Basic and Translational Understandings of Microbial Recognition by Toll-Like Receptors in the Intestine

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Microbial recognition by multicellular organisms is initially accomplished by a group of pattern recognition receptors which are specialized to recognize microbe-associated molecular patterns (MAMPs) such as lipopolysaccharide, bacterial lipoprotein, CpG DNA motif, double strand RNA and flagellin. Toll-like receptors (TLRs) are the representative pattern recognition receptors, and microbial recognition by TLRs elicits innate and inflammatory responses. Ten TLR family members have been presently identified in human genome, and numerous studies discovered that intracellular responses from MAMPs-TLR engagements are mediated by a participation of at least 4 immediate adaptor molecules such as myeloid differentiation primary response gene-88 (MyD88), MyD88 adaptor-like (Mal) (also known as Toll/IL-1 receptor domain-containing adaptor protein [TIRAP]), Toll/IL-1 receptor domain-containing adaptor-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) leading to activate transcription factors including nuclear factor κB, activator protein-1 and interferon-regulatory factors. Given that large amounts of commensal microbiota constantly reside in the intestinal lumen, enteric microbial recognition by TLRs at the intestinal epithelium provides a critical impact on regulating intestinal homeostasis. Indeed, aberrant TLR4 and TLR5 activations are etiologically associated with the development and progress of intestinal inflammatory diseases including inflammatory bowel disease and necrotizing enterocolitis. In this review article, we present the molecular mechanism by which TLRs elicit intracellular signal transduction, and summarize the physiological relevance of TLRs related to the gastrointestinal tract.

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Key Words
Flagellin; Immunity, innate; Toll-like receptor

Introduction

In the human gastrointestinal tract, there are up to $1 \times 10^{12}$ bacterial cells per gram of colonic content, and gut microbiota is constituted by 400-1,000 species of bacteria. In the human gastrointestinal tract, there are up to $1 \times 10^{12}$ bacterial cells per gram of colonic content, and gut microflora is constituted by 400-1,000 species of bacteria. Balanced intestinal microflora plays a direct impact on maintaining the gut homeostasis such as regulating bowel motility, modulating intestinal pain, processing nutritional factors and inducing immune responses. These commensal microbes are recognized by pattern recognition receptors (PRRs), which in turn regulate mucosal innate immunity and inflammatory responses. Among intestinal microflora plays a direct impact on maintaining the gut homeostasis such as regulating bowel motility, modulating intestinal pain, processing nutritional factors and inducing immune responses. These commensal microbes are recognized by pattern recognition receptors (PRRs), which in turn regulate mucosal innate immunity and inflammatory responses. Among...
PRRs, Toll-like receptors (TLRs) are the first-discovered and the most well characterized member of PRRs, and detect microbe-associated molecular patterns (MAMPs) present in a wide range of microorganisms. Here we present the molecular and cellular mechanism of TLR-mediated responses and their clinical implication in the gastrointestinal tract. Given the fact that the gastrointestinal tract of mammalian hosts harbors vast range of microorganism and the intestinal epithelial cells constituting the intestinal epithelium constantly contact with enteric microflora at a front-line of host-microbial communication, this review will be mainly focused on microbial recognition in the intestinal epithelial cells.

### Physiological Effects of Intestinal Microflora on Gut Homeostasis

Imbalanced microbiota in the gut is known to be one of the etiological reasons provoking mucosal inflammation such as inflammatory bowel disease (IBD). Moreover, bacterial flagellin, one of many microbial products, was recently described as a dominant antigen in IBD patients. We and others demonstrated that immune responses triggered by microbial products are associated with intestinal inflammation. Moreover, the gut microbiota is an important environmental factor that affects intestinal motility, resulting in irritable bowel syndrome (IBS). For instance, intestinal microflora elicits both structural and functional responses in the sensory and motor apparatus of the gut by increasing the factors mediating neuronal responses in enteric neurons. Bifidobacterium bifidum and Lactobacillus acidophilus are able to promote motility, while Escherichia species can inhibit this effect. In contrast, several studies suggest inhibitory effects of enteric bacteria on regulating gut motility. Moreover, microbial factors such as short-chain fatty acids, bacterial endotoxin or chemotactic peptides (e.g., formyl-methionyl-leucyl-phenylalanine) released from intestinal bacteria are able to stimulate the enteric nervous system in order to affect gut motility. In this context, therefore, disruption of gut microflora is poised to alter gut immunity and motility associated with aberrant bowel physiology.

