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Flagellin: key target of mucosal innate immunity

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The mucosal immune system is charged with defending the host's vast interfaces with the outside world from the enormous and diverse group of microbes that colonizes these surfaces. A key means by which the mucosal immune system protects the host from such diverse microbes is using germ-line-encoded receptors that target structurally conserved motifs that mediate important bacterial functions. This review focuses on one embodiment of this notion, namely, the mucosal innate immune targeting of flagellin, the primary structural component of flagella, which afford bacteria the ability of directed locomotion. Specifically, we discuss the mechanisms by which flagellin is recognized by the innate immune system, their role in host defense, chronic inflammatory disease, and potential approaches to pharmacologically manipulate these pathways to benefit the host. Discussion will focus on the intestinal tract but will also incorporate key findings in other mucosal surfaces.

DEFENDING THE INTESTINE: A COMPLEX PEACEKEEPING MISSION

A general characteristic of mammalian mucosal surfaces is that they are active interfaces at which essential exchange of molecules takes place between the mammal and the outside world. Thus, unlike the skin, which offers protection by forming a barrier impregnable to even the smallest molecules, the mucosal immune system must protect the host from an assortment of dangers without interfering with the complex "commerce" undertaken in its midst. Mucosal defense is an especially daunting phenomenon in the intestine in that such commerce is often quite variable, particularly in terms of the diversity of food ingestion in which different nutrients and unfamiliar antigens need to be deciphered. Finally, of greatest importance, at least to this review, is the fact that mucosal surfaces in general, and the intestine in particular, are not sterile but are populated by an assortment of microbes that, depending on how well they are "managed," can be a benefit or detriment to the health of the host.

These microorganisms that inhabit the human intestine, known collectively as the "microbiota," contain 10^{13} – 10^{15} bacteria, with the greatest microbial densities being present in the distal alimentary tract, particularly the colon.¹ The intestinal microbiota contains numerous species of all the traditional classifications, including aerobic, anaerobic, motile, non-motile, Gram-positive, and Gram-negative bacteria. The majority of these bacteria cannot be cultured and are thus difficult to study functionally but are thought to be strict anaerobes and non-

motile. Moreover, on the basis of a metagenomic analysis of feces and intestinal biopsies, the microbiota is thought to contain over 15,000 distinct species and more than 2×10^6 distinct genes.² Although intestinal bacteria have traditionally been categorized as being either simply commensal (i.e., neither benefitting nor harming the host) or pathogenic, greater appreciation for the complexity of the microbiota indicates that, in fact, intestinal bacteria form a continuum of species the relative characteristics of which range from mutualism to commensalism to opportunistic pathogens to true pathogens (i.e., those that cause disease in "typical" healthy hosts). Much of the mutualism between the host and microbiota involves metabolic functions. The microbiota can itself be viewed as a "metabolic organ" or "bioreactor" that performs physiological functions comparable with that of the liver.³ The benefit of the microbiota metabolism to the host is highlighted by the observation that gnotobiotic or germ-free rodents require 33% more caloric intake to maintain a comparable body weight than do conventional mice.⁴ The microbiota confers both local and systemic benefits to host defense both by niche occupation and by keeping the immune system properly regulated. Despite the benefits it confers on the host, the microbiota can also be quite harmful to its host if it is not properly "managed." For example, disease states are associated with both general intestinal bacterial overgrowth and inappropriate levels of select *Escherichia coli* species, which can be viewed as opportunist pathogens in that they may promote disease in select hosts/conditions but do not seem to cause disease in healthy hosts.⁵ In addition, the gut is occasionally exposed

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to true enteropathogens, i.e. microbes having a high capacity to cause disease in healthy hosts through contaminated food and water. When present, such pathogens generally comprise a tiny fraction of the intestinal bacteria and thus the task of identifying such pathogens is analogous to finding a needle in a haystack. Moreover, pathogens can be quite structurally similar to less harmful members of the microbiota, sometimes differing in only a few genes that make them detrimental to the host. Thus, the gut immune system has the Herculean task of detecting and clearing pathogens, keeping opportunistic pathogens in-check, and doing so without causing excessive harm to beneficial microbes or host tissues.

INNATE MUCOSAL IMMUNITY: TO MAKE PEACE, PREPARE FOR WAR

The complex task of “keeping the peace” between the host and microbes in the intestine is accomplished by a multifaceted approach involving physical barriers, border patrols (i.e., resident immune cells), and an advanced intelligence system (i.e., adaptive immunity). Arguably, the most important component of this system, at least in the sheer numbers of bacteria it excludes, is the physical barrier as formed by epithelial cells themselves, which is covered in a thick layer of mucins as “border fencing” produced by goblet cells interspersed throughout the gut epithelium.⁶ An additional means of keeping vast numbers of microbes in-check is through the production of natural peptide antibiotics, such as defensins and cathelicidins, which are produced by epithelial cells, especially by Paneth cells located at the base of intestinal crypts. The “advanced intelligence system” is mediated by dendritic cells interdigitated among epithelial cells, which sample the intestinal contents and present peptide antigens to T cells present in the lamina propria and lymphoid follicles. In the small intestine, another type of specialized epithelial cells called M (microfold) cells is present in the follicle-associated epithelium above Peyer’s patches. These M cells transport and present luminal antigens to T cells by pinocytosis. The gut is the host’s largest reservoir of immune cells, including macrophages, dendritic cells, B cells, and T cells, existing in organized lymphoid structures and spreading throughout the lamina propria, which together constitute the gut adaptive immune system. This review will focus on the role of “border security patrol,” specifically discussing how epithelial cells, and mucosal macrophages and dendritic cells detect bacterial flagellin to coordinate their responses to protect against true pathogens and keep commensal microbes from running amok.

