TLRs in the Gut.

III. Immune responses to flagellin in Crohn’s disease: good, bad, or irrelevant?

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Gewirtz AT. TLRs in the Gut. III. Immune responses to flagellin in Crohn’s disease: good, bad, or irrelevant? Am J Physiol Gastrointest Liver Physiol 292: G706–G710, 2007. First published October 12, 2006; doi:10.1152/ajpgi.00347.2006.—Recent observations suggest that bacterial flagellin may be a target of the seemingly aberrant innate and adaptive immune responses that are associated with Crohn’s disease. This article discusses the evidence that supports a role for flagellin in Crohn’s disease and considers the potential roles of these responses in the pathophysiology of this disorder.

Toll-like receptor 5; adaptive immunity; epithelial cell

THE HUMAN INTESTINE is colonized by a vast array of commensal microbial species and is occasionally exposed to potentially pathogenic bacteria. A subset of such microbes, both commensal and pathogens, is motile, which is achieved by the expression of one or more flagella, the structural portion of which is largely composed of a polymer of the protein flagellin. In some cases, being motile is absolutely necessary for a bacterium to colonize its mammalian host. In other cases, it presumably provides an advantage in reaching particular niches in one or more of its reservoirs or hosts it colonizes. Bacterial expression of flagella is highly regulated, likely because of both its high metabolic cost and because flagella/flagellin is targeted by the mammalian immune system to detect the presence of motile organisms. Specifically, flagella have long been known to be major antigens of Salmonella and Escherichia coli strains, and such antigenicity serves as the basis of the H serotyping that is used in classifying these organisms. Concomitantly, flagellin is also the dominant T cell epitope in response to infection by Salmonella. More recently, it has been discovered that flagellin is also a potent activator of the innate immune system. Specifically, the mammalian germ line encoded (i.e., innate) cell surface receptor Toll-like receptor (TLR)5 can, upon the detection of picomolar levels of flagellin, trigger a massive induction of host gene expression designed to protect the host from the perturbing microbe. The role of flagellin in host responses to pathogens is discussed in detail in the previous article of this Themes series.

This article will discuss the potential role of flagellin in chronic idiopathic inflammatory bowel diseases (IBD), especially Crohn’s disease (CD). IBD in general, and CD in particular, is widely thought to be driven by mucosal immune responses to enteric microflora. The specific organisms that are the target of this response are not yet well defined, and, thus, there remains debate as to whether disease is driven by an aberrant response to normally commensal microflora or results from pathogen(s) that have yet to be identified. Regardless of which of these scenarios ultimately proves to be correct, there are a number of mechanisms by which flagellin may play a role in the pathophysiology of CD. Thus, this review will discuss these potential mechanisms and the recently accumulating evidence that supports them. Furthermore, this review will discuss the emerging understanding of the basic physiology/immunobiology of host responses to flagellin that will hopefully pave the way for the precise deciphering of the role of flagellin in diseases characterized by chronic inflammation at mucosal surfaces such as CD.

Flagellin May Drive Acute Inflammation in CD

The acute flares of IBD have been observed to correlate closely with the presence of neutrophils in the intestinal mucosa. Such neutrophils are not merely an indicator of active IBD but rather are thought to be responsible for causing much of the clinical manifestations and tissue damage associated with such active inflammation. Consequently, products that promote the recruitment of these neutrophils to the intestinal mucosa might have the potential to drive the active flares of IBD. The observation that, in most murine models, colitis is dependent on the presence of commensal enteric microflora supports such a role of microbial products. Thus, in this context, the finding that flagellin released by commensal E. coli isolates has the potential ability to activate the expression of chemokines such as IL-8 that drive neutrophil movement suggests that flagellin might play a role in promoting acute flares of IBD (4). Specifically, basolateral treatment of model intestinal epithelia with flagellin (purified from Salmonella or E. coli or generated in transfected eukaryotic cells and thus free of any other bacterial products) recapitulates the proinflammatory gene expression induced by colonization with live Salmonella. Importantly, applying flagellin to the apical surface of such epithelia does not induce this induction of proinflammatory gene expression. Salmonella but not commensal E. coli can transcytose its flagellin across the epithelium, thus explaining, in part, why colonization by Salmonella, but not flagellated commensal E. coli, results in potent induction of proinflammatory gene expression in model epithelia. While the degree of the polarity of this response has been reported to vary in different cell lines, the induction of proinflammatory gene expression in response to basolateral but not apical flagellin has also been observed in an ex vivo study (14) utilizing human intestinal mucosa.

