TLRs in the Gut.

IV. Negative regulation of Toll-like receptors and intestinal homeostasis: addition by subtraction

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Shibolet O, Podolsky DK. TLRs in the Gut. IV. Negative regulation of Toll-like receptors and intestinal homeostasis: addition by subtraction. Am J Physiol Gastrointest Liver Physiol 292: G1469–G1473, 2007; doi:10.1152/ajpgi.00531.2006.—Toll-like receptors (TLRs) are a family of transmembrane proteins that recognize conserved molecular motifs on microorganisms. Ligand binding to TLRs initiates signaling cascades that activate NF-κB, MAPK, and interferon response factors. These culminate in cellular responses including activation of antimicrobial killing mechanisms, production of cytokines and chemokines, maturation of antigen presenting cells, and the recruitment of the adaptive immune response. Intestinal epithelial cells represent a unique population of cells that exist in direct contact with a biomass of bacteria. Initiation of TLR signaling is tightly regulated because prolonged and excessive activation of TLRs can lead to uncontrolled inflammation detrimental to the host. Varied mechanisms appear to contribute to control of TLR activation in the intestinal epithelium. These include the collective effects of several negative regulators that include IRAK-M, TOLLIP, SIGIRR, A20, Nod2, and PPARγ. However, it remains to be determined whether they comprise the entire spectrum of negative control mechanisms and how they are bypassed to trigger activation during challenge by pathogens.

Toll-like receptors; innate immunity; inflammation; ligand inhibition

INNATE IMMUNITY IS AN EVOLUTIONARILY ancient form of host defense present in invertebrate as well as vertebrate organisms. Innate immunity is central to host defense against invading pathogens, providing recognition of microorganisms and rapid deployment and activation of effector cells. Activation of innate immunity also initiates subsequent adaptive immune responses.

The ability to recognize microorganisms depends in part on a family of cell surface transmembrane receptors known as the Toll-like receptors (TLRs) (15). There are 13 known mammalian TLRs, and over the last few years much has been learned about their signaling pathways (1).

Although TLRs may play a significant role in defense against pathogens, inappropriate activation of their signaling pathways may lead to deleterious inflammation and tissue injury. Accordingly, normal host mechanisms that minimize the risk of inappropriate activation are essential to sustain mucosal homeostasis. Here we consider recent findings of TLR signal regulation in the intestine with focus on inhibitory mechanisms that control TLR signaling in the gastrointestinal (GI) tract. It is clear that current knowledge does not adequately account for the essential rigorous control of host response.

TLR Signaling Pathways and Mechanisms of Inhibition

The TLR proteins are type I integral transmembrane glycoproteins. Their extracellular domains consist of leucine rich repeats (LRR), whereas their intracellular component contains a TIR (Toll/IL-1 receptor) domain, exhibiting homology with the interleukin-1 receptor (IL-1R) superfamily. Ligand engagement by TLR leads to activation of two pathways. TLR1, 2, 4, 5, 6, 7, 8, and 9 signal via the MyD88 adaptor, whereas TLR3 signaling activates an alternative “MyD88-independent” pathway. TLR4 is the only receptor known to activate both MyD88 dependent and independent pathways.

Negative control mechanisms ensure regulated activation of TLR signaling. Known mechanisms may be broadly categorized by site of action: 1) membrane (receptor), 2) cytoplasm (intracellular signal transduction), and 3) nuclear (modulation of transcription). These are summarized in Tables 1, 2, and 3, respectively, and in Fig. 1.

Inhibition of TLR Signaling in the GI Tract

TLRs have been implicated in the pathogenesis of many GI disorders, including celiac disease (21), inflammatory bowel disease (7), colon cancer (3), and infectious colitis (22). Avoiding inappropriate activation of NF-κB via TLR inhibition is crucial in the GI tract, where epithelial cells are in constant contact with a dense and complex milieu of commensal microorganisms. It remains to be elucidated whether TLR inhibition in the GI tract is tissue or cell specific or whether it is part of a generalized immune anti-inflammatory response. The latter may include factors that decrease direct contact between the TLRs and their ligands, such as integrity of the mechanical mucosal barrier and preepithelial factors such as mucin and intestinal trefoil factor (TFF3). Although, as referenced above, many factors are known to have the ability to attenuate or abrogate TLR signaling, the roles of many of these have not yet been characterized in the intestine. It is also unclear whether additional as yet unidentified inhibitors of TLR that are specific to the GI tract exist. It is similarly important to define the triggers that “switch off” inhibitory mechanisms when pathogens are encountered. Addressing these unanswered questions may then enable assessment of the role of TLR inhibitors in regulating intestinal inflammation.

Although many more inhibitors of TLRs have already been identified, only six, discussed below, have yet been found to act in the GI tract.