INNATE IMMUNE RECOGNITION OF FLAGELLIN: TARGETING CONSERVATION AMIDST DIVERSITY

Despite the aforementioned great genetic diversity among intestinal bacteria, a number of structural motifs used by bacteria are highly conserved. Such conservation likely reflects that these motifs perform essential functions, thus making it difficult for bacteria to avoid expressing them. For example, lipopolysaccharide and peptidoglycan provide bacteria with essential structural elements, whereas bacterial DNA is, even more obviously, required for existence. In accordance with their essential

functions, these motifs are targeted vigorously by innate immune cells (macrophages, neutrophils, and dendritic cells) obtained from blood/spleen as well as by some somatic cells such as endothelial cells. In contrast, cells at mucosal surfaces (both epithelial and immune cells) seem relatively less responsive to these products possibly to avoid excess reactivity to the microbiota in general.⁷ In contrast, as will be discussed below, cells in the intestinal mucosa are quite responsive to the bacterial protein flagellin—the primary component of flagella (a typical flagella is comprised of 30,000–70,000 flagellin monomers)—and are thus essential for motility.⁸ It is less obvious why bacteria require the ability of directed locomotion. Indeed, many bacteria never exhibit motility. Moreover, clinical isolates of classic motile pathogens, such as *Salmonella*, are occasionally found to be lacking in the expression of flagella and consequently are non-motile. Yet it seems quite intuitive that the ability to move in a directed manner, toward a niche/food source or away from danger, would be incredibly advantageous to most bacteria. Thus, we view the importance of motility as self-evident in that, despite the enormous metabolic cost of producing and operating flagella (up to 30% of a bacterium’s total caloric intake)⁹ and the selective immune pressure directed against flagellin, many bacteria that colonize the intestine do indeed express these “expensive,” immunogenic, and complex structures.

If one accepts that it is intuitive that the ability of motility has the potential to help a bacterium colonize a host, it logically follows that organisms might develop multiple mechanisms to detect flagellin at various stages of infection. Indeed, the innate recognition of flagellin is not limited to animals but is also shown by plants and insects.¹⁰ Mammals have evolved at least two distinct pathways of recognizing flagellin monomers. Specifically, extracellular flagellin is recognized through toll-like receptor 5 (TLR5), whereas intracellular flagellin is recognized through a pathway apparently involving both interleukin-converting enzyme protease-activating factor (IpaF) and Nod-like receptor apoptosis-inhibitory protein-5 (Naip5).¹¹ TLR5 is one of 11 toll-like receptors, whereas both Naip5 and IpaF belong to the NLR family of intracellular pattern recognition/signaling molecules.

Toll-like receptor 5 is functionally expressed on many types of epithelial cells, including those isolated from the intestinal, respiratory, and kidney/urogenital tracts and ocular surfaces, in that both primary cells and numerous cell lines respond potently to picomolar concentrations of flagellin. In the polarized epithelia examined to date, in the intestinal, kidney, and airway cell lines and, most importantly, the human colon, TLR5 expression is polarized to the basolateral surface. In contrast, TLR5 does not seem to be expressed at a functionally significant level in typical (i.e., non-mucosal) populations of mouse macrophages and dendritic cells, although it is expressed on such cells in humans.¹² However, TLR5 is apparently expressed on murine intestinal dendritic cells, and functional studies discussed below suggest distinct roles for epithelial vs. immune cell, TLR5. The basic mechanisms by which TLR5 signals appear to be relatively similar to those used by other TLRs. Briefly, the ligation of TLR5 with flagellin results in a MyD88-dependent

activation of MAP kinases (p38 and extracellular signal-regulated kinase) and I κ B kinase, which culminates in the activation of the canonical nuclear factor- κ B pathway.¹³ The overall downstream outcome of such signaling results in the transcriptional activation of an extensive panel of at least 500 genes, the overall function of which seems to be to protect the cells against various challenges.¹⁴ Consequently, TLR5-activated genes include those with direct antibacterial function (e.g., defensins), immune-cell chemoattractants, as well as a number of more general stress-induced genes, such as heat-shock proteins. In addition, TLR5 ligation in epithelial cells results in the activation of a number of genes with an antiapoptotic function.¹⁵ Activation of such antiapoptotic gene expression allows cells to stay alive in response to challenges that would otherwise result in cell death.

