin T-cell responses, whereas infection with a small inoculum did not lead to such responses, even when the ultimate organism burdens were identical (due to dissemination and proliferation). In the case of infection with a large inoculum, DCs are more likely to encounter extracellular-phase bacteria that express flagellin or transcytosed flagellin itself, and the subsequent TLR5 activation would lead to increased antigen presentation to T cells. In contrast, infection with a small inoculum would limit the exposure of lamina propria DCs to flagellated *Salmonella*, leading to a less robust adaptive immune response.

While the importance of flagellin expression has been best studied for *Salmonella* infection, there is evidence that flagellin plays a role in infection with other enteric pathogens as well, although TTSS-dependent translocation like that which occurs in *Salmonella* has not been demonstrated for these organisms. Coworkers and I initially reported that EAEC caused inflammation in infected patients, as well as human epithelial cell cultures, and that the latter required expression of flagellin (49, 50). Harrington et al. found that most of the inflammatory response to EAEC in nonpolarized epithelial cells was due to flagellin but that there was a flagellin-independent inflammatory response in polarized T84 cell monolayers that required expression of aggregative adherence fimbriae (23). There is no rodent model that resembles human EAEC infection, but we found that colonization of mice was independent of flagellin expression (M. So, J. Nataro, B. Vallance, and T. Steiner, unpublished observations). A role for flagellin has also been reported for enteropathogenic *E. coli* and enterohemorrhagic *E. coli*, both of which induce flagellin-dependent epithelial cell inflammation (6, 61). In contrast, *Campylobacter jejuni* flagellin appears to be necessary for autoagglutination and adherence to intestinal epithelial cells but not for inflammation (21, 28). This is consistent with an inability to activate TLR5, as discussed previously. Finally, *Shigella*, while nearly identical genetically to *E. coli*, has not been shown to require flagellin or TLR5 for inflammation, probably due to minimal or no expression of flagella.

### Ipaf-DEPENDENT RECOGNITION OF FLAGELLIN

Recently, flagellin was shown to have a TLR5-independent proinflammatory activity that depends on two related intracellular pattern recognition receptors, Naip5 and Ipaf, which are members of the NACHT-leucine-rich repeat-containing receptor (NLR) family, which also includes Nod and Apaf proteins. In contrast to TLR5, NLR proteins do not act through MyD88, and while NF- $\kappa$ B activation does occur after stimulation, the predominant effect of these receptors appears to be an effect on activation of caspases (leading to maturation of IL-1 $\beta$  and IL-18 and apoptosis).

Ren et al. (40) screened random *Legionella* mutants that survived within macrophages from C57BL/6 mice (which are ordinarily resistant to infection), and all of these mutants were defective in flagellin production. This effect on flagellin was independent of TLR5, since murine macrophages (unlike human macrophages) are deficient in TLR5; moreover, the effects of flagellin were the same in MyD88<sup>-/-</sup> mice. Ren et al. showed that *Legionella* and *Salmonella* flagellins both activated caspase-1 in murine macrophages, leading to cell death. This effect was dependent on expression of Naip5, which had previously been shown to be a marker on chromosome 13 required for macrophage resistance to *Salmonella* (12, 58). Molofsky et al. reported similar findings and also demonstrated that flagellin-deficient *Legionella* was more virulent in a murine tracheal infection model (37).

Miao et al. and Franchi et al. reported that control of intracellular *Salmonella* also requires Ipaf (17, 34). Macrophages from Ipaf-deficient mice were resistant to flagellin-dependent killing, and overexpression of Ipaf enhanced the flagellin effect. In contrast, TLR5<sup>-/-</sup> mice were not defective in *Salmonella*-dependent macrophage killing. This effect was dependent on SPI-1, suggesting that enough flagellin can be exported through the TTSS to activate caspase-1 even when motility is suppressed (such as during intracellular infection).

