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TOPIC HIGHLIGHT

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Toll-like receptors in inflammatory bowel disease-stepping into uncharted territory

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Abstract

Ulcerative colitis and Crohn's disease are chronic relapsing-remitting inflammatory processes of the intestinal tract. The etiology of these diseases is currently unknown. However, inflammation is hypothesized to result from inappropriate activation of mucosal immunity by luminal antigens in genetically susceptible individuals. Toll-like receptors (TLRs) are a family of transmembrane proteins that act as microbial pattern recognition receptors. They are crucial initiators of innate immune responses. The role of TLRs in the pathogenesis of inflammatory bowel disease (IBD) has not been fully elucidated. In this review, we aim to analyze the available data connecting individual TLRs to intestinal inflammation and IBD.

Key words: Toll-like receptors; Inflammatory bowel disease; Intestinal inflammation

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INTRODUCTION

Inflammatory bowel disease (IBD) is comprised of two major forms of chronic inflammation of the gastrointestinal tract, ulcerative colitis (UC) and Crohn's disease (CD). These two entities differ in their location (colon only *vs* the whole length of the intestinal tract), pattern of distribution (continuous *vs* patchy), depth of involvement (mucosal *vs* transmural) and histology (crypt abscesses *vs* granulomas). The onset of IBD typically occurs in the second and third decades of life, and a majority of affected individuals progress to relapsing and chronic disease[1].

The etiology of IBD is currently unknown. Inflammation is hypothesized to result from inappropriate activation of mucosal immunity by environmental factors in genetically susceptible individuals[2]. There is strong evidence to support the role of intestinal microflora in the pathogenesis of IBD. Mice raised under germ-free conditions do not develop spontaneous colitis in several experimental models[3]. Additionally, antibiotic treatment and probiotic bacteria were shown to induce remission in IBD patients[4-6]. Inappropriate activation of innate immunity is the other arm involved in the pathogenesis of IBD[7,8]. Activation of innate immunity relies at least partially on recognition of conserved microbial motifs known as pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs)[9]. There are two major families of PRRs known as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) receptors.

TLRs

There are currently 11 known mammalian TLRs. They are transmembrane receptors that are found either on the cell membrane (TLR1, 2, 4, 5 and 9) or on intracellular organelles (TLR3, 7 and 8)[10,11]. TLRs are expressed throughout the gastrointestinal (GI) tract on intestinal epithelial cells (IECs), myofibroblasts, enteroendocrine cells, and on immune cells within the lamina propria, such as T cells, and dendritic cells (DCs)[12-16]. Extracellular domains of TLRs consist of leucine-rich repeats (LRRs), whereas their intracellular component contains a TIR (Toll/IL-1 receptor) domain, exhibiting homology with the interleukin-1 receptor (IL-1R) superfamily. Most TLR signal *via* the MyD88

adaptor (TLR1, 2, 4, 5, 6, 7, 8 and 9), whereas TLR3 signaling activates an alternative "MyD88-independent" pathway. TLR4 is the only receptor known to activate both MyD88-dependent and -independent pathways[17]. Ligand binding to TLRs initiates signaling cascades that activate NF-κB, MAPK, and interferon response factors. In this review, we will address the role of the TLR family in the pathogenesis of IBD.