The recognition of a pathway of intracellular flagellin came from observations that, although “typical” populations of macrophages lack TLR5 expression and do not activate nuclear factor- κ B in response to soluble flagellin, such cells can distinguish between wild-type (WT) pathogens and isogenic aflagellate mutants in that they are much better at controlling intracellular replication of flagellated pathogens relative to isogenic aflagellate mutants. Such an ability to control flagellate pathogens was lost in mice carrying a natural variant of Naip5 and an engineered loss of Ipaf.^{16–18} Mechanistic studies of both of these NLRs revealed that macrophages respond to flagellin by activation of the inflammasome, a multiprotein complex, in a manner completely dependent on Ipaf and Naip5.¹⁹ Use of a viral transduction system showed that this pathway can be activated by the expression of monomeric flagellin, or a fraction therein, and does not require the presence of assembled flagella.¹⁹ The extent to which Naip5 is necessary for inflammasome activation is an area of discrepancy in this field and is beyond the scope of this review. Thus, the pathway will simply be referred to here as the Ipaf pathway. Ipaf-mediated activation of the inflammasome results in the activation of the cysteine aspartyl protease, caspase-1, which cleaves both pro-IL-1 β and pro-IL-18 into their mature active forms and drives cell death, a process known as pyroptosis (**Figure 1**).¹¹ Although the consequences of this pathway may seem somewhat limited in scope relative to those activated by TLR5, these inflammasome cytokines are increasingly appreciated as playing key roles in numerous biological processes, from intestinal inflammation to basic regulation of energy metabolism. Moreover, the death of circulating infected cells may play a pivotal role in limiting the scope of some infections.

Co-immunoprecipitation studies suggest that TLR5 is likely to directly associate with flagellin,²⁰ whereas the actual sensor of cytosolic flagellin in the Ipaf pathways has not been carefully addressed. In any case, both pathways are thought to recognize flagellin monomers rather than polymerized flagellin. TLR5 recognizes a conserved motif present on the surface of flagellin monomers but which is buried inside the polymerized flagella. The motif is comprised of highly conserved regions of the flagellin molecule present in both the N and the C terminus of the molecule, which form a region thought to be required for the polymerization of flagellin into flagella.²¹ Activation of

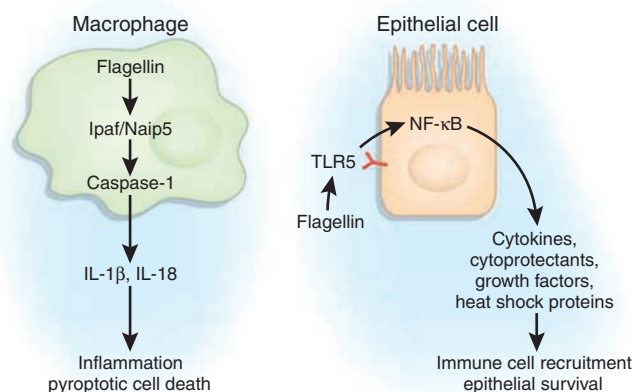


Figure 1 Epithelial cells and macrophages use markedly different signaling pathways to respond to flagellin. As detailed in the text, epithelial cells activate a panel of host defense genes on sensing basolateral flagellin through TLR5. Although the extent to which macrophages respond to flagellin appears to vary depending on the species and location within the host, “typical” populations of murine macrophages respond to intracellular flagellin through pyroptotic cell death mediated by Ipaf.

Ipaf requires only the C-terminal 35 amino acids of the flagellin protein, which is also not accessible on flagella.¹⁹ In contrast, the region of the flagellin molecule recognized by adaptive innate immunity arises on the outside of polymerized flagella and is highly variable even among closely related bacteria,²² thus providing the serospecificity that is the basis of H serotyping used to classify *E. coli* and *Salmonella* isolates. TLR5’s targeting of the conserved regions of the flagellin monomer affords this receptor the ability to recognize flagellins from a wide variety of bacteria including *Salmonella*, *E. coli*, *Pseudomonas*, *Listeria*, *Legionella*, *Clostridia*, and *Vibrio*. Yet, some bacteria, notably *Helicobacter pylori* and *Campylobacter jejuni*, express flagellins that are indeed functional and afford motility but yet avoid activating TLR5.^{23,24} To date, the Ipaf pathway has only been shown to be activated by flagellin from *Salmonella* and *Legionella* although, given the conservation of the flagellin C terminus recognized by the Ipaf pathway, broad recognition of various flagellins seems likely.

The source of extracellular flagellin monomers that can activate TLR5 might simply be shed flagellin monomers, which may be an inherent consequence of flagella depolymerizing at the distal end at a substantial distance from the bacteria.²⁵ Thus, there will likely be flagellin monomers wherever there are flagellated bacteria. Interestingly, some bacteria, notably *Helicobacter* and *Vibrio* species, encase their flagella in a sheath and thus avoid shedding flagellin monomers possibly to reduce immune detection. Alternatively, some bacteria, particularly the *Salmonella* species, are reported to have a distinct mechanism that allows them to release flagellin monomers not physically associated with the flagellum on detection of the host lysophospholipids,²⁶ suggesting that at least an enhanced activation of TLR5 might be a consequence of a pathogenic mechanism. In contrast to TLR5 activation, free flagellin monomers do not seem to be an efficient means of activating the Ipaf pathway, but rather activating this pathway requires flagellin to enter the cytosol.¹⁹ Activation of Ipaf by *Salmonella* requires a type-III secretion system,

suggesting that this structure is required for flagellin to attain such a location and, potentially providing a means by which macrophages may make inflammasome cytokines in response only to pathogens. However, our recent study indicates that, *in vivo*, even purified flagellin monomers alone may be sensed by the Ipaf pathway, suggesting that at least some cells have the means of cytosolic uptake of free flagellin.²⁷