The mechanisms of Naip5- or Ipaf-dependent flagellin signaling and/or recognition remain unknown, but the findings described above suggest that detection of flagellin is important enough to innate immunity to encourage the evolution of two independent recognition systems. It is particularly interesting that flagellin recognition by NLRs was found in murine macrophages, which do not express TLR5 but are highly responsive to LPS; in contrast, high levels of TLR5 are expressed on intestinal epithelial cells, which are generally unresponsive to LPS. This suggests that compartmentalization of innate immune responses to LPS and flagellin, which are coexpressed on many bacteria, is necessary for appropriate detection and control of enteric infections.

### CONTROVERSIES AND FUTURE STUDIES

Despite the advances in our understanding of flagellin structure as it relates to TLR5 recognition, there is still not enough experimental evidence to allow sequence-based prediction of whether a particular bacterial flagellin is able to activate TLR5. This is partially due to the fact that flagellin-dependent TLR5 signaling is seldom an “on/off” process but has many shades of gray that reflect stepwise loss of potency with different flagellin

mutations. Phenotypic assays of motility and TLR5-dependent signaling thus remain the “gold-standard” tests for the inflammatory potency of various flagellins. This fact has made it impossible to answer several critical questions, such as whether pathogens differ from commensals in the ability to activate TLR5 in the absence of pathogenic cofactors.

One example of this problem involves the inflammatory effects of *Helicobacter pylori* flagellin. Based on its primary sequence, *H. pylori* flagellin (encoded by the *flaA* gene) should not activate TLR5. Indeed, weak or no inflammatory activity of FlaA has been observed in several studies. For example, Gewirtz et al. showed that *H. pylori* infection of AGS gastric epithelial cells caused IL-8 release but that culture supernatants did not cause release; moreover, a mutant lacking *flaA* was not attenuated (20). Recombinant flagellin from *H. pylori* 60190 (an ATCC strain) caused weak IL-8 release from T84 colonocytes and no IL-8 release from AGS cells. Similarly, Lee et al. reported that purified FlaA from various *H. pylori* strains caused little IL-8 release from gastric epithelial cells and no release from Caco-2 colonocytes (30). These workers found, however, that deletion of flagellin genes from the N6 strain did partially attenuate IL-8 release from infected gastric epithelial cells.

In contrast, convincing inflammatory activity of *H. pylori* flagellin was described in two different reports. Smith et al. found that *H. pylori* patient isolates caused TLR2- and TLR5-dependent inflammatory signaling in gastric epithelial cells and HEK 293T cells overexpressing these TLRs (47). In addition, purified flagellin from these strains also caused NF- $\kappa$ B activation in TLR5-expressing HEK cells. However, the inflammation in response to flagellin was significantly weaker than the inflammation induced by Pam3Cys (a TLR2 agonist) and required quite high concentrations of FlaA (10  $\mu$ g/ml). Similarly, Torok et al. showed that both TLR2 and TLR5 are required for *H. pylori*-induced mitogen-activated protein kinase phosphorylation and IL-8 release (52).

One explanation for these different findings is that there is expression of a second bacterially derived TLR5 agonist by *H. pylori*, although no such agonist has ever been identified. A more likely explanation is that FlaA, while clearly less potent than *Salmonella* FliC, still has weak TLR5 agonist activity (as suggested by the findings of Smith et al.). The amount of flagellin released into culture supernatants might be below the threshold for TLR5 activation, while the amount of flagellin delivered during live bacterial infection could be greater than this threshold. This would make sense if flagellin does not bind directly to TLR5 but rather binds to another cell surface structure via a low-affinity interaction, as suggested for *Pseudomonas* flagellin. Another intriguing possibility is that *H. pylori* flagellin is active in its polymerized state, in contrast to *Salmonella* flagellin. Specific studies need to be undertaken to test these hypotheses.

