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Toll-like receptor signaling and regulation of intestinal immunity

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The intestine is a complex organ that must maintain tolerance to innocuous food antigens and commensal microbiota while being also able to mount inflammatory responses against invading pathogenic microorganisms. The ability to restrain tolerogenic responses while permitting inflammatory responses requires communication between commensal bacteria, intestinal epithelial cells and immune cells. Disruption or improper signaling between any of these factors may lead to uncontrolled inflammation and the development of inflammatory diseases. Toll-like receptors (TLR) recognize conserved molecular motifs of microorganisms and, not surprisingly, are important for maintaining tolerance to commensal microbiota, as well as inducing inflammation against pathogens. Perturbations in individual TLR signaling can lead to a number of different outcomes and illustrate a system of regulation within the intestine in which each TLR plays a largely non-redundant role in mucosal immunity. This review will discuss recent findings on the roles of individual TLRs and intestinal homeostasis.

Introduction

Our intestine contains 100 trillion commensal bacteria,[1] outnumbering our human cells by a ratio of 10:1. Surprisingly, our intestine is able to maintain tolerance to this incredible antigenic burden, yet still provide protective inflammatory responses against invading enteric pathogens. Homeostasis refers to the dynamic balance in the intestine between tolerance and inflammation suggesting the existence of specific mechanisms that are able to restrain inflammation, during steady-state conditions. Perturbations of these mechanisms may result in dysregulation of intestinal responses leading to dysbiosis of commensal populations, aberrant immune responses and ultimately development of inflammatory disease.

One can consider that the gastrointestinal tract contains three distinct compartments. The microbial component encompasses luminal or mucosal-associated commensal bacteria and, in humans is composed of 6–10 phyla and approximately 5,000 distinct species.[2] Changes in the microbiota, termed dysbiosis, have been associated with chronic intestinal diseases such as inflammatory bowel disease (IBD),[3] as well as with extraintestinal diseases such as diabetes[4] or multiple sclerosis.[5,6] The immune compartment is contained within the lamina propria and the Peyer's patches. In these sites naïve T cells are induced to become regulatory T cells or pro-inflammatory effector cells via specialized dendritic cells (DC).[7] Separating the immune cells and the commensal microbes is a single layer of cells comprising the intestinal epithelium. The intestinal epithelium is made up of a number of cell types with functions that range from cytokine secretion, IgA secretion, production of anti-microbial peptides and mucus production.

Due to the diverse functions and complicated nature of these intestinal compartments the regulation and maintenance of homeostasis is important to prevent dysregulated immune responses and development of inflammatory disease. Given the intimate association between the microbial world and the gut, it is not surprising that innate signals are critical for maintenance of intestinal homeostasis. TLRs comprise a set of innate molecules that recognize conserved molecular motifs on bacteria and viruses and are present on both epithelial and immune cells.[8] Activation of a TLR by its ligand induces several intracellular signaling cascades resulting in the production of cytokines, chemokines and the transcription of other genes important for initiating and controlling infection. The myeloid differentiation primary response gene (MyD88) adaptor molecule is involved in many signaling pathways including TLR signaling (excluding TLR3) and non-TLR pathways such as IL-1R signaling pathway.[9] Studies using mice deficient for MyD88 have shown that signaling via TLRs plays an important role in intestinal homeostasis. Recognition of commensal microbiota in a MyD88-dependent manner has been shown to be required for epithelial cell homeostasis,[10] response to injury,[11] and induction of antimicrobial peptides.[12,13]

Since MyD88 is a common signaling molecule for most TLRs, one may reason that individual effects of a single TLR would either be represented in the phenotype of MyD88-deficient mice or would be redundant among all the TLRs. However, the reported effects of individual TLRs on mucosal homeostasis seem to point to distinct and non-redundant roles. This review will highlight the impact of those TLRs that recognize bacteria, fungi, and parasites on intestinal homeostasis and disease.

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TLR2 and Its Co-Receptors, TLR1 and TLR6

TLR2 recognizes molecular patterns associated with Gram-negative and Gram-positive bacteria and yeast, i.e., lipoproteins,[14,15] lipoteichoic acid[16] and zymosan,[17] respectively. Ligand-induced activation of TLR2 leads to recruitment of toll-interleukin receptor domain containing adaptor protein (TIRAP) and MyD88, which results in activation of nuclear factor kappa b (NFκB), and production of cytokines and chemokines.[18,19] TLR2 is functionally expressed by a number of distinct cell types in the intestinal mucosa and is constitutively expressed in the murine gastrointestinal epithelium, but expression is restricted to the crypts.[20] In the healthy gut, intestinal epithelial cells restrain potential pro-inflammatory responses to ubiquitous commensal lipoproteins through modulation of TLR2 signaling.[21] These alterations in TLR2 signaling occur as a result of increased expression of negative regulators, such as Tollip and A20,[22] and activation of cell signaling pathways[23] that induce production of anti-inflammatory IL-10, which inhibits macrophage and DC effector functions and limits immune responses.[24] Alterations in TLR2-mediated NFκB activation in antigen-presenting cells(APC), as seen in CARD15 (NOD2) mutations, leads to mucosal inflammation through exaggerated IFN-γ, IL-12 and IL-23 production.[25]