FLAGELLIN/TLR5: A DOMINANT INNATE IMMUNE INTERACTION AT MUCOSAL SURFACES

Perhaps the most striking property of flagellin relative to other innate immune activators is its ability, at very low concentrations, to potentially activate a wide variety of epithelial cells to make immune-cell-recruiting chemokines—a bioactivity not yet observed to be shared by any other microbial ligand. For example, the addition of picomolar concentrations of purified flagellin to model gut epithelia is sufficient to recapitulate all of the pro-inflammatory gene expressions exhibited by epithelial cells on infection with the gastroenteritis-causing pathogen, *Salmonella typhimurium*. Furthermore, flagellin is necessary for the activation of epithelial pro-inflammatory gene expression induced by bacteria in that deletion of the flagellin gene(s) from *S. typhimurium* ablates its ability to activate pro-inflammatory gene expression in these cells. In contrast, the fact that these cells neither display pro-inflammatory gene expression in response to purified lipopolysaccharide (LPS) nor a flagellate *Salmonella* suggests that this TLR4 ligand is neither necessary nor sufficient to activate gut epithelia. By similar logic, other bacterial activators of innate immunity, such as peptidoglycan and CpG DNA, are also not major players in activating intestinal epithelial cells. The extent to which observations apply to various epithelial cell lines from the intestine and other sources exhibits some variance depending on the particular cell line studied but, overall, supports the notion that flagellin is a dominant activator of epithelial cells. The fact that flagellin-induced activation in these studies was generally based on rapid secretion of interleukin-8 (IL-8) or other nuclear factor- κ B-mediated cytokines and typically used low concentrations of flagellin suggests that such responses were mostly, if not entirely, mediated by TLR5, although a potential role for Ipaf or other possible flagellin-recognition pathways has not been formally extensively studied.

Given that epithelial cells are the most numerous cells at any mucosal surface and generally the first cells to be encountered by microbes seeking to colonize these surfaces, we propose that flagellin is a dominant innate immune recognition pathway in mucosa in general. *In vivo* studies indeed support the notion that flagellin recognition plays an important role in host defense at such surfaces but, interestingly, this seems not to be mediated entirely by epithelia but rather, in contrast to their systemic counterparts, by mucosal innate immune cells that are also responsive to flagellin. Specifically, in the intestine, CD11⁺C-expressing lamina propria cells, presumed DC, exhibit robust TLR5-dependent production of the pro-inflammatory cytokines, IL-12 and IL-6, in response to flagellin but are unresponsive to LPS.²⁸ Moreover, in contrast to bone-marrow-derived macrophages, which are quite unresponsive to flagellin,

alveolar macrophages exhibit TLR5-dependent production of tumor necrosis factor- α in response to ng ml⁻¹ concentrations of flagellin albeit less tumor necrosis factor- α than produced in response to LPS.²⁹ In accordance with a role for select populations of immune cells responding directly to flagellin, studies from bone-marrow chimeric mice in which only hemopoietic or non-hemopoietic cells have the capacity to respond to flagellin revealed that both cell compartments play distinct roles in this response. Specifically, epithelial cells were responsible for most of the chemokines induced by flagellin, whereas pro-inflammatory cytokines, such as tumor necrosis factor- α and IL-12, were from hemopoietic cells.³⁰ This pattern may reflect the ability of epithelia to recruit immune cells to mucosal infections, whereas immune cells likely make the more general “command decisions” of regulating the production of these master pro-inflammatory cytokines. In any case, the high degree of responsiveness to flagellin of both epithelial and immune cells at mucosal surfaces suggests an important role for the recognition of this product in mucosal defense.

Although the expression of TLR5 at mucosal surfaces logically positions it to detect flagellated mucosal pathogens, it also poses a potential problem, namely, how to avoid inappropriate activation of this receptor in response to flagellin released by non-pathogenic bacteria, especially in the intestinal lumen, given the large load of such microbes. However, studies in several polarized epithelial cell lines indicate that the expression of TLR5 is polarized to the basolateral surface. The degree of such polarity varies depending on which cell line is being studied and likely the specific culture conditions, with not all cell lines exhibiting highly polarized responses. However, perhaps most importantly, human colon, studied *ex vivo*, was only responsive to flagellin administered to the serosal (basolateral) surface.³¹ Thus, TLR5 may provide a means of detecting whether the epithelium has been breached by both flagellated pathogens and/or non-pathogenic bacteria that opportunistically managed to reach a subepithelial locale. Importantly, such a compartmentalization of TLR5 suggests that this receptor may play a role in driving inflammation in states of epithelial barrier dysfunction—a condition associated with inflammatory bowel disease (IBD).³² In accordance with this notion, intracolonic administration of flagellin enhanced mucosal inflammation when given to mice with ulcerated mucosa, but had no such effect on healthy mice.^{31,33} Thus, studies using purified flagellin support the notion that TLR5 may drive gut inflammation in response to enteric pathogens and/or epithelial barrier dysfunction.