TLR4

The Toll-like receptor 4 (TLR4) gene is located on the long arm of human chromosome 9. Its ligand is lipopolysaccharide (LPS), and signal initiation requires the presence of CD14. In mouse models, TLR4 is involved in regeneration of IECs. TLR4 KO and MyD88 KO mice show impaired mucosal healing, and disturbed barrier function in response to administration of the colitis inducing dextran sulfate sodium (DSS) which leads to an increase in intestinal bleeding, colonic damage, bacterial translocation and to increased mortality. A similar aggravating effect of DSS was observed when natural ligands of TLR4 were eliminated either by broad spectrum antibiotic treatment or by raising the mice in a germ-free environment[18]. TLR4 signaling was shown to play a role in the initiation of intestinal inflammation. Treatment with CRX-526, a TLR4 antagonist, inhibited the development of moderate-to-severe colitis in MDR 1a-deficient mice, and TLR signal abrogation by MyD88 KO prevented development of spontaneous colitis in IL-10 KO mice[19]. Human IECs normally express TLR3 and TLR5, whereas TLR2 and TLR4 are only minimally expressed[20]. However, TLR4 expression is upregulated in both CD and UC. In pediatric IBD patients, higher levels of TLR4 mRNA and protein were found in the inflamed colonic mucosa, but not in non-inflamed controls[21].

Epidemiological studies show an association between TLR4 polymorphism and susceptibility to IBD. In a German cohort, the CD14 promoter 1-260C>T single-nucleotide polymorphism (SNP) was associated with UC, but not CD, while the opposite was found in a Hungarian cohort. No association with IBD of the TLR4 896A>G SNP was found in either cohort[14]. In a Belgian study, the allele frequency of the TLR4 A299G polymorphism, affecting the extracellular domain of TLR4 that is associated with an abrogated response, was significantly higher in CD (11% *vs* 5%, $P = 0.004$ in one cohort and 12% *vs* 5%, $P = 0.007$ in another cohort) and in UC patients (10% *vs* 5%, $P = 0.027$) compared with controls[22]. The same SNP was exclusively related to CD in another study[23] and a third study found no association of this SNP with IBD[24]. Another TLR4 polymorphism, T399IL, was exclusively associated with UC, and not with CD[25].

TLR5

TLR5 is highly expressed in colonic epithelial cells (CECs). The bacterial ligand of TLR5 is flagellin, which is present on most motile bacteria. Expression of TLR5 appears to be basolateral in healthy individuals, and CECs in the intact colon do not respond to flagellin. It is suggested that the response of CECs to flagellin is specifically elicited under inflammatory conditions with epithelial barrier disruption[26,27]. Consistent with these findings, rectal administration of flagellin to control mice did not elicit an inflammatory response, but was able to aggravate DSS-induced colitis[26]. Interestingly, flagellin derived from *Salmonella* species, but not commensal bacteria was able to stimulate proinflammatory chemokines secretion by IECs[28]. In CD patients, tolerance to commensal-derived flagellin is lost and serum reactivity to flagellin can be demonstrated[29]. Additionally, CD patients carrying a susceptibility NOD2 mutation exhibit an enhanced flagellin reactivity which was independently associated with distinct CD phenotypes[30]. TLR5 KO mice develop spontaneous colitis, and a marked elevation in pro-inflammatory cytokine levels. This colitis is mediated *via* TLR4 signaling as TLR5 and TLR4 double KO fail to develop spontaneous colitis[31].

Data connecting TLR5 polymorphism to IBD is limited. Recently a TLR5-stop polymorphism in which a point mutation at nucleotide 1174 generates a stop codon rendering TLR5 inactive was described[32]. It is a relatively common polymorphism with a 5% allele frequency. Healthy subjects carrying the TLR5-stop SNP have significantly lower levels of flagellin-specific IgG and IgA, but similar levels of total and LPS-specific immunoglobulins. Moreover, it was observed that the carriage rate of the TLR5-stop SNP was significantly lower in CD patients as compared with unaffected relatives and unrelated controls[33].

TLR9

Unmethylated cytosine-guanosine dinucleotides (CpG), which are frequently recognized in the DNA of bacteria and viruses, are the ligands for TLR9[34,35]. The role of TLR9 signaling in pathogenesis of IBD is under intense investigation.