### CONCLUSIONS

Despite the proliferation of scientific discoveries about inflammatory responses to flagellin, significant questions remain. For example, can TLR5 bind flagellin directly, and if so, can it distinguish subtle differences in the conserved domains in different bacteria? What is the evolutionary advantage or disadvantage

vantage to bacteria of evading TLR5-dependent flagellin recognition (which occurs in certain  $\alpha$ -proteobacteria)? Is TLR5 expressed on any luminal surfaces in the human gut, and if so, how are inflammatory responses to ubiquitous commensal bacteria kept in check? What features of TLR5 signaling are unique, and how do the resulting responses aid the host in mounting appropriate innate and adaptive immune responses? At the current rate of investigation, it might not be long before these and other important questions are answered.

ACKNOWLEDGMENTS

I was supported by operating grants from the Canadian Institutes for Health Research (grant III 64355) and the Crohn's and Colitis Foundation of Canada, as well as by a career award in the biomedical sciences from the Burroughs-Wellcome Fund and a CIHR New Investigator award.

I thank Artem Cherkasov for assistance with flagellin modeling and figure generation.

REFERENCES

1. Adamo, R., S. Sokol, G. Soong, M. I. Gomez, and A. Prince. 2004. *Pseudomonas aeruginosa* flagella activate airway epithelial cells through asialoGM1 and toll-like receptor 2 as well as toll-like receptor 5. *Am. J. Respir. Cell Mol. Biol.* **30**:627–634.
2. Alaniz, R. C., L. A. Cummings, M. A. Bergman, S. L. Rassoulian-Barrett, and B. T. Cookson. 2006. *Salmonella typhimurium* coordinately regulates FliC location and reduces dendritic cell activation and antigen presentation to CD4<sup>+</sup> T cells. *J. Immunol.* **177**:3983–3993.
3. Andersen-Nissen, E., K. D. Smith, K. L. Strobe, S. L. Barrett, B. T. Cookson, S. M. Logan, and A. Aderem. 2005. Evasion of Toll-like receptor 5 by flagellated bacteria. *Proc. Natl. Acad. Sci. USA.* **102**:9247–9252.
4. Bambou, J. C., A. Giraud, S. Menard, B. Begue, S. Rakotobe, M. Heyman, F. Taddei, N. Cerf-Bensussan, and V. Gaboriau-Routhiau. 2004. In vitro and ex vivo activation of the TLR5 signaling pathway in intestinal epithelial cells by a commensal *Escherichia coli* strain. *J. Biol. Chem.* **279**:42984–42992.
5. Barthel, M., S. Hapfelmeier, L. Quintanilla-Martinez, M. Kremer, M. Rohde, M. Hogardt, K. Pfeffer, H. Russmann, and W. D. Hardt. 2003. Pretreatment of mice with streptomycin provides a *Salmonella enterica* serovar Typhimurium colitis model that allows analysis of both pathogen and host. *Infect. Immun.* **71**:2839–2858.
6. Berin, M. C., A. Darfeuille-Michaud, L. J. Egan, Y. Miyamoto, and M. F. Kagnoff. 2002. Role of EHEC O157:H7 virulence factors in the activation of intestinal epithelial cell NF-kappaB and MAP kinase pathways and the upregulated expression of interleukin 8. *Cell. Microbiol.* **4**:635–648.
7. Ciacci-Woolwine, F., I. C. Blomfield, S. H. Richardson, and S. B. Mizel. 1998. *Salmonella* flagellin induces tumor necrosis factor alpha in a human promonocytic cell line. *Infect. Immun.* **66**:1127–1134.
8. Coburn, B., Y. Li, D. Owen, B. A. Vallance, and B. B. Finlay. 2005. *Salmonella enterica* serovar Typhimurium pathogenicity island 2 is necessary for complete virulence in a mouse model of infectious enterocolitis. *Infect. Immun.* **73**:3219–3227.
9. Coombes, B. K., B. A. Coburn, A. A. Potter, S. Gomis, K. Mirakhor, Y. Li, and B. B. Finlay. 2005. Analysis of the contribution of *Salmonella* pathogenicity islands 1 and 2 to enteric disease progression using a novel bovine ileal loop model and a murine model of infectious enterocolitis. *Infect. Immun.* **73**:7161–7169.
10. Crellin, N. K., R. V. Garcia, O. Hadifar, S. E. Allan, T. S. Steiner, and M. K. Levings. 2005. Human CD4<sup>+</sup> T cells express TLR5 and its ligand flagellin enhances the suppressive capacity and expression of FOXP3 in CD4<sup>+</sup> CD25<sup>+</sup> T regulatory cells. *J. Immunol.* **175**:8051–8059.
11. Cunningham, A. F., M. Khan, J. Ball, K. M. Toellner, K. Serre, E. Mohr, and I. C. MacLennan. 2004. Responses to the soluble flagellar protein FliC are Th2, while those to FliC on *Salmonella* are Th1. *Eur. J. Immunol.* **34**:2986–2995.
12. Diez, E., S. H. Lee, S. Gauthier, Z. Yaraghi, M. Tremblay, S. Vidal, and P. Gros. 2003. Birle1 is the gene within the Lgn1 locus associated with resistance to *Legionella pneumophila*. *Nat. Genet.* **33**:55–60.
13. Donnelly, M. A., and T. S. Steiner. 2002. Two nonadjacent regions in enteroaggregative *Escherichia coli* flagellin are required for activation of toll-like receptor 5. *J. Biol. Chem.* **277**:40456–40461.
14. Eaves-Pyles, T., K. Murthy, L. Liaudet, L. Virag, G. Ross, F. G. Soriano, C. Szabo, and A. L. Salzman. 2001. Flagellin, a novel mediator of *Salmonella*-induced epithelial activation and systemic inflammation: I kappa B alpha degradation, induction of nitric oxide synthase, induction of proinflammatory mediators, and cardiovascular dysfunction. *J. Immunol.* **166**:1248–1260.
15. Eaves-Pyles, T. D., H. R. Wong, K. Odoms, and R. B. Pyles. 2001. *Salmonella*