MUCOSAL TLR5: QUICK TRIGGERING OF THE ALARM PUTS THE FIRE OUT FAST

The role of innate immune recognition of flagellin in response to flagellated pathogens can be studied by both deleting flagellin gene(s) from bacteria and/or deleting innate immunity genes from the host. The difficulties in interpreting studies in which flagellin genes are deleted from a particular pathogen former approach are the following: (1) Alterations in the bacterium's pathogenicity may reflect a role for motility or even loss of adherence, which can be mediated by flagella rather than by

the loss of flagellin *per se*.³⁴ (2) Deleting some of the most highly expressed/regulated genes may have relatively broad effects on gene expression as, for example, the deletion of flagellin genes in *Salmonella* reduces the expression of its type 3 secretion system. (3) Deleting flagellin genes will not shed light on which host genes mediate its recognition. The primary negative aspect to relying only on alteration of host genes is that deletion of some genes can result in a variety of indirect or compensatory phenotypes, thus altering virulence in a nonspecific manner. Thus, as highlighted by results from us and others studying *Salmonella*, it is important to use both approaches to address the role of flagellin in infection.

The relative role of flagella in *Salmonella* pathogenicity has long been studied by comparing the relative virulence of WT vs. aflagellate strains. Such studies have generally used low-dose oral infection, which results in a systemic disease reminiscent of typhoid fever. Thus, LD₅₀ of a strain in this model can be viewed as reflecting, in part, how well it breaches the mucosal defenses. Such studies have generally observed that the deletion of flagellin genes results in increased virulence (lower LD₅₀), suggesting that innate immune recognition of flagellin helps protect against bacterial dissemination.³⁵ Although the actual differences in LD₅₀ in these studies tend to be relatively mild (2- to 10-fold), the fact that the increased virulence occurs despite loss of an attribute, namely, motility, which would normally presumably benefit the pathogen, suggests that the degree of increased virulence resulting from reduced innate immune activation may actually be considerably greater. In accordance, mutants that actually express flagellin but are not motile because of defects in other proteins that make-up or power flagella, and thus lose the advantage of motility but do not avoid innate immune mechanisms targeting flagellin, have reduced virulence relative to WT strains. Thus, it seemed quite surprising that mice engineered to lack TLR5 were observed to be relatively resistant to bacterial dissemination and death when orally infected with *Salmonella*.²⁸ However, subsequent studies revealed that such protection was not specific to flagellated *Salmonella* but was also observed in response to aflagellate *Salmonella*. Their resistance to this infection model appears to be attributable to alterations in basal gene expression and/or altered gut microflora. Thus, although these observations serve as a caveat to interpreting studies in which genetically engineered mice are used in infection models, they may not be especially informative as to the role of innate immune recognition of flagellin in *Salmonella* infection *per se*.³⁶

In contrast, in a model of *Salmonella*-induced enteritis reminiscent of the majority of food-borne *Salmonella* infections in developed countries, the approach of deleting flagellin genes “phenocopied” that of deleting TLR5.³⁶ Specifically, blocking the flagellin/TLR5 by either method resulted in loss of the early recruitment of neutrophils that occurred in the first 12 h of infection. The reason that the basal phenotype of TLR5KO mice did not appear to affect this infection model may be that, in this model, mice are subjected to a large bolus of antibiotics 48 h before infection, which may remove any differences in microflora between WT and TLR5KO mice and, consequently,

reduce relative differences in basal gene expression, thus “leveling the playing field.” In any case, the absence of such an early immune-cell recruitment, when either *Salmonella* lacked flagellin genes or mice lacked TLR5, resulted in greater bacterial loads, which eventuated in an ultimately greater amount of neutrophil recruitment by 48 h. Such increased immune-cell recruitment, combined with the loss of flagellin-induced cytoprotective gene expression in epithelial cells, ultimately resulted in much greater tissue damage at both gross and microscopic levels. Such enhanced tissue damage was dependent on signaling by other TLRs in that mice lacking MyD88 lacked significant inflammatory pathology. Although the finding that ablating the receptor/ligand interaction thought to trigger neutrophil recruitment eventuates in greater neutrophil recruitment may seem somewhat paradoxical, this conclusion is somewhat analogous to the notion that an efficient fire detection system, specifically, an appropriate fire alarm, will allow for quicker recruitment of fire fighters, thus making it possible for a relatively small number of them to put out a fire quickly before it spreads, thereby preventing a “multi-alarm” fire in which many fire fighters are called in from multiple precincts.

