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TLR Signaling in the Gut in Health and Disease

Maria T. Abreu, Masayuki Fukata, and Moshe Arditi

The human intestine has evolved in the presence of diverse enteric microflora. TLRs convert the recognition of pathogen-associated molecules in the gut into signals for antimicrobial peptide expression, barrier fortification, and proliferation of epithelial cells. Healing of injured intestinal epithelium and clearance of intramucosal bacteria require the presence of intact TLR signaling. Nucleotide oligomerization domain (Nod)1 and Nod2 are additional pattern recognition receptors that are required for defense against invasive enteric pathogens. Through spatial and functional localization of TLR and Nod molecules, the normal gut maintains a state of controlled inflammation. By contrast, patients with inflammatory bowel disease demonstrate inflammation in response to the normal flora. A subset of these patients carry polymorphisms in TLR and CARD15/NOD2 genes. A better understanding of the delicate regulation of TLR and Nod molecules in the gut may lead to improved treatment for enteric infections and idiopathic inflammatory bowel diseases. The Journal of Immunology, 2005, 174: 4453–4460.

The intestinal immune system has developed under the dual pressure of protecting the host from pathogenic infections and coexistence with the myriad commensal organisms in the lumen. These same commensal bacteria and yeast elicit a potent inflammatory response across other mucosal surfaces such as the lung and bladder. The purpose of this review is to examine the adaptations made by the intestinal innate immune system to the presence of commensal bacteria to fulfill these dual responsibilities. In particular, we will focus on the role of TLRs and Nucleotide oligomerization domain (NOD) proteins as receptors for pathogen-associated molecular patterns in the gut environment.

Components of the intestinal innate immune response

The intestinal innate immune system consists of multiple cell types. The first layer of defense is the intestinal epithelial cells that line the luminal surface of the gastrointestinal tract (Fig. 1). Interdigitated among the intestinal epithelial cells are dendritic cells that sample the luminal contents and may present Ags to T cells present in the lamina propria and lymphoid follicles (1). In the small intestine, specialized epithelial cells called M cells reside in the follicle-associated epithelium overlying Peyer’s patches. These M cells transport Ags through pinocytosis to the Peyer’s patches where APCs process the Ags and present these to naive T cells. Immediately beneath the layer of intestinal epithelial cells, smooth muscle cells provide the structural support for epithelial cells. All of these cell types may contribute to the innate immune response to bacteria (Tables I–IV). The lamina propria is also populated by macrophages and dendritic cells that participate in Ag presentation to the B and T cells (both CD4 and CD8) that form the adaptive mucosal immune system.

TLR signaling in the gut in health

The principal role of TLR signaling in the intestine is the same as in other tissues—defense against pathogens. In chickens, the TLR4 gene maps to the region of the genome previously identified to confer protection against Salmonella infection (2) supporting a role for TLR4 and likely other TLRs in protection against enteric pathogens. However, because of the close proximity and high density of pathogen-associated molecular patterns (PAMPs) in the intestinal lumen, a variety of mechanisms have evolved to protect against dysregulated inflammation in the absence of pathogens (Table V).

Location, location, location

The single layer of epithelial cells lining the intestine, especially in the colon, forms an impermeable barrier to luminal bacteria and PAMPs including LPS through tight junctions (3). This is critical given that this organ contains approximately 10^11–10^13 bacteria per gram of stool (4).

We and others (5–7) have shown that colonic epithelial cells express low levels of TLR4 and MD-2 and are poorly responsive to LPS. Fig. 2 demonstrates the cellular localization of TLRs in a colonic epithelial cell. The response to TLR2 ligands is also muted (8). The teleological explanation for this relatively low responsiveness to these PAMPs may be the inability to discriminate pathogens from commensal bacteria based on the presence of PAMPs alone without cellular invasion or other signs of
stimulate secretion of the chemokine CCL20 by IEC, which in turn recruits immature dendritic cells to the intestinal epithelium (13). TLR5 activation also stimulates secretion of the chemokines IL-8 and MIP3α (14). Thus signaling by TLR5 in response to pathogen-derived flagellin results in recruitment of inflammatory cells and initiation of an adaptive immune response to pathogens.