This proposed mechanism of how the human intestinal epithelium distinguishes between pathogenic and commensal motile bacteria suggests some potential mechanisms whereby flagellin might drive the acute flares of inflammation in CD. Here, we discuss two such possible mechanisms that are supported with evidence from human studies. One such possi-
bility is that the increased epithelial permeability that has long been associated with IBD results in flagellin from commensal bacteria gaining access to the basolateral surface of the epithelium, thus activating proinflammatory gene expression. As such alterations in permeability occur early in disease progression, often preceding clinical disease appearance, this potential means of aberrantly inducing inflammation can be envisioned to drive both the initial flares of acute disease or the acute inflammatory flares associated with chronic IBD. Although the permanent absence of disease in some patient’s relatives who also display abnormal permeability suggests that such alterations in gut permeability may not be sufficient to result in flagellin driving acute inflammation. It should also be noted that most observations of increased permeability in CD have been made using relatively small molecules (250–5000 Da) that are considerably smaller than flagellin (about 50 kDa). Nonetheless, the observation that, in mice, bolus rectal administration of flagellin was not observed to activate immune responses (15) but repeat administration of rectal flagellin during the onset of dextran sodium sulfate (DSS)-induced colitis exacerbated inflammation (14) supports the possibility that, at least in cases of severe epithelial damage, flagellin can drive the acute flares of intestinal inflammation that define active IBD.

Another possibility is that, in CD, flagellin may access the basolateral surface of the epithelium via bacteria attaining inappropriate locations, which has been observed to occur in CD. Specifically, whereas examination of the healthy mucosa has generally found bacteria to be attached to the mucous layer and not in direct contact with the apical membrane of the epithelium, a study (18) in IBD patients observed bacteria very closely opposed and/or directly adherent to the apical membrane and in some cases within, beyond, or between epithelial cells. Whether such bacteria express flagellin in these locales is not known, but some of these bacteria are E. coli that have genes for flagellin, thus supporting this possibility. While the unresponsiveness of the healthy human intestine to flagellin given apically ex vivo suggests that apical/mucosal bacteria would need to generate a high local concentration of flagellin to activate the epithelium, it is also quite possible that such adherent/invaded bacteria result in increased intestinal permeability, further aiding the possibility of flagellin reaching the basolateral membrane. In scenarios of either increased epithelial permeability and/or interepithelial/subepithelial bacteria, one might expect that, given the ability of host germ line receptors to recognize a variety of bacterial products, a number of products would be involved in promoting acute inflammation.

While it seems likely that products other than flagellin are involved, some findings nonetheless argue against a role for some of the other well-defined bacterial activators of innate immunity. Specifically, whereas all tested intestinal epithelial cell lines have been observed to express proinflammatory genes in response to flagellin, most of these cell lines do not respond similarly to LPS, peptidoglycan, or bacterial DNA. While the lamina propria is populated by classes of immune cells (monocytes, macrophages, and dendritic cells) that, when tested from peripheral blood, respond well to these other bacterial agonists, when isolated from the intestine, these cells have been observed to not exhibit proinflammatory gene expression in response to these products (i.e., “inflammatory anergy”) (17). Thus, while other bacterial products are likely capable of activating epithelial proinflammatory gene expression, speculating that flagellin is the dominant bacterial activator does not seem unreasonable.

If, indeed, flagellin activates epithelial gene expression in IBD, as we speculate, a key question to be answered is whether this response benefits or harms the host. One could imagine that flagellin’s effects on the epithelium should promote killing of mucosal bacteria, thus reducing the levels of the microbes/molecules that drive disease. Furthermore, as discussed in the previous article in this series, in addition to promoting neutrophil influx, flagellin also activates antiapoptotic/cytotrophic gene expression that may protect epithelial cells from a variety of conditions that would otherwise result in epithelial cell death (12). However, flagellin-induced neutrophil infiltration can itself drive clinical manifestations of inflammation and cause lasting damage to the gut mucosa and thus may be largely detrimental to the host. Additionally, as will be discussed below, flagellin-induced gene expression and the subsequent immune cell infiltration may also promote the adaptive immune responses that maintain the chronically inflamed state. While the overall role of flagellin-induced gene expression is not yet clear, the recent tantalizing finding that, in individuals of Ashkenazi Jewish ethnicity, carriage of a dominant negative TLR5 stop polymorphism was negatively associated with CD suggests that reduced TLR5 signaling (the polymorphism is thought to result in a 75% reduction in TLR5 function) may offer some protection against the development of CD (5). In contrast, carriage rates of the TLR5 stop codon were not reduced in ulcerative colitis. Thus, it may be reasonable to consider pharmacological inhibition of TLR5 signaling as a means to treat or prevent CD.