TLR5 also defends against mucosal flagellated pathogens in the lung and urinary tract in a manner quite reminiscent of that observed in the intestine. Specifically, mice lacking TLR5 exhibit a delayed neutrophil recruitment in response to *Legionella pneumophila* that ultimately results in greater immune-cell recruitment and, consequently, greater inflammatory pathology in the lung.²⁹ Loss of TLR5 led to a similar reduction in early neutrophil recruitment in response to uropathogenic *E. coli*, which allowed for greater numbers of bacteria to escape the mucosa and reach the kidney.³⁷ Thus, in addition to helping quench a fire before it spreads, activation of TLR5 may also allow for a fire to be contained, thus preventing it from spreading to other locales. Our general assessment of the literature in this area, and our unpublished studies, indicate that the increase in infectious inflammatory pathology is greater when bacterial flagellin is deleted rather than when TLR5 is deleted, suggesting that the Ipaf pathway also plays an important role in host defense against intracellular flagellated mucosal pathogens. Studies in this field in the next few years with Ipaf-deficient mice should shed light on this notion.

ROLE OF INNATE IMMUNITY TO FLAGELLIN IN IBD

Given that altered bacterial–host interactions are likely to be central to IBD, that flagellated bacteria are abundant in the gut, and that epithelial cell lines and human colon respond robustly to purified flagellin, it seems quite likely that TLR5, and perhaps Ipaf, will interact with flagellin at some point in the course of IBD. However, whether this interaction will benefit or harm the host is proving to be a complex question, with the answer depending substantially on the particular circumstances that might result in the innate immune system recognizing flagellin. Considering that the potential role of flagellin in IBD was identified on the basis of its potent ability to induce neutrophil chemoattractants, particularly IL-8, it seems likely that flagellin may play a role in driving neutrophil recruitment in active IBD.

Neutrophil infiltration to the mucosa is not only the defining hallmark of active inflammation but is also thought to cause many of the clinical manifestations and tissue damage associated with this state. Thus, it seems reasonable to hypothesize that activation of TLR5 by flagellin might be a detriment to the host particularly if occurring in the absence of a pathogen, as can be envisaged to happen in IBD. In support of this view, bacterial isolates from Crohn's disease patients exhibit an increased ability to elicit the production of IL-8 from cultured epithelial cells apparently driven by increased expression/secretion of flagellin.³⁸ However, as the above-described studies on flagellin have reminded us, perhaps it is better to presume that immune responses are in fact beneficial until proven detrimental. Indeed, one can easily imagine a number of mechanisms by which activation of TLR5 might mitigate IBD. First, flagellin-induced gene expression should promote the killing of mucosal bacteria, thus reducing the levels of potentially perturbing microbes/molecules that may drive disease. Second, activation of TLR5 induces the expression of genes with direct antibacterial activity such as defensins, thus providing another means by which host recognition of flagellin may help keep bacteria in-check. Furthermore, ligation of TLR5 upregulates epithelial heat-shock protein expression³⁹ and potentially activates antiapoptotic/cytoprotective gene expression,¹⁵ which together may protect epithelial cells from a variety of conditions that would otherwise result in epithelial-cell damage and/or death.⁴⁰ Analogous to the case for TLR5, one can envisage that activation of Ipaf would not only promote inflammation but also protect against inflammation by mediating the death of bacteria that might cause inflammation in the first place. This discussion of the role of innate immune recognition of flagellin in IBD will primarily focus on the role of TLR5 as there is simply little that is known about Ipaf in this regard.

Studies using the dextran sodium sulfate model of colitis supported the notion that activation of TLR5 exacerbates the severity of colitis and worsens the overall health of the host. Pothoulakis and co-workers³¹ observed that mucosal administration of flagellin to a dextran sodium sulfate-injured colon, but not to an intact colon, increased the severity of colitis, enhanced immunohistopathological damage, and worsened mortality, thus supporting the idea that flagellin activates TLR5 (or perhaps Ipaf in macrophages) on breach of the epithelium, which can occur in colitis. Furthermore, these results indicate that, in this context, in which pathogens are not present, activation of TLR5 is a detriment to the host. A recent study by Darfeuille-Michaud and co-workers, which used a more physiological approach to present flagellin to the mucosa, further supports this conclusion. Specifically, these investigators administered dextran sodium sulfate in mice colonized by adherent-invasive *E. coli* that were isolated from Crohn's disease patients. They observed that adherent-invasive *E. coli*, but not typical commensal *E. coli*, exacerbated colitis and that this activity was lost in adherent-invasive *E. coli* flagellar mutants.⁴¹ Thus, in the context of gut barrier dysfunction or other possible perturbations, flagellin from bacteria that are not true pathogens, but can colonize IBD patients, may drive the acute

inflammation seen in IBD. Although there is not much data from human studies regarding the role of innate immunity to flagellin in IBD, the notion that activation of TLR5 promotes colitis is supported by a genetic study in which we examined, in IBD patients and in control subjects, the rate of carriage of a dominant-negative TLR5-stop polymorphism, which is thought to result in a 75% reduction in TLR5 function.⁴² We observed that, in individuals of Ashkenazi Jewish ethnicity, carriage of this TLR5 polymorphism was negatively associated with Crohn's disease, but not with ulcerative colitis, suggesting that reduced TLR5 function may offer some protection against developing CD.⁴³