A recent study has demonstrated that colitic animals and patients with Crohn’s disease express serum reactivity against flagellin derived from commensal bacteria (15). The response to flagellin was directed against a specific peptide sequence derived from a limited array of bacterial species. The titer of these Abs correlated with the degree of inflammation in the colitic mice. These data suggest that TLR5-dependent recognition of flagellin may play a role in the inappropriate immune response to commensal bacteria in idiopathic inflammatory bowel disease (IBD). The cell types expressing TLR5 were not determined in this study.

Conditions in the small intestine are inherently different from those in the colon. The density of bacteria increases from $10^2$ bacteria in the proximal small intestine to $10^8$ in the distal ileum. Whereas colonic epithelial cells are generally poorly responsive to LPS, small intestinal epithelial cells may not be (Table I). Studies by Hornef et al. (16, 17) have demonstrated that in a small intestinal enterocyte line, LPS is taken up by a clathrin-dependent mechanism where it interacts with TLR4, MyD88, and IRAK-1 in the Golgi. These data suggest the possibility that small intestinal villous enterocytes can respond to the presence of PAMPs. Studies of primary small intestinal epithelial cells have not been done.

The lamina propria of the intestine is rich with hemopoietic cells including macrophages, dendritic cells, B cells, and T cells. Staining of lamina propria macrophages has shown that expression of TLR2 and TLR4 is low in uninflamed intestine (18). In addition, isolated lamina propria macrophages do not express CD14 and are LPS unresponsive (19). Human lamina propria dendritic cells in the intestine are difficult to isolate and characterize but functional studies suggest that intestinal dendritic cells respond to TLR3 and TLR4 ligands (Table III) (20). Together, these data suggest the possibility that regulated expression of TLRs by macrophages and dendritic cells in the small intestine might contribute to tissue homeostasis and prevent inflammatory bowel disorders.

### Table I. Expression and function of TLRs and TLR-associated molecules by the intestinal innate immune system

<table>
<thead>
<tr>
<th>TLRs</th>
<th>Large intestine (in vivo or primary cells)</th>
<th>Small intestine</th>
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<tbody>
<tr>
<td>1</td>
<td>+ RNA (8, 9) (function unknown)</td>
<td>+ RNA (88)</td>
</tr>
<tr>
<td>2</td>
<td>+ RNA (8, 9) (function unknown)</td>
<td>+ Ileum protein (89), RNA functional (6)</td>
</tr>
<tr>
<td>3</td>
<td>+ Protein (89) RNA (9) (function unknown)</td>
<td>+ Protein (89)</td>
</tr>
<tr>
<td>4 (MD2)</td>
<td>+ RNA (5), protein (89), RNA (9) (function unknown)</td>
<td>+ Ileum protein (89)</td>
</tr>
<tr>
<td>5</td>
<td>+ Protein (89), RNA (9) (function unknown)</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>+ RNA (8, 9) (function unknown)</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>+ RNA (9) (function unknown)</td>
<td>+ RNA (90)</td>
</tr>
<tr>
<td>8</td>
<td>+ RNA (9) (function unknown)</td>
<td>+ RNA (90)</td>
</tr>
<tr>
<td>9</td>
<td>+ RNA (9) (function unknown)</td>
<td>+ Ileum protein (31)</td>
</tr>
<tr>
<td>10</td>
<td>Not expressed (91)</td>
<td>ND</td>
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*In most cases, expression of specific TLRs and signaling molecules have been assessed but the function in vivo has not been ascertained. +, TLR has been shown to be expressed. We have indicated whether expression is positive at the RNA, protein level, or both. An asterisk (*) denotes that protein and RNA are expressed, and the receptor has been shown to be functional in response to the appropriate ligand.*
demonstrate that peroxisome proliferator-activated receptor- and reduces inflammation in animal models of colitis. These studies suggest that expression and activity of inhibitors of TLR signaling are an important mechanism to limit intestinal inflammation and that reduced expression of inhibitors of TLR signaling provide another mechanism by which to limit TLR signaling in the intestine. Tollip is a Toll/TIR domain-containing inhibitory protein that is bound to IRAK (21). We have shown that Tollip expression is high in intestinal epithelial cells that are poorly responsive to LPS (22). Furthermore, Tollip expression increases in LPS- or LTA-treated intestinal epithelial cells and is associated with hyporesponsiveness to PAMPs (9).