### Microbial Recognition by Pattern Recognition Receptors

Since living organisms are in a continuous contact with microbial milieu including commensal and pathogenic microbes, the host-microbial communication such as recognizing the presence of microbes and responding to microbial factors is one of the critical measures for innate immunity and commensal relationship between host and microflora. The host-microbial interaction is accomplished by a molecular sensor of microbial recognition in which microbial sensory system specifically perceives a characteristic molecular pattern (e.g., lipopolysaccharide [LPS], flagellin, CpG DNA, single or double strand RNA) from various microorganisms. Since the microbial sensory system specifically recognizes and binds to a microbial pattern molecule, the sensory system is regarded as PRRs. PRRs recognize microbes in multicellular organisms, resulting in activating cellular responses against the microorganism: activation of innate and adaptive immunity and inflammatory responses.

### Toll-Like Receptors

TLRs are the type I transmembrane protein, which is composed of leucine rich repeat domain at the extracellular region, transmembrane domain and cytoplasmic Toll/IL-1 receptor (TIR) domain. While leucine rich repeat domain is specialized to recognize a specific microbial ligand, TIR domain is responsible for mediating the intracellular signaling generated by the engagement between TLRs and MAMPs. In human genome, so far ten TLRs have been identified: TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9 and TLR10. TLRs specifically recognize and bind to MAMPs: for instance, TLR4 and TLR5 are the receptor for LPS and flagellin, respectively (Table). Based on the cellular localization,

### Table. Representative Toll-Like Receptors and Their Specific Ligands

| TLRs | MAMPs                                                                 |
|------|-----------------------------------------------------------------------|
| TLR2 | Bacterial lipoproteins (eg, Pam3Cys and MALP-2)                       |
|      | Lipoteichoic acid (LTA)                                               |
|      | Peptidoglycan (PGN)                                                   |
|      | Zymosan                                                               |
| TLR3 | double strand RNA (dsRNA) (eg, Poly [I:C])                           |
| TLR4 | Lipopolysaccharide (LPS)                                              |
| TLR5 | Flagellin                                                             |
| TLR7 | Single strand RNA (ssRNA),                                           |
|      | Small anti-viral compounds                                            |
| TLR9 | Unmethylated CpG DNA                                                 |

TLRs, toll-like receptors; MAMPs, microbe-associated molecular patterns; Pam3Cys, a synthetic ligand of TLR2; MALP-2, Macrophage-activating lipopeptide-2.
TLRs are categorized into 2 groups: plasma membrane TLRs including TLR1, TLR2, TLR4, TLR5 and TLR6 residing in the plasma membrane and endosomal TLRs including TLR3, TLR7, TLR8 and TLR9 present at intracellular endosomes.22

TLRs make a homo- or hetero-complex (eg, TLR1/TLR2 and TLR2/TLR6) to expedite microbial sensing, and recruit an array of immediate adaptor molecules to its cytoplasmic TIR domain upon a ligand recognition.21,23 Intensive studies identified at least 4 adaptor molecules such as myeloid differentiation primary response gene-88 (MyD88), Mal/TIRAP (Mal is also called TIR domain-containing adaptor protein [TIRAP]), TIR domain-containing adaptor-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) interacting with TLRs in response to ligand stimulation.21,22 These adaptor molecules harbor a TIR domain responsible for the interaction with the cytoplasmic TIR domain of TLRs. When TLR recognizes a microbial ligand, it recruits a single or a combination of adaptor molecule(s) to its cytoplasmic TIR domain.22 Subsequently, these adaptor molecules associate with IL-1 receptor-associated kinases (IRAKs) to mediate the signaling to a member of TNF receptor-associated factor (TRAF) family (eg, TRAF6). Therefore, TLR-induced signaling leads to activation of inhibitory κ B kinases (IKKs) and mitogen-activate protein kinases (MAPKs) to activate transcription factors such as nuclear factor κ B (NF-κB), activator protein-1 (AP-1) and interferon-regulatory factors (IRFs), followed by inducing a pleiotropic gene expression involved in immune and inflammatory responses.22,24,25