In light of these findings, we reasoned that mice lacking TLR5 would likely be resistant to developing colitis (in the absence of pathogens). In contrast, we observed that TLR5KO mice had a tendency to develop spontaneous colitis.⁴⁴ The most severe manifestation of spontaneous colitis was rectal prolapse, which was exhibited by about 10–12% of mice lacking TLR5, whereas, in total, about 40% of TLR5KO mice had gross, histopathological, and serological evidence of colitis. Importantly, it should be noted that such severe manifestations of colitis have not been observed in TLR5KO mice housed at other facilities, suggesting that, similar to other spontaneously colitic mice, e.g., IL-10KO, their phenotype may be significantly affected by environmental conditions, particularly their microflora. In accordance, rederivation of the mice by embryonic transplant into mice purchased from Jackson Labs (Bar Harbor, ME) markedly attenuates the severity of their disease (our unpublished observations). Although, on the basis of gross or histopathological analysis, about 60% of TLR5KO appeared relatively normal, analysis of their intestinal gene expression by microarray indicated that they had substantial elevation in pro-inflammatory gene expression and thus may be poised to develop colitis. In accordance, when bred onto an IL-10-deficient background, loss of TLR5 resulted in severe colitis with 100% penetrance in the double-knockout mice (about 60% of IL-10-deficient littermates get colitis). Regardless of whether they exhibited colitis, loss of TLR5 resulted in inability to control the enteric microflora. Specifically, non-colitic TLR5KO mice exhibit moderately elevated (about fivefold) numbers of total culturable bacteria in their feces and, moreover, have many bacteria that are tightly associated with the colonic surface, which contrasts to WT mice that have most bacteria in the intestinal lumen. TLR5KO mice also exhibited a mild translocation of bacteria to the liver and spleen. More interestingly, in TLR5KO mice, Ipaf signal is intact as both WT and TLR5KO mice induced serum IL-18 levels to a similar extent and yet could not protect against colitis.²⁷ We are currently endeavoring to define the role of Ipaf in TLR5KO spontaneous colitis. In addition, we are actively investigating the role of adaptive immunity in TLR5KO colitis. We have preliminarily observed that breeding TLR5KO onto a Rag^{-/-} background, and thus having no adaptive immune system, markedly attenuates disease severity, suggesting that adaptive immunity is indeed very important for colitis in these mice.

A variety of approaches, including much of the studies discussed above, suggest that the colitis exhibited by TLR5KO

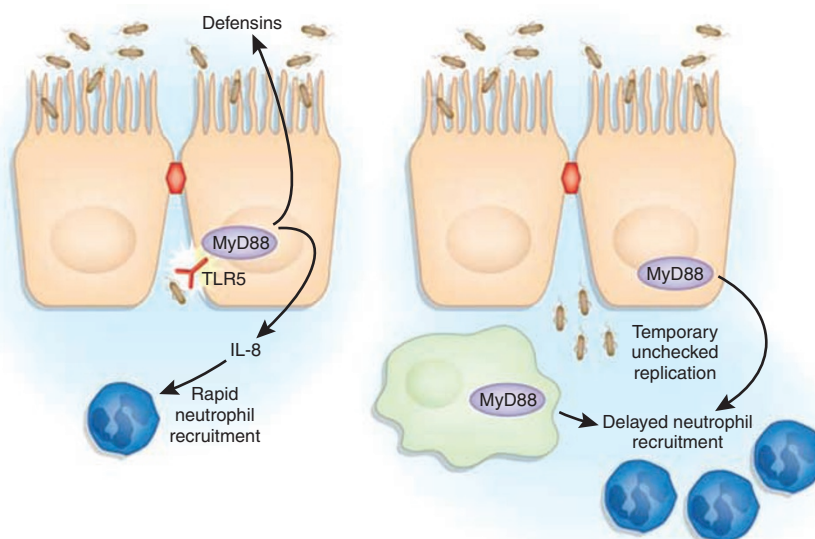


Figure 2 Loss of TLR5-mediated neutrophil recruitment delays pathogen clearance eventuating in increased inflammation. As described in the text, TLR5 activation recruits neutrophils within hours to efficiently contain perturbing bacteria. In the absence of TLR5, other MyD88-dependent signaling pathways ultimately get activated and will drive inflammation. However, the relative delay in triggering recruitment of inflammatory cells appears to result in a greater degree of inflammation being necessary to contain the expanded infection.

mice results from loss of TLR5 function on epithelial cells, which underlies TLR5KO colitis. Loss of epithelial TLR5 may reduce the epithelial secretion of antibacterial mediators and/or reduced recruitment of immune cells. In any case, the resulting failure to manage the enteric microbiota likely results in colitis through the activation of other pathways of TLR signaling. In support of this notion, loss of TLR5 resulted in an enhanced expression of the LPS receptor, TLR4, and other proteins involved in LPS-induced signaling. Moreover, mice lacking both TLR4 and TLR5 were free of colitis indicating that the colitis of TLR5KO mice was dependent on TLR4. Moreover, mice lacking MyD88 that had been bred/housed with TLR5KO also do not develop spontaneous colitis. Thus, together, these results indicate that, in mice, loss of TLR5 results in the ability to control the normally non-pathogenic microbiota resulting in the activation of MyD88-dependent signaling pathways, such as TLR4, which eventuates in colitis (**Figure 2**). However, it should be noted that it is not yet clear which cells mediate such MyD88-dependent responses in that, in contrast to macrophages isolated from the peritoneum or derived from bone marrow or blood monocytes, intestinal macrophages do not normally make inflammatory cytokines in response to LPS. We hypothesize that perhaps TLR4, with the ability to trigger inflammation, is basally present on an undefined lamina propria cell type or that it is upregulated on some cell type on the dysregulated microbial–host interactions that may occur on loss of TLR5. Although loss of functional TLR5 from humans does not appear to result in colitis in humans, these results are nonetheless an example of how a loss of innate immune function, which in Crohn’s patients may result from mutations in *Nod2*, can eventuate in increased immune activity possibly because of the failure to put out “fires” quickly.