Recently, Wald et al. (23) have shown that intestinal epithelial cells express high levels of a novel, TIR-containing inhibitory molecule called SIGIRR (single Ig IL-1R-related molecule) or Tir8. Animals that are deficient in TIR8/SIGIRR are more susceptible to colitis induced by dextran sodium sulfate (DSS) (24, 25). Although not formally a TLR inhibitor, peroxisome proliferator-activated receptor-γ inhibits NF-κB activation and reduces inflammation in animal models of colitis. Data demonstrate that peroxisome proliferator-activated receptor-γ expression is at least partially under the control of TLR4 and luminal bacteria (26). These studies suggest that expression and activity of inhibitors of TLR signaling are an important mechanism to limit intestinal inflammation and that reduced expression of these inhibitors might contribute to IBD.

Expression of inhibitors of TLR signaling

Inhibitors of TLR signaling provide another mechanism by which to limit TLR signaling in the intestine. Tollip is a Toll/TIR domain-containing inhibitory protein that is bound to IRAK (21). We have shown that Tollip expression is high in intestinal epithelial cells that are poorly responsive to LPS (22). Furthermore, Tollip expression increases in LPS- or LTA-treated intestinal epithelial cells and is associated with hyporesponsiveness to PAMPs (9).

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Secretion of anti-microbial peptides

Paneth cells are specialized epithelial cells located in the base of small intestinal crypts and characterized by their electron-dense granules containing defensins. Defensins are small cationic peptides containing sulfide bonds that exert their effect by damaging the bacterial cell membrane (27). Besides demonstrating broad anti-microbial properties, defensins have chemokine properties as well (28). The α-defensins, HD5 and HD6, are expressed by Paneth cells (29), and either whole bacteria or PAMPs such as LPS, LTA, and muramyl dipeptide stimulate release of defensins (30) suggesting that Paneth cells express a wide range of TLRs. Paneth cells express TLR9 in their secretory granules and degranulate their contents in vivo in response to administration of CpG oligodeoxynucleotides (ODNs) (31). Administration of CpG ODNs also protects against subsequent challenge with Salmonella typhimurium. In addition to defensins, the work of Hooper et al. (32) has demonstrated that luminal bacteria induce expression of microbicidal angiogenins by Paneth cells. These data directly implicate TLRs in the antimicrobial function of Paneth cells.

In addition to α-defensin expression by Paneth cells, IEC may express β-defensins 1, 2, and 3 (33). Investigators have demonstrated that Salmonella enteriditis flagellin can stimulate...
expression of β-defensin-2 in intestinal epithelial cells (34, 35). We have recently demonstrated that TLR4- and TLR2-dependent pathways can stimulate β-defensin-2 expression by intestinal epithelial cells (36). These data suggest that, like Paneth cells, intestinal epithelial cells respond to PAMPs by secreting antimicrobial peptides. Thus secretion of various antimicrobial peptides by Paneth cells and enterocytes is likely to be regulated by TLR-mediated recognition of PAMPs.

**Homeostatic function of bacterial-epithelial interactions**

Since the innate immune system of the intestine has evolved in the presence of luminal bacteria, it is reasonable to hypothesize that normal intestinal function may be regulated by bacteria through TLRs. Hooper et al. (37) has elegantly demonstrated that the introduction of bacteria into the intestine of a gnotobiotic mouse results in the induction of a complex pattern of gene expression. The genes expressed include junctional proteins, enzymes involved in digestion, and metabolism.

Exposure of colonic epithelial cell lines to bacterial lipopeptide or peptidoglycan results in apical tightening and sealing of the tight junctional protein ZO-1 and increased transepithelial electrical resistance (38). These data may tie together previous observations that certain probiotic preparations increased barrier function in vitro (39, 40) and attenuate inflammation in animal models of colitis (41–43). However, in an animal model of necrotizing enterocolitis, LPS inhibited migration of intestinal epithelial cells across a wound through a TLR4-dependent mechanism (44). Thus depending on the scenario, TLR signaling may aid in certain aspects of barrier function but not others.