Toll-Like Receptors in the Gastrointestinal Tract and Their Clinical Relevance

In order to appreciate the impact of enteric microbiota on gut physiology, thus, it is very critical to understand how intestinal epithelial cells recognize enteric microbiota and what are the consequences resulted from microbial recognition by the intestinal epithelial cells. Having found that TLRs are the representative microbial recognition sensor at the cell membrane as well as inside a cell, TLRs at intestinal epithelial cells should be a key sensory system in recognizing enteric microbes, which in turn alters the intracellular signaling within the gut epithelial cells and leads to activating innate and inflammatory responses.

intriguingly, most of intestinal epithelial cell lines are potently responsive to flagellin which is a specific ligand for TLR5,26,27 and hypo-responsive to TLR2 and TLR4 (eg, Pam3Cys and LPS, respectively) activation.28,29 Despite of the hypo-responsiveness of TLR4 in various intestinal epithelial cells, uncontrolled TLR4 activation is clearly responsible for necrotizing enterocolitis which is a well known pediatric intestinal inflammatory disease.30 Preterm infants show a higher expression level of TLR4 at the intestine than normal term infants, which renders preterm infants highly susceptible to the intestinal inflammation due to TLR4 activation by enteric microbes obtained after delivery.30,31 Moreover, baby formula has been known to upregulate TLR4 expression in the intestine, which is probably associated with one of the reason for pediatric intestinal inflammation.32 In addition, TLR4 deficiency makes the mouse susceptible to dextran sulfate sodium-induced colitis, while feeding LPS to normal mice provides protective effects against dextran sulfate sodium-induced colitis.33 These studies suggest that TLR4 activation by LPS in the intestine may provide beneficial effects such as promoting epithelial cell proliferation resulting in enhanced wound healing at the intestinal epithelium. Therefore, LPS/TLR4 engagement is believed to be one of the most important factors governing the intestinal physiology.

Given the fact that most intestinal epithelial cells are strongly responsive to flagellin,26,27 TLR5 should be considered as an important microbial sensor providing a significant impact on intestinal epithelial cells which are not only critical for maintaining the intact epithelial barrier function, but highly associated with the activation of lamina propria cells at the submucosa. Indeed, aberrant TLR5 activation appears to be etiologically associated with the development and progress of IBD.34,35 Intriguingly, TLR5 seems to be asymmetrically localized at the basolateral side of epithelial mucosa.4,34 Therefore, impaired barrier function of the epithelium escalates responsiveness to flagellin, which may be responsible for exacerbated intestinal inflammation.35 Despite many studies suggesting that flagellin/TLR5 engagement promotes intestinal inflammation,3,8,11,36-39 some studies imply that flagellin protects mucosal epithelium against mucosal insults (eg, radiation).40,41 Interestingly, Gewirtz’s group reported that 10% of TLR5-knockout (KO) mice exhibited spontaneous colitis in a TLR4-dependent manner,42,43 indicating that TLR5 may protect from mucosal inflammation. However, they also observed that such spontaneous colitis in TLR5-KO mice was not observed in “rederived” TLR5-KO mice.44 Moreover, the other TLR5-KO mice independently generated by Flavell’s group have normal growth, size, fertility and lifespan, and show no obvious abnormalities.44 Thus, further studies are needed to determine whether TLR5 promotes or protects from colitis. In addition to the involvement of TLR5 in mucosal inflammation, our recent
study showed that TLR5 activation could elicit potent anti-tumor immunity against colon cancer by promoting neutrophil infiltration to tumor legions.45

TLR3 specifically recognizing double-strand RNA from viruses elicits potent innate immune responses by inducing IFN-β production against rotavirus which is one of well known enteric viruses.46 TLR9 can also be activated in intestinal epithelial cells transfected with CpG DNA motifs.47 Since bacterial DNA harbors a large amount of CpG DNA motifs, TLR9 should be activated by CpG DNA released from enteric bacteria in the intestine.