- flagellin-dependent proinflammatory responses are localized to the conserved amino and carboxyl regions of the protein. *J. Immunol.* **167**:7009–7016.
16. Felix, G., J. D. Duran, S. Volko, and T. Boller. 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* **18**:265–276.
17. Franchi, L., A. Amer, M. Body-Malapel, T. D. Kanneganti, N. Ozoren, R. Jagirdar, N. Inohara, P. Vandenberg, J. Bertin, A. Coyle, E. P. Grant, and G. Nunez. 2006. Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages. *Nat. Immunol.* **7**:576–582.
18. Gewirtz, A. T., T. A. Navas, S. Lyons, P. J. Godowski, and J. L. Madara. 2001. Cutting edge: bacterial flagellin activates basolaterally expressed tlr5 to induce epithelial proinflammatory gene expression. *J. Immunol.* **167**:1882–1885.
19. Gewirtz, A. T., P. Simon, Jr., C. K. Schmitt, L. J. Taylor, C. H. Hagedorn, A. D. O'Brien, A. S. Neish, and J. L. Madara. 2001. *Salmonella typhimurium* translocates flagellin across intestinal epithelia, inducing a proinflammatory response. *J. Clin. Investig.* **107**:99–109.
20. Gewirtz, A. T., Y. Yu, U. S. Krishna, D. A. Israel, S. L. Lyons, and R. M. Peek, Jr. 2004. *Helicobacter pylori* flagellin evades toll-like receptor 5-mediated innate immunity. *J. Infect. Dis.* **189**:1914–1920.
21. Guerry, P., C. P. Ewing, M. Schirm, M. Lorenzo, J. Kelly, D. Pattarini, G. Majam, P. Thibault, and S. Logan. 2006. Changes in flagellin glycosylation affect *Campylobacter* autoagglutination and virulence. *Mol. Microbiol.* **60**:299–311.
22. Hapfelmeier, S., B. Stecher, M. Barthel, M. Kremer, A. J. Muller, M. Heikenwalder, T. Stallmach, M. Hensel, K. Pfeffer, S. Akira, and W. D. Hardt. 2005. The *Salmonella* pathogenicity island (SPI)-2 and SPI-1 type III secretion systems allow *Salmonella* serovar typhimurium to trigger colitis via MyD88-dependent and MyD88-independent mechanisms. *J. Immunol.* **174**:1675–1685.
23. Harrington, S. M., M. C. Strauman, C. M. Abe, and J. P. Nataro. 2005. Aggregative adherence fimbriae contribute to the inflammatory response of epithelial cells infected with enteroaggregative *Escherichia coli*. *Cell. Microbiol.* **7**:1565–1578.
24. Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill, and A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* **410**:1099–1103.
25. Huang, F. C., A. Werne, Q. Li, E. E. Galyov, W. A. Walker, and B. J. Cherayil. 2004. Cooperative interactions between flagellin and SopE2 in the epithelial interleukin-8 response to *Salmonella enterica* serovar Typhimurium infection. *Infect. Immun.* **72**:5052–5062.