It was reported that intragastric and subcutaneous administration of CpG oligodeoxynucleotides (CpG-ODNs) reduced the severity of DSS and TNBS-induced acute colitis and of chronic colitis in IL-10 KO mice[36]. It was further shown that the beneficial therapeutic effect of probiotic bacteria in murine colitis models was mediated *via* the effect of CpG on intact TLR9 signaling[37]. Later, it was suggested that type I interferons may act as immunomodulatory effectors of the TLR9 pathway[38]. Further support for the immunosuppressive role of CpG-ODNs came from studies in which CD4+CD62L+ T lymphocytes from CpG-ODN pretreated donor mice were unable to induce colitis in recipient SCID mice in a transfer model, and were able to suppress CD4+ T cell-mediated colitis when co-transferred. Furthermore, CD4+CD62L+ cells from TLR9 deficient mice induced a significantly more severe colitis in SCID recipients than cells from controls[39]. These data would suggest that TLR9 signaling suppresses intestinal inflammation.

In contrast, a pro-inflammatory effect of TLR9 signaling in colitis models has also been demonstrated.

Intraperitoneal administration of CpG-ODN increased the severity of DSS-induced acute and chronic colitis[40]. Induction of DSS colitis in TLR9-deficient mice resulted in markedly reduced intestinal inflammation and proinflammatory cytokine production. Additionally, treatment with adenoviral ODN, known to block CpG effects, resulted in a significant amelioration of colitis[41].

Recently, a novel mode of TLR9 pathway regulation was described which could, at least in part, explain some of the above mentioned discrepancies. Basolateral TLR9 signaling was shown to activate the NF-κB pathway in CECs, whereas apical TLR9 signaling inhibited its activation by inducing accumulation of ubiquitinated I κB in the cytoplasm. Moreover, apical stimulation of TLR9 prevented activation of NF-κB signaling by other TLRs[8]. These data suggest that different routes of CpG-ODN delivery (intraluminal *versus* systemic) may result in anti- or pro-inflammatory effects, respectively. Additionally, this report provides a possible explanation of the divergent effects of CpGs on colitis depending on the time-point of application. Administration of CpG-ODNs when the epithelial barrier is intact results in a protective effect, while the same administration, when the epithelium, is disrupted leads to aggravation of inflammation[42].

The effect of CpG-ODN stimulation in IBD was assessed using *ex vivo* colonic mucosal biopsies from active UC patients and healthy controls. CpG-ODNs significantly inhibited colonic TNF-α and IL-1β generation in a TLR9-dependent manner in UC, and not in controls[43].

In epidemiological studies, the frequency of the -T1237C SNP of the TLR9 promoter region was significantly increased in patients with Crohn's disease as compared with controls[44].

TLR 1, 2, 6

TLR2 is required for recognition of Gram positive and mycobacterial PAMPs including bacterial lipopeptide, lipotechoic acid (LTA) and peptidoglycan (PGN). Following ligand association, TLR2 and TLR6 ± TLR1 form hetero-dimers that may control signal specificity and enhance signal transduction[45, 46].

TLR2 KO mice display increased susceptibility to DSS-induced colitis. In mice, TLR2 stimulation effectively augments tight junction barrier assembly against stress-induced damage through the activation of PI3K/Akt pathway. Oral treatment of mice with the TLR2 ligand Pam3CSK prior to DSS colitis induction significantly suppressed mucosal inflammation and apoptosis and restored epithelial integrity[47]. In contrast to its anti-inflammatory effect, another report suggested that Pam3CSK administration may actually augment colitis. Suppression of colitis by regulatory T lymphocytes in the CD45RB^{high} T cell transfer model was significantly delayed when the cells were pretreated with Pam3CSK[48].