PPARγ. Peroxisome proliferator-activated receptor-γ (PPARγ) is a member of a nuclear receptor family and has been proposed as a therapeutic target in inflammatory bowel disease (6).
### Themes

#### G1470

**INHIBATORY MECHANISMS OF TOLL-LIKE RECEPTORS**

**Themes**

**Signals from TLR4 and luminal bacteria may regulate PPAR-G1470 inflammatory response genes, whereas Kelly et al. (11) demonstrated that PPAR expression leading to inhibition of NF-**

**B and is responsible for its nuclear export limiting of NF-**

**B-dependent genes.** Collectively, these data suggest that PPAR possesses anti-inflammatory activity that may be mediated through its effect on TLR pathways. The possible ability of PPAR agonists to ameliorate GI inflammation in humans is consistent with these data.

**A20.** A20 is a cytoplasmic zinc finger protein that is induced by inflammatory stimuli including TNF, NF-κB, and IL-1 to confer resistance to apoptosis. A20 regulates TLR3 signaling via interaction with TRIF as well as Sendai virus-induced TLR3-dependent ISRE activation (24). A20 null mice are runted and die a few weeks after birth, exhibiting severe inflammation and damage to multiple organs including the intestine and the liver. A20 is essential for the termination of TLR response and this function protected mice from endotoxin shock (13). Consistent with the downregulatory function attributed to it, A20-deficient mice are markedly sensitive to LPS and TNF injection. These effects suggest that A20 plays a regulatory role in TLR signaling. However, it is presently unclear whether the anti-inflammatory effect of A20 is mediated primarily via TLR regulation or TNF-induced signaling pathways. It remains to be seen whether A20 plays a specific role in GI inflammation or whether the GI manifestation observed in A20-deficient mice are part of a general disinfibited inflammatory response.

**NOD2.** Nod2 (also known as CARD 15) is a cytoplasmic protein with a LRR domain that recognizes the minimal structure muramyl dipeptide component of bacterial cell wall peptidoglycan (16). Germline mutations of the LRR region increase susceptibility to ileal Crohn’s disease (10, 18). Some recent findings suggest that Nod2 may act as an inhibitor of TLR2 signaling and that mutations that reduce Nod2 function lead to increased TLR signaling by removing this inhibition (25). Nod2-deficient mice adoptively transferred with OVA-expressing T-cells and challenged with OVA expressing *Escherichia coli* developed colitis. This colitis was not observed in wild-type Nod2 mice. Nod2-TLR2 double-deficient mice were developed colitis. This colitis was not observed in wild-type Nod2 mice. Nod2-TLR2 double-deficient mice were resistant to induction of colitis consistent with the conclusion that Nod2 mediates its action via regulation of TLR2, leading to a TH1 skewed response (26).

Although the results obtained in these studies suggest that TLR2 possesses a unique ability to signal to NF-κB and to induce colonic inflammation, it should be noted that other investigators have not observed increased IL-12 induction by TLR2 ligand in Nod2 null mice (12). Indeed, some studies have demonstrated that TLR2 may have a protective effect in the GI tract (2, 19). These discrepancies await further clarification.

**IRAK-M.** IRAK-M is a member of the IL-1 receptor associated kinases (IRAK) family of adaptor molecules. In contrast to other IRAK family members that are ubiquitously expressed, IRAK-M expression is limited to monocytes (27). IRAK-M blocks the formation of IRAK1-TRAF6 complexes, preventing dissociation of IRAK1–IRAK4 from the TLR

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### Table 1. TLR inhibitory molecules affecting receptor-ligand association at the cell membrane

<table>
<thead>
<tr>
<th>Inhibitory Mechanism</th>
<th>Protein</th>
<th>Mode of Action</th>
<th>Model Tested</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><strong>Soluble factors</strong></td>
<td>sTLR4</td>
<td>Inhibition of TLR4 signaling, possibly by interfering with receptor ligand association</td>
<td>Mouse T-lymphocyte cell line, CHO-1 (hamster ovary cell line)</td>
<td>Iwami et al. <em>J Immunol</em> 165: 6682–6686, 2000.</td>
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<tr>
<td></td>
<td>sTLR2</td>
<td>Interferes with TLR2 signaling possibly via interaction with CD14, sTLR2 may homodimerize with cell surface TLR2 or act as a decoy receptor by binding to the microbial components recognized by TLR3</td>
<td>Monocel 6 human monocyte cell line and human monocytes</td>
<td>LaBouder et al. <em>J Immunol</em> 171: 6680–6689, 2003.</td>
</tr>
<tr>
<td><strong>Decoy receptors</strong></td>
<td>SIGIRR</td>
<td>Interfering with the recruitment of downstream adaptors to the TLR complex</td>
<td>Intestinal and kidney epithelial cell lines. SIGIRR deficient mice, PBMC from human patients</td>
<td>Wald et al. <em>Nat Immunol</em> 4: 920–927, 2003.</td>
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</tbody>
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**TLR, Toll-like receptors; IEC, intestinal epithelial cells.**
Table 2. TLR inhibitory molecules affecting cytoplasmic signal transduction