26. Igimi, S., F. Amano, and S. Kumagai. 2000. Inhibition of *Salmonella enteritidis* binding to human intestinal epithelial cells with anti-flagella antibody, p. 62. Abstr. 100th Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, DC.
27. Jacchieri, S. G., R. Torquato, and R. R. Brentani. 2003. Structural study of binding of flagellin by Toll-like receptor 5. *J. Bacteriol.* **185**:4243–4247.
28. Johanesen, P. A., and M. B. Dwinell. 2006. Flagellin-independent regulation of chemokine host defense in *Campylobacter jejuni*-infected intestinal epithelium. *Infect. Immun.* **74**:3437–3447.
29. Lee, S. E., S. Y. Kim, B. C. Jeong, Y. R. Kim, S. J. Bae, O. S. Ahn, J. J. Lee, H. C. Song, J. M. Kim, H. E. Choy, S. S. Chung, M. N. Kweon, and J. H. Rhee. 2006. A bacterial flagellin, *Vibrio vulnificus* FlaB, has a strong mucosal adjuvant activity to induce protective immunity. *Infect. Immun.* **74**:694–702.
30. Lee, S. K., A. Stack, E. Katzowitsch, S. I. Aizawa, S. Suerbaum, and C. Josenhans. 2003. *Helicobacter pylori* flagellins have very low intrinsic activity to stimulate human gastric epithelial cells via TLR5. *Microbes Infect.* **5**:1345–1356.
31. Lyons, S., L. Wang, J. E. Casanova, S. V. Sitaraman, D. Merlin, and A. T. Gewirtz. 2004. *Salmonella typhimurium* transcytoses flagellin via an SPI2-mediated vesicular transport pathway. *J. Cell Sci.* **117**:5771–5780.
32. Maaser, C., J. Heidemann, C. von Eiff, A. Lugerling, T. W. Spahn, D. G. Binion, W. Domschke, N. Lugerling, and T. Kucharzik. 2004. Human intestinal microvascular endothelial cells express Toll-like receptor 5: a binding partner for bacterial flagellin. *J. Immunol.* **172**:5056–5062.
33. McDermott, P. F., F. Ciacci-Woolwine, J. A. Snipes, and S. B. Mizel. 2000. High-affinity interaction between gram-negative flagellin and a cell surface polypeptide results in human monocyte activation. *Infect. Immun.* **68**:5525–5529.
34. Miao, E. A., C. M. Alpuche-Aranda, M. Dors, A. E. Clark, M. W. Bader, S. I. Miller, and A. Aderem. 2006. Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nat. Immunol.* **7**:569–575.
35. Mizel, S. B., A. N. Honko, M. A. Moors, P. S. Smith, and A. P. West. 2003. Induction of macrophage nitric oxide production by gram-negative flagellin involves signaling via heteromeric toll-like receptor 5/toll-like receptor 4 complexes. *J. Immunol.* **170**:6217–6223.
36. Mizel, S. B., A. P. West, and R. R. Hantgan. 2003. Identification of a sequence in human toll-like receptor 5 required for the binding of Gram-negative flagellin. *J. Biol. Chem.* **278**:23624–23629.