During acute intestinal injury or inflammation, stimulation of TLR2/1 heterodimers by Pam_3CysSK_4, a synthetic triacylated lipoprotein analog, preserves zonula occludens-1-associated barrier via activation of PI3K and Akt.[10,24] However, in chronic intestinal inflammation, such as that induced by adoptive transfer of naïve $CD4^+$ T cells into mice lacking adaptive immunity (RAG1-deficient mice), TLR2 signaling does not affect gut pathology.[26] Therefore, TLR2 signaling may confer protection against acute mechanical injury through maintenance of tight junction integrity while having minimal effects on regulation of sustained inflammatory processes.

Apart from its role in barrier defense and immune responses in the mucosal compartment, TLR2 signaling has direct and indirect effects on T cell function. TLR2 signaling in APC induces expression of enzymes involved in the metabolism of vitamin A and allows the APC to imprint a gut-homing phenotype on T cells.[27] Direct activation of TLR2/1 on human $CD4^+$ T cells promotes T_H17 responses,[28] and TLR2/1 signaling on committed $CD4^+$ T_{Reg} cells reduces their suppressive activity by promoting a shift toward IL-17 production.[29] However, polysaccharide A (PSA), a product of the commensal bacteria *B. fragilis*, binds TLR2 independent of TLR1 or TLR6 and promotes IL-10 production in $CD4^+$ T cells, thereby restraining T_H17 responses and enhancing its own colonization of the gut,[30] suggesting that commensal bacteria are able to exploit TLR pathways to suppress immunity, thereby establishing host-microbial symbiosis. These seemingly contradictory effects are likely due to differences in co-receptor engagement and highlight the multiple and opposing functions attributed to TLR2.

The ability of TLR2 signaling to produce pro- and anti-inflammatory responses may be due to its ability to interact with multiple co-receptors,[31] including TLR1,[32] TLR6,[32] Dectin-1,[33] CD36[34] and CD14.[35] However, this characteristic, which sets it apart from other TLRs, makes it difficult to study.[36] Our group has shown that TLR2/6 ligands educate DC to become tolerogenic and promote the polarization of IL-10-producing regulatory T cells (Tr1) in vitro and in vivo.[37] On the other hand, activation of TLR2/1 educates DC to produce greater amounts of IL12p40 and low levels of IL-10, promoting the differentiation of T_H1[37] or T_H17 cells.[31] These findings demonstrate that engagement of a specific co-receptor by TLR2 can promote either an inflammatory or a regulatory response. Alteration in the inflammatory outcome is due to the difference in activation of cell signaling pathways. Release of IL12p40 by TLR2/1-activated DC is caused by P38-MAPK activation, whereas the regulatory response by TLR2/6 DC is dependent on JNK activation.[37]

It is becoming increasingly evident that not only microbial signals but also the tissue microenvironment contribute to the type of immune response generated. Interestingly, our work and the work of others show an important role for the generation of mucosal immune responses via TLR2/1 signaling. Infection or elevated levels of IL-6 have been shown to help induce mucosal IL-17 responses in conjunction with mucosal production of TGF-β.[38,39] We have identified that specific TLR2/1 signaling in mucosal DC during oral infection with *Yersinia enterocolitica* induces an increase in IL-6 and IL-23 and prime T_H17 responses that clear the infection.[31] On the other hand, TLR2/1 signaling in the spleen during intravenous infection induces IL-12p70 and primes T_H1 immunity.[31] Within the gut, the epithelial cells express TLR1[21] and TLR1 signaling in the epithelial cells indirectly impacts the generation of T_H17 priming via the production of chemokines, which recruit DC to the site of infection (manuscript in review). Also unique to the gut tissue is the production of IgA, and TLR1 signaling is critical for the generation of Yersinia-specific secretory IgA in the feces of mice.[31] Interestingly, TLR1 signaling in DC outside of the gut can imprint a gut-homing phenotype on T cells via the induction of enzymes that promote the metabolism of vitamin A.[27]