**Table V. Mechanisms to control deleterious TLR-mediated inflammation in the gut**

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**FIGURE 2.** TLR signaling pathways in intestinal epithelial cells. TLR4 and MD-2 expression is low in intestinal epithelial cells, especially in the colon. In small intestinal epithelial cell lines, TLR4 has been found associated with the Golgi apparatus resulting in intracellular recognition of LPS. TLR2, TLR1, and TLR6 are expressed but may be reduced in their ability to signal either because of diminished expression at the cell surface or because of increased expression of inhibitory molecules such as Tollip or SIGIRR. TLR5 has been shown to be expressed either at the basolateral or apical surfaces of the intestinal epithelial cell. NOD2 and TLR9 are both intracellular pattern recognition receptors that have been demonstrated to be expressed in the cytoplasm of intestinal epithelial cells. Not shown is the MyD88-independent pathway of signaling, which has not been specifically studied in intestinal epithelial cells.

Another dimension of TLR function in the gut environment relates to protection from epithelial injury. Oral administration of DSS results in epithelial injury and exposure of the lamina propria to luminal bacteria. Rakoff-Nahoum et al. (45) have shown that administration of DSS to TLR4, TLR2, or MyD88 knock-out mice resulted in higher mortality than wild-type mice. Administration of broad-spectrum antibiotics to wild-type mice has a similar deleterious effect as MyD88 insufficiency suggesting that signals provided by the luminal bacteria via TLRs protect against DSS damage. Before the discovery of TLR signaling pathways, studies with germ-free mice showed that these mice had greatly reduced intestinal epithelial proliferation (46). More recent studies suggest a molecular explanation: Rakoff-Nahoum and colleagues reported that intestinal epithelial cell proliferation is markedly decreased in MyD88−/− and TLR4−/− mice, suggesting that TLR signals are important for maintenance of normal epithelial barriers in the intestine.

Results from our laboratory are consistent with this interpretation. Using the DSS model, we have found that TLR4−/− and MyD88−/− mice have increased rectal bleeding in response
to DSS (47). DSS colitis is characterized by an influx of neutrophils to the lamina propria. Histologic examination of DSS-treated TLR4−/− or MyD88−/− mice reveals a dramatic reduction in the number of neutrophils compared with control mice secondary to diminished chemokine expression by lamina propria macrophages. The consequences of the decreased neutrophil recruitment include Gram-negative bacterial translocation to mesenteric lymph nodes in knockout but not wild-type mice. These data demonstrate that TLR signaling in response to the presence of bacteria in the lamina propria following epithelial injury is critical for healing of the gut. Others have also shown that LPS induces cytoprotective heat shock protein expression by IEC and protects against radiation-induced injury (48, 49).

Role of TLRs in the regulation of the adaptive immune response in the gut

Intestinal bacteria are essential for the normal development of GALT. In the absence of luminal bacteria, B cells and T cells do not home to the lamina propria of the intestine, and IgA is not secreted (50). T cells home to the intestine through expression of the αββ2 integrin, which interacts with mucosal addressin cell adhesion molecule-1 on mucosal high endothelial venules (51) as well as through expression of the CCR9 chemokine receptor, which responds to the thymus-expressed chemokine ligand secreted by small intestine epithelial cells (52, 53). Recent studies have demonstrated that dendritic cells isolated from mesenteric lymph nodes but not those from spleen result in the expression of the chemokine receptor CCR9 and the integrin αββ2 by T cells (54, 55). Administration of LPS or poly(I:C) dramatically enhances CCR9 and αββ2 expression by CD8+ T cells, suggesting TLR4 and TLR3, respectively, may be involved in vivo in the generation of gut-tropic T cells (54). These data demonstrate that TLR signaling by dendritic cells is important in the recruitment of T cells to the intestine.

The normal intestinal adaptive immune system does not respond to commensal bacterial Ags as measured by T cell proliferation, Th1 cytokine secretion, or IgG secretion (56, 57). Rather the response to commensal bacteria is characterized by development of regulatory T cells and secretion of IgA. By contrast, patients with IBD have aberrant T cell responses to the host flora (57, 58) and express anti-microbial Abs that correlate with severity of disease (59). At least part of the reason for the normal hyporesponsiveness to commensal flora is the presence of regulatory CD4+CD25+ T cells that secrete IL-10 (60, 61). LPS from enteric bacteria but not pathogenic bacteria results in development of regulatory T cells (62). These studies suggest that the gut environment uses signals from luminal bacteria to develop tolerance by a mechanism involving regulatory T cells.