37. **Molofsky, A. B., B. G. Byrne, N. N. Whitfield, C. A. Madigan, E. T. Fuse, K. Tateda, and M. S. Swanson.** 2006. Cytosolic recognition of flagellin by mouse macrophages restricts *Legionella pneumophila* infection. *J. Exp. Med.* **203**:1093–1104.
38. **Murthy, K. G., A. Deb, S. Goonesekera, C. Szabo, and A. L. Salzman.** 2004. Identification of conserved domains in *Salmonella muenchen* flagellin that are essential for its ability to activate TLR5 and to induce an inflammatory response in vitro. *J. Biol. Chem.* **279**:5667–5675.
39. **Pietila, T. E., V. Veckman, P. Kyllonen, K. Lahteenmaki, T. K. Korhonen, and I. Julkunen.** 2005. Activation, cytokine production, and intracellular survival of bacteria in *Salmonella*-infected human monocyte-derived macrophages and dendritic cells. *J. Leukoc. Biol.* **78**:909–920.
40. **Ren, T., D. S. Zamboni, C. R. Roy, W. F. Dietrich, and R. E. Vance.** 17 March 2006, posting date. Flagellin-deficient *Legionella* mutants evade caspase-1- and Naip5-mediated macrophage immunity. *PLoS Pathog.* **2**:e18. [Epub ahead of print.]
41. **Rhee, S. H., E. Im, M. Riegler, E. Kokkotou, M. O'Brien, and C. Pothoulakis.** 2005. Pathophysiological role of Toll-like receptor 5 engagement by bacterial flagellin in colonic inflammation. *Proc. Natl. Acad. Sci. USA.* **102**:13610–13615.
42. **Samatey, F. A., K. Imada, S. Nagashima, F. Vonderviszt, T. Kumasaka, M. Yamamoto, and K. Namba.** 2001. Structure of the bacterial flagellar protofilament and implications for a switch for supercoiling. *Nature* **410**:331–337.
43. **Schmitt, C. K., S. C. Darnell, and A. D. O'Brien.** 1996. The attenuated phenotype of a *Salmonella typhimurium* *flgM* mutant is related to expression of *FliC* flagellin. *J. Bacteriol.* **178**:2911–2915.
44. **Schmitt, C. K., J. S. Ikeda, S. C. Darnell, P. R. Watson, J. Bispham, T. S. Wallis, D. L. Weinstein, E. S. Metcalf, and A. D. O'Brien.** 2001. Absence of all components of the flagellar export and synthesis machinery differentially alters virulence of *Salmonella enterica* serovar Typhimurium in models of typhoid fever, survival in macrophages, tissue culture invasiveness, and calf enterocolitis. *Infect. Immun.* **69**:5619–5625.
45. **Smith, K. D., E. Andersen-Nissen, F. Hayashi, K. Strobe, M. A. Bergman, S. L. Barrett, B. T. Cookson, and A. Aderem.** 2003. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. *Nat. Immunol.* **4**:1247–1253.
46. **Smith, K. D., and A. Ozinsky.** 2002. Toll-like receptor-5 and the innate immune response to bacterial flagellin. *Curr. Top. Microbiol. Immunol.* **270**:93–108.
47. **Smith, M. F., Jr., A. Mitchell, G. Li, S. Ding, A. M. Fitzmaurice, K. Ryan, S. Crowe, and J. B. Goldberg.** 2003. Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for *Helicobacter pylori*-induced NF-kappa B activation and chemokine expression by epithelial cells. *J. Biol. Chem.* **278**:32552–32560.
48. **Stecher, B., S. Hapfelmeier, C. Muller, M. Kremer, T. Stallmach, and W. D. Hardt.** 2004. Flagella and chemotaxis are required for efficient induction of *Salmonella enterica* serovar Typhimurium colitis in streptomycin-pretreated mice. *Infect. Immun.* **72**:4138–4150.