TLR2/6 signaling leads to the induction of IL-10 and immunosuppression via a secreted virulence factor of *Yersinia* species[31,37] and the synthetic diacylated ligand FSL-1.[37] Unlike TLR2/1 signaling, TLR2/6 induction of IL-10 is ubiquitous, found both in mucosal[31] and systemic tissues,[31,37] suggesting that TLR6 may be important for regulating or dampening immune responses. Overall, these studies emphasize that interaction of TLR2 with either TLR1 or TLR6 leads to dual and opposing effects. Given that TLR2/1 and TLR2/6 ligands are triacylated[40] and diacylated[41] lipoproteins, respectively, this suggests that the bacteria can modulate the immune response and can evade host immunity depending upon its acylation status.[42]

TLR2 activation by commensal bacteria has been shown to be involved in extraintestinal diseases. For example in experimental encephalomyelitis (EAE), a mouse model of multiple sclerosis, a lipid derived from *Porphyromonas gingivalis* and other bacteria commonly found in the gastrointestinal tract are capable of enhancing autoimmunity in a TLR2-dependent manner.[43] However, the role of TLR2 in direct regulation of

the enteric microbiota has not yet been examined; therefore it is unclear whether the shifts in microbiota influence extra-intestinal diseases. Even less described is the interaction of TLR1 or TLR6 with TLR2 that could affect outcomes of extraintestinal diseases.

TLR4

TLR4 has been shown to be involved in defense against pathogens as well as establishing commensal colonization and maintaining tolerance to commensal bacteria. However, despite a common signaling pathway that includes recruitment of MyD88, phosphorylation of IL1-receptor-associated kinase (IRAK) and tumor necrosis factor receptor-associated factor 6 (TRAF6), and subsequent release of NFκB and IFNβ,[44,45] the downstream effects of TLR4 are varied. This range of responses may depend on the inflammatory status of the mucosal microenvironment.

Though barely detectable at baseline, TLR4 expression and sensitivity to its ligand, lipopolysaccharide (LPS), are increased in the setting of intestinal injury associated with Crohn disease and ulcerative colitis,[46,47] and in the presence of inflammatory cytokines such as IFN-γ and TNF-α.[48] Upon disruption of the epithelium, activation of TLR4 elicits inflammatory cytokine and chemokine expression with recruitment of innate and adaptive immune cells to limit bacterial invasion. For instance, in enteric *Toxoplasma gondii* infection and in dextran-sodium sulfate (DSS) colitis, TLR4 signaling leads to increased IL-6 and IL-12 expression[49] and neutrophil recruitment,[50] respectively, which contain bacterial translocation. Similarly, B cell recruitment and IgA production via induction of CCL20, CCL28 and proliferation-inducing ligand (APRIL) occur during constitutive activation of TLR4[45] to assist in clearance of pathogens.[51] The absence of TLR4 signaling during injury results in a pattern of severe mucosal damage with impaired epithelial proliferation, attenuated inflammatory response and marked bacterial translocation.[50] Though maladaptive in the short-term, the restrained proliferation associated with aberrant TLR4 signaling confers protection from malignancy associated with chronic colonic inflammation.[44,52]

TLR4 signaling has also been shown to affect the intestinal flora. Regulation of the microbiota by TLR4 appears to be attributable to alterations in gastrointestinal motility, which may assist in clearance of pathogens and maintenance of commensal populations,[53] differentiation of goblet cells,[54] and expression of antimicrobial peptides. TLR4 is constitutively expressed in the mouse gastrointestinal crypts[20] where its signaling directly regulates transcription of α-defensin genes[13] and β-defensin-2 genes[55] in response to alterations in the microbial communities of the gut. TLR4 signaling is also involved in the development of antibodies to biliary epithelial cells in primary sclerosing cholangitis, a disease that is closely associated with IBD and is thought to arise from impaired intestinal mucosal integrity. This finding demonstrates that defective TLR4 signaling may cause a shift in intestinal microbiota and the development of extraintestinal diseases.[56]

TLR5

TLR5 recognizes flagellin, the main protein of bacterial flagella and is crucial for the detection of invasive flagellated bacteria at the mucosal surface.[57] TLR5 plays an important role in maintaining intestinal homeostasis by regulating host defense against enterobacterial infections. This is in part because of the differential expression pattern of TLR5. Mucosal but not splenic DC express TLR5 due to different host environments including the presence of retinoic acid and other host stromal factors that alter TLR5 expression.[58] Activation of TLR5 signaling induces mucosal production of IL-17 and IL-22, which promote antimicrobial defense important for clearance of the pathogen. Production of TLR5-dependent IL-17, and IL-22 can occur via DC-dependent activation of $CD3^{-}CD127^{+}$ lymphoid tissue inducer cells (LTi),[59,60] activation of T_H1/T_H17 cells, decreased expression of T_{Reg} cells[60] and decreased induction mucosal IgA production.[61]