Baseline expression level of TLR2 on enterocytes was reported to be low, but its levels increased with inflammation. Immunohistochemical analysis showed either a significant increase or no change in TLR2 expression in the terminal ileum of patients with inactive and active IBD compared to controls[20,49]. Another study showed that monocytes isolated from patients with active IBD had higher expression levels of TLR2 on their cell surfaces, and a significantly increased TNF-α production in response to TLR2 agonist stimulation as compared to inactive patients and healthy controls[50]. It was recently suggested that NOD2, which is mutated in 15% of CD patients, and was the first CD susceptibility gene to be discovered, is involved in TLR2 signal regulation. Specifically, it was shown that muramyl-dipeptide (MDP), which is the ligand for NOD2 activation, negatively regulates TLR2 signaling. NOD2 deficient mice adoptively transferred with OVA-specific CD4+ T cells, and stimulated with OVA expressing *E. coli* (ECOVA) developed colitis, whereas wild type controls did not. Importantly, this colitis was TLR2 dependent, since inflammation was suppressed in NOD2-TLR2 double deficient mice[51]. It was later reported that administration of MDP protects mice from the development of experimental colitis by downregulating multiple TLR responses, not just TLR2[52].

Epidemiological data assessing the role of TLR2 in the pathogenesis of IBD are scarce. None of the nonsynonymous SNPs of TLR1, 2 or 6 were involved with IBD susceptibility. However, a number of variants were found to be associated with disease phenotypes. The TLR2 R753G and TLR1 R80T SNPs were found to be associated with pancolitis in UC. The relative risks for heterozygous patients to develop pancolitis were 5.8 and 3.3 for R80T and R753G, respectively[53]. There was a negative association between TLR6 S249P SNP and proctitis in UC patients. In CD there was a negative association between ileal disease involvement and TLR1 S602I SNP.

TLR3

This less studied TLR in the pathogenesis of IBD, signals upon activation by double stranded RNA through a Myd88-independent pathway *via* the adaptor TRIF. Double stranded RNA is produced during viral replication as an intermediate of the replication cycle or as part of the viral RNA genome, and is also produced during apoptosis.

In wild type mice, subcutaneous administration of poly (I:C), a synthetic TLR3 agonist, protected against DSS-induced colitis. In contrast, intragastric administration of poly (I:C) offered no protection in this colitis model nor did its administration activate the innate immune system as assessed by serologic parameters[54].

Activation of TLR3 signaling induced by poly(I:C) was shown to cause an increase in IL-15 secretion leading to mucosal damage in the small intestine. IL-15 is a key regulatory cytokine involved in mucosal homeostasis. IL-15 secretion increased the percentage and number of CD3+NK1.1+ intestinal intraepithelial lymphocytes (IELs) and caused their enhanced cytotoxicity[55]. TLR3 signaling was also shown to induce IEC expression of

Rae1 (a ligand for NKG2D), which mediates epithelial destruction and mucosal injury by interacting with NKG2D expressed on intestinal intraepithelial lymphocytes [56].

In humans, TLR3 expression by IECs of UC patients is comparable to that of healthy controls, while TLR3 expression was significantly downregulated in CD patients, both in inflamed and non-inflamed tissue[20].

CONCLUSION

Inflammatory bowel disease is a chronic relapsing disease of the gastrointestinal tract. Although the etiology is unknown, both innate immunity and the commensal bacterial flora are hypothesized to play a major role in its pathogenesis. The gastrointestinal innate immune system has to recognize, sort and respond to a vast array of microbial products present in the intestinal lumen. TLRs have evolved as the major innate immune surveillance, recognition and response receptors central to efficient host defense and homeostasis of the intestinal mucosa.

The factors directing TLR-regulated immune response in IBD remain poorly understood. TLRs may either enhance or suppress intestinal inflammation. Membrane localization (either basolateral or apical), expression pattern (IECs *versus* regulatory T lymphocytes), parallel signaling by additional TLRs, cytokine combinations and interactions with specific intestinal flora all determine the type and balance of the immune response.

In mouse models, knockout of a single TLR usually does not result in spontaneous colitis. Only after additional genetic and pharmacological interventions does a clear gastrointestinal phenotype emerge. These data suggest that mutations in a single TLR are insufficient to explain the complex pathogenesis of IBD. However, they do suggest that TLRs are crucial for initiation and progression of IBD and play a major role in its pathogenesis.

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