<table>
<thead>
<tr>
<th>Inhibitory Mechanism Targeting TIR Domain-Containing adaptors</th>
<th>Protein</th>
<th>Mode of Action</th>
<th>Model Tested</th>
<th>Reference</th>
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Table 3. TLR inhibitors affecting transcriptional output

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<tr>
<th>Inhibitory Mechanism</th>
<th>Protein</th>
<th>Mode of Action</th>
<th>Model Tested</th>
<th>Reference</th>
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<tr>
<td>Ubiquitin-mediated regulation</td>
<td>CYLD</td>
<td>Interferes with TLR2 signaling through inhibition of TRAF6 and TRAF7</td>
<td>Human IEC</td>
<td></td>
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</table>
suggesting that Tollip is involved in endotoxin tolerance in a TLR-specific manner. However, Tollip-deficient mice exhibit normal NF-κB, as well as MAPK, signaling when stimulated with IL-1β or LPS. Production of IL-6 and TNF-α was significantly reduced compared with wild-type mice, suggesting that Tollip controls the magnitude of inflammatory cytokine production in response to IL-1β and LPS. The discrepancies between the observed phenotype in mice and in vitro observations might stem from titration of signaling cascades by Tollip overexpression leading to signal blockade (4).

Concluding Remarks and Questions

Although progress has been made in defining mechanisms that may enable the mucosa to control activation of innate immune responses despite continuous exposure to commensal bacteria, a number of questions remain unanswered.

Are the above-mentioned "intestinal specific TLR inhibitors" enough to exert the rigorous control of homeostasis needed in the intestine? Several observations suggest that this is not the case. IEC express several TLRs including TLR2, TLR3, TLR4, TLR5, and TLR9 (14). The six inhibitory molecules shown to regulate intestinal inflammation seem to exert their effect through TLR2, TLR3, and TLR4. However, a recent report suggests that TLR9 signaling is important for maintaining intestinal homeostasis. Lee et al. have shown that apical but not basolateral stimulation of TLR9 in IEC induces inhibition of NF-κB activation (14). The authors did not find a role for PPARγ in this differential inhibition, but it is plausible that another inhibitor(s), possibly PI3K, or maybe a novel inhibitor may be involved. TLR5 has also been shown to mediate intestinal inflammation. A recent report showed that inhibition of TLR5 is rapid and does not require protein synthesis, suggesting that an inhibitory molecule and/or mechanism is already in place when cells come in contact with flagellin (20). However, that inhibitor was not identified. Collectively these observations lead us to believe that additional inhibitors of TLR signaling remain to be identified.

Is TLR inhibition tissue or cell specific? The finding that several TLR inhibitory molecules are active in immune cells but not in IEC or the converse (IRAK-M and SIGIRR, respectively) suggests that cell and tissue inhibitory specificity exists. However, the mechanisms conferring such specificity are poorly understood. Many of the inhibitory factors known to
affect TLR signaling have yet to be assessed for their expression in IEC and their role in intestinal homeostasis. Finally, how are the inhibitory mechanisms switched off when a pathogen is encountered? The underlying presumption is that the governor on innate immune response involves bacterial and/or cellular components. Studies of the bacterial role in inhibition suggest that commensal bacteria possess mechanisms to induce inhibition of inflammation, through constituents not present in pathogenic bacteria. These include prevention of IkB degradation by nonpathogenic salmonella (17) or active export of NF-kB from the nucleus by Bacteroides (11). Others suggest that LPS from pathogenic bacteria is recognized differentially by TLR4 to induce inflammation, although the structural differences between pathogenic and nonpathogenic LPS remain uncertain (9). However, TLR ligands are shared by commensal and pathogenic bacteria, suggesting that cellular mechanisms such as compartmentalization (TLR5) or differential activation (TLR9) are likely to play a role. Both mechanisms might be important.

TLR inhibition acts to avoid potentially deleterious activation despite the omnipresent luminal flora that produces products that can act as TLR ligands. TLR inhibition may be backstopped by mechanisms that ensure the integrity of the mechanical mucosal barrier as well as preepithelial factors such as mucus and TFF3 that decrease direct contact between the receptors and their ligands. Studies are needed to enhance understanding of the role of TLR inhibitor molecules in preventing inappropriate activation of NF-kB and other inflammatory responses and potential alterations in their function that might contribute to GI inflammatory disorders. Current evidence suggests that additional TLR inhibitor molecule(s) that play a specific role in intestinal signaling have yet to be identified.

GRANTS
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REFERENCES