The recognition of flagellin by TLR5 is the dominant means by which model intestinal epithelia activate pro-inflammatory gene expression in response to *Salmonella enterica*. However, TLR5 knockout (TLR5KO) mice are resistant to Salmonella, and this resistance is attributed to changes in the basal phenotype of TLR5KO mice.[62] The small intestine and colon of TLR5KO mice exhibit elevated levels of host defense genes that mediate innate and adaptive immunity in the gut. This includes changes in the basal phenotype of antimicrobial peptides and an increase in serum and fecal IgA and IgG and transport proteins in the gut.[63] TLR5KO mice also have a homeostatic shift in microbiota composition with an increase in Proteobacteria, more specifically enterobacterial species including *E. coli*, which was observed in proximity to the gut epithelium.[64] Whether the change in microbiota composition (due to absence of TLR5 signaling) or the absence of TLR5 directly contributes to the change in basal phenotype is not clearly understood.

The absence of TLR5 signaling leads to increased resistance to infection, dysbiosis, alterations in gene expression and also impacts host metabolism. Naïve TLR5KO mice exhibit the hallmark features of metabolic syndrome including increases in body mass, visceral fat, triglycerides, cholesterol, blood pressure and low-grade chronic inflammation. TLR5KO mice also have insulin resistance even when on a calorie-restricted diet. Loss of TLR5-signaling in RAG1-deficient mice, which lack T and B lymphocytes, still results in impaired glucose regulation, demonstrating that development of TLR5KO metabolic syndrome occurs independently of the adaptive immune system. Transfer of TLR5KO microbiota to wild-type germ-free mice conferred many aspects of the TLR5KO phenotype, suggesting that the altered microbiota contributes to the development of metabolic syndrome.[65] However, whether the altered microbiota is the cause or the effect in TLR5KO mice remains yet to be determined.

TLR9

TLR9 is localized intracellularly in the endosomal compartment and recognizes intracellular bacteria by binding unmethylated

cytosine phosphate guanine (CpG) dinucleotides.[66] These nucleotides are expressed at high levels in prokaryotic DNA found within the commensal microbiome. Studies examining the localization of TLR9 in intestinal epithelial cells have suggested that activation can occur via basolateral and apical surface domains of TLR9.[67] However, the possibility of an endosomal response in epithelial cells cannot be overruled, as differential sorting of the endosomes may occur.[68] These studies suggest that the signaling of TLR9 on the apical or basolateral surfaces determine whether the response is tolerogenic or inflammatory, respectively. Apical activation of TLR9 does not induce NFκB. However, it induces expression of Frizzled 5, a regulator or Paneth cell maturation, and is for the production of antimicrobial peptides.[67] In contrast, basolateral activation of TLR9 activates NFκB activation and ultimately induces IL-8 production.[67] Regulating tolerance and inflammation upon the surface of the epithelial cells makes sense as the apical surface faces the intestinal lumen and comes into contact with commensal bacteria, and probiotic DNA suppresses inflammation and is protective in models of colitis.[69,70] Furthermore the maintenance of Paneth cells and their secreted antimicrobial peptides appears to be important for homeostatic control of the commensal bacteria. In contrast to commensal bacteria, pathogenic bacteria that have breached the epithelium would stimulate basolateral TLR9 to produce inflammatory mediators and initiate the immune response.

During inflammation the gut must control the differentiation of T_{Reg} cells, which have the potential to limit the inflammatory response. In the absence of TLR9 there is an increase in T_{reg} cells within the small intestine, leading to an inability to protect from infection.[71] The administration of CpG to antibiotic treated mice either infected with *Toxoplasma gondii*[71] or during chemical-induced colitis[72] acts as an adjuvant and contributes to the inflammatory response.

Conclusion

Altogether, these data demonstrate that TLR signaling in the intestine has important and non-redundant effects on regulating the commensal microbiota, inducing inflammatory responses and restraining or promoting tolerance. Globally, pathogens contain ligands for multiple TLR, and in any infection or inflammatory state multiple signaling pathways will be activated and will influence each other. As discussed in this review, TLR expression can be compartmentalized to specific cell types and locations in the gut, and individual TLR may induce a different set of cytokines (e.g., TLR6 and the production of IL-10). Thus there are many levels of regulation that allow specific TLR engagements to fine-tune the immune response. This fine-tuning of the immune response by TLR ligands can be manipulated by commensal microbiota in order to allow colonization, promote tolerance and limit inflammatory disease. In turn, manipulation of TLR signaling by pathogens represents an important evasion strategy. Understanding how unique TLR signals can impact different cellular responses is important for the generation of potential oral vaccines or in the treatment of inflammatory disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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