THERAPEUTIC USE OF FLAGELLIN: PUTTING THE HOST ON RED ALERT

Although excessive activation of TLR5 may indeed ultimately prove to play a pathogenic role in some chronic inflammatory processes, its above-described role in protecting the host in various scenarios makes it clear that activating this receptor can protect the host, thus suggesting that, perhaps pharmacologically activating TLR5 might be beneficial in certain circumstances. In support of this notion, we observed that the severe inflammatory pathology caused by aflagellate *Salmonella* could be rescued by the systemic administration of purified flagellin. Moreover, the intestinal pathology induced by WT *Salmonella* could be markedly reduced by such a treatment, suggesting that the protection normally conferred by this receptor could be boosted by further TLR5 activation.⁴⁵ Exogenous administration of flagellin also protected against systemic *Salmonella* infection in both mice and chickens.^{46,47} Kumar *et al.*⁴⁸ showed that the administration of *Pseudomonas aeruginosa* flagellin through the subconjunctival and intraperitoneal routes 24 h before *Pseudomonas* infection reduced inflammatory pathology in a mouse model of *P. aeruginosa*-induced keratitis.⁴⁸ Finally, prophylactic administration of flagellin to mice made them resistant to rotavirus infection.⁴⁷ Thus, therapeutic administration of flagellin may be a means of providing temporary broad protection against a variety of microbial pathogens. The mechanism of such a protection is not well worked out but may involve immune-cell recruitment to mucosal surfaces and induce the epithelial expression of genes that repel bacteria (e.g., defensins), allowing them to withstand various challenges (i.e., antiapoptotic genes and heat-shock proteins). However, given the robust and systemic cytokines induced by such treatments of flagellin, many possible alternate mechanisms may underlie these observations.

Flagellin can also protect against non-microbial challenges. For example, systemic flagellin administration protects against oral dextran sodium sulfate, a chemical colitogen that induces intestinal inflammation.⁴⁷ Moreover, a study from multiple labs showed that systemic flagellin administration protects against γ -radiation in a TLR5- and MyD88-dependent manner. Such protection was manifest at the level of survival and in protecting against radiation-induced intestinal mucositis.^{47,49} Again, likely mechanisms involve cytoprotective gene expression and effects on immune cells. In addition, studies have shown that flagellin possesses highly effective mucosal adjuvant activity, and its incorporation in vaccine formulations to promote mucosal innate and adaptive immunity is being explored.⁵⁰ Finally, a recent study from Pothoulakis and co-workers⁵¹ suggests that flagellin promotes necrosis of tumors *in vivo* and thus may have significant activity against some cancers, especially epithelial-based cancers (i.e., carcinomas), possibly by promoting immune-cell recruitment to the tumor site. Thus, although there remains much basic work on understanding the host response to flagellin, clinical trials investigating its therapeutic efficacy in humans are underway. It seems likely that many of flagellin's potential beneficial actions will be shared by other TLR agonists, particularly those primarily resulting from systemic cytokine production, whereas some may prove to be specific for the preferential mucosal activation conferred by flagellin. Although any strategy seeking to activate innate immunity will need to consider the potential to induce inflammatory pathology, the fact that flagellin, at least in mice, does not induce high levels of master pro-inflammatory cytokines (i.e., tumor necrosis factor and IL-12p70) induced by other TLR agonists, notably LPS, suggests that flagellin may be relatively amenable to being used for this purpose.⁴⁷

CONCLUSION

Although the means by which the mucosal immune system protects the host from the prokaryotes that inhabit mucosal surfaces is highly complex and multifaceted, select prokaryotic elements are especially targeted presumably because of their function being very important to those microbes that use them. This review has focused on the role of the innate immune targeting of bacterial flagellin especially in the intestine. One theme we have tried to convey is that, because it is very important, mammalian hosts have at least two distinct means of recognizing this molecule although the relative role of one of these, namely, Ipaf, has only recently been appreciated. Interestingly, flagellin is also a dominant target of adaptive immunity both in response to infection and in Crohn's disease, which both highlight the interrelatedness of innate and adaptive immunity, and the immune system uses multiple approaches to target this molecule. We envisage that a better understanding of the precise roles of TLR5, flagellin, and adaptive immunity in protecting the host against bacteria should aid our understanding of bacterial pathogenesis and IBD, and may allow for therapeutic manipulations of these pathways to treat and/or prevent a variety of disease states.

DISCLOSURE

The authors declared no conflict of interest.

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