Adaptive Immune Responses to Flagellin May Drive CD

While neutrophils in the intestinal mucosa are the best histopathologic correlate of the acute flares of disease, a cardinal histopathological feature of CD biopsies not taken during an active flare is the presence of increased numbers of mucosal lymphocytes. Observations that CD patients exhibit increased adaptive immune responses to their own commensal microflora suggest that these lymphocytes are not just indicators but may be functionally associated with CD. Moreover, in a variety of mouse models, transfer of CD4 T cells, but not other subsets of lymphocytes, to immunodeficient mice results in colitis in such recipient mice. Development of such colitis is dependent on the presence of commensal microflora. Together, these and other results suggest that the recognition of antigens from commensal microflora by CD4 T cells and the generation of inappropriate immune responses thereafter may be an underlying event in CD and that the functional presence of these cells ultimately results in the triggering of the neutrophil influx that drives acute flares of disease. In light of their potential role in driving CD, the antigens that CD4 T cells recognize are strong candidates to play an essential role in CD. Thus, recent findings that indicate that flagellin is the dominant antigen in CD suggest that, irrespective of its potential role in driving acute flares of CD, acquired immune responses to flagellin play an important role in CD.

The notion that flagellin is the dominant antigen in CD springs from a study (7) that used the technique of serological
expression cloning to identify the proteins of commensal microflora that are reactive with antisera from a naturally arising spontaneously colitic mouse strain. Specifically, of the 55 antigens that were immunoreactive with sera from colitic C57Bl/6J or colitic mice but not a closely related healthy strain, 15 antigens had amino acid sequences that indicated they were flagellin genes that, although not previously sequenced, were particularly similar to flagellins from Clostridia species. Similar results were obtained with other spontaneously colitic mouse strains including Mdr-null mice, which are thought to develop colitis as a result of impaired barrier function, and IL-10-null mice, which develop colitis due to a primary defect in immune regulation. Moreover, in wild-type mice, the induction of colitis via the addition of DSS to drinking water, which results in acute colitis via the induction of ulcerations in the epithelium, results in the rapid induction of serum immunoreactivity to Clostridia-like flagellins within 2 wk following the administration of DSS (15). The speed of this response suggests that the Clostridia flagellin-like antigen is normally present in the intestine. Thus, the development of flagellin immunoreactivity in these models suggests that adaptive immune responses to flagellin may be a unifying event in intestinal inflammation resulting from a variety of underlying causes. While these observations are largely based on measurements of anti-flagellin antibodies, whereas it is CD4 T cells that are able to drive murine models of immune-mediated colitis, generation of anti-flagellin antibodies is absolutely T cell dependent and thus levels of anti-flagellin antibodies can be viewed as an indirect measure of functional levels of flagellin-specific T cells (15).

Increased flagellin immunoreactivity has also been observed in CD patients. Specifically, CD patients have been observed to have elevated levels of serum IgG and IgA to both Clostridia-like flagellin and E. coli flagellin (7, 16). Such increases were not observed in ulcerative colitis. Such immunoreactivity against E. coli was not strictly limited to flagellin, but, nonetheless, flagellin was clearly one of the major targets of this increased immune response observed in CD patients, supporting the notion that the flagellin’s immunodominance discovered in mice extends to humans with CD. Interestingly, it was recently observed that colectomized individuals, referred to as “short gut” patients, also have increased levels of serum anti-flagellin antibodies (20). Such elevations, which were substantially greater than those observed in CD, were observed regardless of whether persons had their colons removed due to underlying CD or unrelated reasons (e.g., acute trauma). As short gut patients have also been suggested to have abnormal permeability and bacterial overgrowth, this observation is again consistent with the notion that increased exposure to flagellin may, in part, drive the elevated immunoreactivity to flagellin observed in CD.