49. **Steiner, T. S., A. A. Lima, J. P. Nataro, and R. L. Guerrant.** 1998. Enteropathogenic *Escherichia coli* produce intestinal inflammation and growth impairment and cause interleukin-8 release from intestinal epithelial cells. *J. Infect. Dis.* **177**:88–96.
50. **Steiner, T. S., J. P. Nataro, C. E. Poteet-Smith, J. A. Smith, and R. L. Guerrant.** 2000. Enteropathogenic *Escherichia coli* expresses a novel flagellin that causes IL-8 release from intestinal epithelial cells. *J. Clin. Investig.* **105**:1769–1777.
51. **Tallant, T., A. Deb, N. Kar, J. Lupica, M. J. de Veer, and J. A. DiDonato.** 2004. Flagellin acting via TLR5 is the major activator of key signaling pathways leading to NF-kappa B and proinflammatory gene program activation in intestinal epithelial cells. *BMC Microbiol.* **4**:33.
52. **Torok, A. M., A. H. Bouton, and J. B. Goldberg.** 2005. *Helicobacter pylori* induces interleukin-8 secretion by Toll-like receptor 2- and Toll-like receptor 5-dependent and -independent pathways. *Infect. Immun.* **73**:1523–1531.
53. **Tsujita, T., A. Ishii, H. Tsukada, M. Matsumoto, F. S. Che, and T. Seya.** 2006. Fish soluble Toll-like receptor (TLR)5 amplifies human TLR5 response via physical binding to flagellin. *Vaccine* **24**:2193–2199.
54. **Uematsu, S., M. H. Jang, N. Chevrier, Z. Guo, Y. Kumagai, M. Yamamoto, H. Kato, N. Sougawa, H. Matsui, H. Kuwata, H. Hemmi, C. Coban, T. Kawai, K. J. Ishii, O. Takeuchi, M. Miyasaka, K. Takeda, and S. Akira.** 2006. Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c<sup>+</sup> lamina propria cells. *Nat. Immunol.* **7**:868–874.
55. **Verma, A., S. K. Arora, S. K. Kuravi, and R. Ramphal.** 2005. Roles of specific amino acids in the N terminus of *Pseudomonas aeruginosa* flagellin and of flagellin glycosylation in the innate immune response. *Infect. Immun.* **73**:8237–8246.
56. **Weinstein, D. L., M. Carsiotis, C. R. Lissner, and A. D. O'Brien.** 1984. Flagella help *Salmonella typhimurium* survive within murine macrophages. *Infect. Immun.* **46**:819–825.
57. **West, A. P., B. A. Dancho, and S. B. Mizel.** 2005. Gangliosides inhibit flagellin signaling in the absence of an effect on flagellin binding to toll-like receptor 5. *J. Biol. Chem.* **280**:9482–9488.
58. **Wright, E. K., S. A. Goodart, J. D. Growney, V. Hadinoto, M. G. Endrizzi, E. M. Long, K. Sadigh, A. L. Abney, I. Bernstein-Hanley, and W. F. Dietrich.** 2003. Naip5 affects host susceptibility to the intracellular pathogen *Legionella pneumophila*. *Curr. Biol.* **13**:27–36.
59. **Wyant, T. L., M. K. Tanner, and M. B. Sztein.** 1999. Potent immunoregulatory effects of *Salmonella typhi* flagella on antigenic stimulation of human peripheral blood mononuclear cells. *Infect. Immun.* **67**:1338–1346.
60. **Wyant, T. L., M. K. Tanner, and M. B. Sztein.** 1999. *Salmonella typhi* flagella are potent inducers of proinflammatory cytokine. *Infect. Immun.* **67**:3619–3624.
61. **Zhou, X., J. A. Giron, A. G. Torres, J. A. Crawford, E. Negrete, S. N. Vogel, and J. B. Kaper.** 2003. Flagellin of enteropathogenic *Escherichia coli* stimulates interleukin-8 production in T84 cells. *Infect. Immun.* **71**:2120–2129.

Editor: J. B. Kaper