**Toll-Like Receptor-Induced Signaling in the Intestinal Epithelial Cells**

Although TLR3+, TLR4+, TLR5- and TLR9-induced responses have been demonstrated in the intestinal epithelial cells,26,27,48-50 among various TLRs are TLR4 and TLR5 which have been intensively investigated as it pertains to the gastrointestinal diseases. In this review, therefore, TLR-activated molecular mechanism will be primarily addressed on TLR4- and TLR5-mediated responses. TLR4 makes a heteromeric receptor complex with CD14 and myeloid differentiation-2 at the plasma membrane in order to expedite LPS recognition.21,23 Mal/TIRAP adaptor molecule is then recruited to the plasma membrane and binds with the cytoplasmic TIR domain of TLR4, followed by recruiting MyD88 adaptor molecule. Recruiting Mal/TIRAP and MyD88 to TLR4 at the plasma membrane leads to activating serine-threonine kinase IRAKs which subsequently interact with TRAF6. TRAF6 is a cytosolic adaptor molecule mediating IKKs and MAPKs, which subsequently activates transcription factors NF-κB and AP-1, respectively.51,52

Meanwhile, LPS binding to the heteromeric complex of TLR4 also causes additional adaptor recruitment of TRAM. The complex is then endocytosed to endosome in which TRIF adaptor molecule is recruited to TLR4 complex. Like Mal/TIRAP for MyD88, TRAM adaptor appears to play a bridging role for TRIF to be recruited to TLR4.51,52 Once TRIF is associated with TLR4, it primarily induces transcription factor IRF-3 activation mediating IFN-β production. Collectively, the combinations of Mal/TIRAP-MyD88 and TRAM-TRIF adaptors are involved in TLR4-induced signaling pathways, in which the former is called the MyD88-dependent pathway mostly associated with NF-κB and AP-1 activation, and the latter is the MyD88-independent pathway mainly linked to IRF-3 activation for anti-viral defense by producing IFN-β (Figure).

TLR5 residing in the plasma membrane has been known to utilize only MyD88 adaptor, resulting in activating NF-κB and AP-1.53,54 Our recent study, however, showed that TRIF is recruited to TLR5 at least in the intestinal epithelial cells to regulate NF-κB and AP-1 activation, while TRIF associated with TLR3 does not appear to elicit IRF-3 activation for IFN-β production.55 Our study also determined that TRAM is not re-
Microbial Recognition by Non-pattern Recognition Receptors in Enterochromaffin Cells

Like intestinal epithelial cells, enterochromaffin (EC) cells residing in the mucosa are in a continuous contact with gut microflora, and communicate with microflora in the gut, and a wide variety of receptor expression (eg, 5-hydroxytryptamine [5-HT] receptors, pituitary adenylate cyclase-activating polypeptide receptor, α-adrenergic receptor, β-adrenergic receptor, cholinergic receptor, corticotropin-releasing hormone receptor, γ-aminobutyric acid receptor and TLRs) have been suggested in these cells.57,58 EC cells act as mucosal sensory transducers and secrete 5-HT in response to various physiological and pathological luminal stimuli. Engagements of these receptors by microbial factors or bacterial toxins seem to trigger a delicate mechanism for the production of 5-HT in EC cells,59-61 thereby modulate the gut motility. Unfortunately, microbial factors stimulating receptors on EC cells and the molecular and cellular mechanisms have yet to be studied.

Nonetheless, although microbial recognition is primarily mediated by PRRs like TLRs, some bacterial products are able to interact with its own specific receptor which is not a member of PRRs. For instances, cholera toxin, a secretory enterotoxin from Vibrio cholerae, binds to its receptor GM1 ganglioside in EC cells. A recent study showed that odorants present in the luminal environment stimulate olfactory receptors expressed in EC cells, resulting in 5-HT production.62

Conclusion

TLRs specifically recognize the presence of microorganisms, and there are large numbers of microbes in the gut. Thus, the gastrointestinal tract is the primary organ in which microbial recognition by TLRs should be essential to maintain the homeostasis. Indeed, impaired microbial recognition by either altered gut microflora or genetic defects of PRRs turned out to be responsible for certain type of gastrointestinal disease.2,7 Moreover, emerging evidence suggests that microbial recognition in the gut elicits potent innate immunity against pathogenic microbes or tumors. Therefore, studying TLRs in the gastrointestinal tract is now attracting more research interest than any other research areas. While numerous studies have been focused on determining the complex intracellular signaling pathways and the involvement of signaling molecules in TLR-dependent responses, studies on clinical relevance of TLRs in the gastrointestinal tract still remain unclear. Thus, during the next decade, translational and clinical researches on this area will be aimed to determine the disease relevance of TLRs or TLR-dependent signaling pathways, which in turn pave the way to develop new therapeutic approaches of the gastrointestinal disease including IBD and IBS.

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