While there is a clear association of adaptive immune responses to flagellin in both CD and a variety of murine models of colitis, the role of this response in disease pathophysiology is not yet well defined. Perhaps the most basic question is does acquired immunity to flagellin promote, or protect against, clinical manifestations of CD? Potentially, one could imagine that CD4 T cells recognizing flagellin might activate effector cells of the innate and adaptive immune system, thus resulting in an inflammatory response upon the detection of flagellin. This possibility is supported by the observation that transfer of flagellin-specific CD4 T cells to an immunodeficient mouse results in colitis in such recipient mice showing that, under some conditions, flagellin-specific T cells can cause acute colitis (7). However, the extent to which this observation applies to mice or humans that have an endogenous adaptive immune system, populated by effector and regulatory cells, is not clear. For example, normal mice injected with flagellin make a robust adaptive immune response to this molecule but do not show signs of developing colitis. Conversely, one might imagine that adaptive immune responses to flagellin play a role in protecting the host against the microbes that are driving the disease in the first place. Unfortunately, there is relatively little evidence to indicate whether the adaptive immune responses to flagellin in CD patients drive, attenuate, or have no effect on their disease. One observation that is consistent with the possibility that these responses might be harmful is that persons carrying the above-mentioned TLR5 stop codon polymorphism and thus seem to have some protection from developing CD are also protected from naturally acquiring immune responses to flagellin (5), but such associations are far from proof of this notion. Thus, we anticipate that studies in mice in which it is possible to positively and negatively manipulate adaptive immune responses to flagellin in various models of colitis will be necessary to shed substantial light on whether acquired immune responses to flagellin drive CD or is a helpful adaptation that ameliorates disease.

Flagellin May Promote Adaptive Immunity in CD

In addition to potentially serving as an antigenic target of the CD-associated adaptive immune responses, flagellin may also promote these responses to both itself and other bacterial antigens that are thought to drive the chronic phase of CD. The basis of this speculation is that, at least when administered systemically, flagellin is known to serve as an adjuvant both for T cell and antibody responses (2, 8). Flagellin can activate innate immunity as both acquired immune responses to flagellin and flagellin’s adjuvanticity are abolished in mice lacking the TLR signaling adaptor MyD88. In this regard, responses to flagellin may bridge innate and adaptive immunity in the gut. In light of the general relative gut hyporesponsiveness of the intestine to other innate immune activators, flagellin may be particularly important in mediating the natural acquisition of acquired immune responses in the gut. In support of this concept, it has recently been observed that carriers of the aforementioned dominant negative TLR5 allele display reduced levels of flagellin-specific antibodies (5).

Like other TLR-mediated adjuvants, flagellin presumably promotes adaptive immune responses by inducing the expression of cytokines and costimulatory molecules on antigen-presenting cells. However, in contrast to other TLR agonists, flagellin does not seem to be a potent activator of classical antigen presenting cells at least in mice, where its adjuvanticity has been best demonstrated. Specifically, flagellin seems not to activate murine splenic dendritic cells or peritoneal macrophages in vitro, presumably due to lack of expression of TLR5 (9), although at least the former are activated by flagellin in vivo (8). One possible mechanistic explanation for this difference is that flagellin-induced dendritic cell activation is indirect, particularly that driven by flagellin-induced cytokines.
Another possibility is that the activated dendritic cells that can be found in the spleen 6 h following the injection of flagellin represent a distinct dendritic cell population that migrated to the spleen in response to flagellin. In support of this latter possibility, intestinal lamina propria dendritic cells appear to both express TLR5 and respond to flagellin (19). We are hopeful that the mechanistic studies performed over the next several years will sort out the role of various cell types in flagellin’s promotion of adaptive immune responses and their subsequent role in IBD.

**Advances in Immunobiology of Flagellin Should Help in the Understanding of Its Role in CD**

As discussed above, substantially more research is needed to define the role of flagellin in IBD. Much of this research will likely take the form of association-based studies in humans and mechanistic/interventionist studies in murine models of infection and colitis. Additionally, there is still much to be learned of the basic biology of how innate and adaptive immune responses to flagellin are generated. For example, as mentioned above, it is not yet clear which cell types are responding to purified flagellin in vivo let alone the role of the responses such cell types in various physiological contexts such as infection and/or gut barrier dysfunction. While the recent generation of mice that lack the flagellin receptor TLR5 provide a key tool in answering these questions (19), the role of the recently discovered pathway(s) of intracellular recognition of flagellin also need to be considered. Specifically, whereas recognition of flagellin shed by bacteria appears to be recognized entirely by TLR5, two Nod-like receptors (NLRs), namely, neuronal apoptosis inhibitory protein 5 (Naip5) and IL-1β-converting enzyme protease-activating factor (IPAF), have been reported to function in recognizing flagellin on phagocytosed bacteria (3, 10, 11, 13). These NLR seem less important for induction of classic proinflammatory gene expression, but their key role in the regulation of activation of caspase-1 appears very important for the innate immune control of bacteria. Thus, it is not hard to imagine a role for this pathway in regulating the innate immune activity associated with IBD or a role in regulating the adaptive immune responses to flagellin. Deciphering the roles of TLR5 and these NLRs in IBD is thus an important challenge in this area.