Dendritic cells play a critical role in the generation of regulatory T cells in the gut (63, 64). One mechanism by which dendritic cells participate in tolerance is through the selective induction of IgA secretion in response to commensal bacteria (65). In these experiments, whole bacteria were found within dendritic cells; however, the role of TLRs was not examined. The TLR inhibitory molecule TIR8/SIGIRR is expressed by both intestinal epithelial cells and dendritic cells. The increased sensitivity of TIR8-deficient mice to DSS-induced colitis may therefore be due to a defect in dendritic cell function to limit Th1-dependent damage (24).

Animal models of IBD are generally characterized by a dominant Th1 phenotype (66). Mice with a myeloid cell-specific deletion of Stat3 are deficient in IL-10 production and develop enterocolitis (67). This enterocolitis can be prevented by crossing animals to TLR4−/− mice, suggesting that TLR4 signaling is required to set in motion IL-12 production and the Th1 cytokine diathesis required for chronic inflammation. However, the balance between the requirement of TLR4 or other TLRs to initiate and sustain acute and chronic inflammation vs its role in healing of the epithelium is probably contextual and depends on other host factors (e.g., over-production of IL-12), as well as the nature of the injury (e.g., DSS vs spontaneous colitis).

Another data implicating TLR5 in the generation of a regulatory T cell response to commensal flora relate to the observation that oral or systemic administration of CpG ODN, the ligand for TLR9, ameliorates inflammation in several animal models of colitis (68) (69). Genetic evidence also supports a role for TLRs in bowel inflammation, since polymorphisms in TLR9 have been linked with Crohn’s disease (70). The role of TLR9 and bacterial DNA in the gut to suppress inflammation requires further mechanistic studies to exploit this approach at the bedside.

Finally, food allergies are an important health concern, given the mortality from anaphylactic shock following accidental Ag exposure. In an animal model of food allergy, TLR4 signaling and luminal bacteria were protective against the development of allergy (71). Stimuli such as CpG ODN that could shift the immune response toward Th1 development could rescue the allergic phenotype. Together, these data suggest that TLR signaling in the gut along with a full complement of commensal flora are important for balancing extremes of Th1 or Th2 cytokine production associated with inflammation or allergy, respectively.

NOD proteins and their role in intestinal defense

NOD proteins are pattern recognition receptors with homology to plant disease resistance proteins (72, 73). Nod1 and Nod2 confer responsiveness to peptidoglycan through Rip2/RICK kinase, a mediator of NF-κB activation (74–76). Nod1 is expressed by intestinal epithelial cells and is required for recognition of invasive Gram-negative bacteria such as Shigella flexneri (77) and enteroinvasive Escherichia coli (78). The specific ligand recognized by Nod1 is found only in Gram-negative bacterial peptidoglycan. In addition to TLRs, invasive organisms may elicit an innate immune response from intestinal epithelial cells through intracellular Nod1.

By contrast, the ligand for Nod2/CARD15 is muramyl dipeptide derived from peptidoglycan common to both Gram-positive and Gram-negative bacteria (79) (Fig. 3). Nod2 is highly expressed by monocytes and Paneth cells (80). Expression of Nod2 can be induced by IFN-γ and TNF-α in intestinal epithelial cells (81, 82) and is seen in the inflamed colon in Crohn’s disease (83). Polymorphisms in the ligand-binding domain of Nod2 are associated with Crohn’s disease (84, 85). A recent study in which the most common Crohn’s disease-associated mutation was introduced into mice demonstrated that these mice had increased NF-κB activation in response to muramyl dipeptide and increased secretion of IL-1β (86) (Fig. 3). Patients homozygous for the 3020insC frameshift mutation in the Nod2 gene demonstrate defective release of IL-10 from PBMC after stimulation with TLR2 ligands (87). Thus, in the setting of Crohn’s disease-associated mutations of Nod2, bacterial signaling is associated with
ditions for its largest inhabitants—the commensal flora. The intestine is still not clear. Polymorphisms in TLRs and NODs may play a primary or secondary role in idiopathic intestinal inflammation. Whether aberrant TLR signaling change the ability of the adaptive immune response to become hyporesponsive to Toll-like receptor bacterial ligands in intestinal epithelial cells. J. Exp. Med. 195:1299.