Another area that requires basic better understanding is that of how structural differences in flagella/flagellins from various bacterial species affect recognition by the innate and adaptive immunity; or, as the question, is often asked “is flagellin flagellin?” There has been some recent progress in this area, and, at present, the answer to this question appears to be “yes and no.” Specifically, in terms of TLR5-mediated innate immune recognition of flagellin, which is directed at flagellin monomers rather than flagella, it appears that most “common” typical flagellins such as *E. coli*, *Salmonella*, *Pseudomonas*, *Listeria*, and *Clostridia* will activate TLR5 (1). In contrast, some bacteria, such as *Campylobacter* and *Helicobacter* species, are able to produce flagellins that evade TLR5 detection, which might be important for their ability to colonize their hosts (6). Of the more typical flagellins that have some ability to activate TLR5, the relative potencies of these various flagellins as well as the relative amounts of flagellin these bacteria shed has yet to be defined. Based on the large differences in relative potencies of LPS from various bacteria, we speculate there will be substantial differences. It will also be important to understand what features of TLR5 recognition of flagellin are shared by NLRs and other potential receptors that recognize flagellin. Of particular importance will be understanding the relative amounts/potency of various flagellins such as *E. coli* and *Clostridia* in the gut in states of health and colitis.

Use of an invariant germ line encoded receptor such as TLR5 to recognize many different flagellins seems to rely on this receptor targeting a necessary conserved structural motif that is encoded in several conserved nonlinear regions of the flagellin gene. Conserved regions of the flagellin protein are also likely targeted by the adaptive immune system in that mice injected with *E. coli* flagellin monomers make antibodies that can recognize flagellin monomers from *Salmonella* or, to a lesser but significant degree, flagellin from *Clostridia* species (15). Yet, such flagellins are certainly not immunologically identical in that mice injected with *Clostridia*-like flagellins did not make detectable antibodies to *E. coli* flagellin. Furthermore, whereas humans with CD showed elevated immunoreactivity to flagellin from *E. coli* or *Clostridia*-like bacteria, colitic mice only displayed elevated immunoreactivity to only the latter. Additionally, recognition of flagella by specific antibodies is highly specific, even among bacterial strains of

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**Fig. 1.** The three primary ways by which flagellin may play a role in inflammatory bowel diseases (IBD). Flagellin from commensal microbes may cross the altered epithelial barrier that occurs in IBD. Such flagellin can, via Toll-like receptor 5 (TLR5), induce the epithelium to secrete cytokines that recruit polymorphonuclear neutrophils (PMN). Such cytokines may promote adaptive immunity and/or, alternatively, flagellin may activate dendritic cells (DC) and thus directly promote adaptive immune immunity. Flagellin is also targeted by the Crohn’s disease-associated adaptive immune response. TC, T cell.
the same species; hence, the basis of H serotyping. Thus, while adaptive immune recognition of flagellin targets some conserved regions of the molecule, and such cross-reactivity may play a role in broadening the immune response in IBD, there are also various regions of flagellin unique to specific classes of flagellated microbes. Thus, it remains an important challenge in the study of IBD pathogenesis to determine to which flagellins are CD-associated immune response generated and which flagellins will the resulting response recognize.

Concluding Thoughts

As summarized in Fig. 1, host innate and adaptive immune responses to flagellin may play both distinct and interrelated roles in CD. At present, it seems clear that this molecule is present at the crime scene and is perhaps involved in both the acute and chronic phases of this disorder. Much less clear is whether host responses to flagellin are part of a healthy immune response that benefits the host, albeit not enough to prevent clinical indicators of disease, or are part of an aberrant immune response that should be targeted by immune and/or traditional pharmacological therapy. We speculate that some aspects of the immune response to flagellin might be detrimental, whereas others are beneficial. Thus, we are hopeful that, over the next several years, it will be possible to define the roles of various aspects of the innate and adaptive immune response to flagellin and, in doing so, develop treatment strategies for this disorder.

REFERENCES