The Association Between Gut Microbiota, Toll-Like Receptors, and Colorectal Cancer

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ABSTRACT: The large number of microbes found in the gut are involved in various critical biological processes in the human body and have dynamic and complex interactions with the immune system. Disruptions in the host’s gut microbiota and the metabolites produced during fermentation promote the development of intestinal inflammation and colorectal cancer (CRC). Toll-like receptors (TLRs) recognize specific microbial-associated molecular patterns specific to microorganisms whose signaling is involved in maintaining intestinal homeostasis or, under certain conditions, mediating dysbiosis-associated intestinal inflammation. The signaling pathways of TLRs are described first, followed by a discussion of the interrelationship between gut microbes and TLRs, including the activation of TLRs by gut microbes and the effect of TLRs on the distribution of gut microbiota, particularly the role of microbes in colorectal carcinogenesis via TLRs. Finally, we discuss the potential roles of various TLRs in colorectal cancer.

KEYWORDS: Toll-like receptors, gut microbes, colorectal cancer, NF-κB, pattern recognition receptor

Background
Colorectal cancer (CRC) is one of the most common cancers, ranking third in malignancy diagnoses and second in cancer deaths globally, and tends to increase at younger ages (before age 50). CRC is a multifactorial disease, and it is considered that inheritance, age, environment, lifestyle and other factors influence its occurrence.3,4 Evidence that high-fat diets, high-protein diets, and consumption of red and processed meats are high-risk factors for cancer, while a high-fiber diet significantly reduces the incidence of CRC. As high-throughput sequencing technology advances and more research is conducted, intestinal flora has become a focal point for CRC causation and prevention studies.

Many microbes, including bacteria, viruses, archaea, and fungi, colonize the human gut, with bacteria being the most common. The gastrointestinal ecosystem is formed at birth and changes continuously throughout life, with the microbial composition of the gut of healthy people being highly specific and in dynamic equilibrium. The gastrointestinal tract is the most colonized part of the human body by microbes, accounting for more than 70% of the total. Microbes play an important role in the gastrointestinal tract that affects not only metabolism and nutrient absorption, but also the human immune system and the development of colorectal cancer.11-14 Intestinal microbes influence colorectal cancer progression by producing virulence factors, metabolites, and effects on host inflammation and immunity.15,16

Toll-like receptors (TLRs) are members of the pattern recognition receptor family that recognize microorganism-specific molecular patterns. Bacterial antigens, such as peptidoglycan and lipopolysaccharide (LPS), are involved in the TLR signaling pathway. The dynamic nature of the gut microbial community necessitates host tolerance and monitoring to prevent pathogenic colonization and to protect symbiotic microbes. Any imbalance in the microbiome can disrupt TLR signaling pathways, resulting in uncontrolled inflammation and diseases like inflammatory bowel disease (IBD), which can eventually lead to CRC.18,19

TLR-mediated interaction between the microbiota and the intestinal mucosa is essential in maintaining dynamic intestinal homeostasis. TLR is increasingly implicated in tumor progression, particularly in microbiota-associated CRC. Specific TLRs overexpressed in tumor cells can inhibit growth, whereas other TLRs aid tumorigenesis and progression. When these TLRs are activated, they can cause inflammation, tumor cell

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proliferation, immune evasion, local invasion, and distant metastasis. In this review, we focus on the TLR signaling pathway, the interaction between the gut microbiota and TLR, and their role in the development of CRC.

**TLR Family and Its Pathways**

TLR is a type 1 transmembrane receptor expressed on epithelial and lamina propria cells. It contributes to host cell recognition and response to microbial pathogens by recognizing multiple pathogen-associated molecular patterns and damage-associated molecular patterns. Ten TLRs have been identified in humans to date, and the TLR family is divided into two sub-types based on their location in the cell. TLRs 1, 2, 4, 5, 6, and 10 are expressed on the cell surface and recognize extracellular microorganisms; TLRs 3, 7, 8, and 9 are thought to detect the presence of viral particles.

TLRs on cell membranes or nuclear endosomes recognize ligands such as structures, flagellin, and single- and double-stranded RNAs from Gram-positive and Gram-negative bacteria and induce receptor dimerization, with most TLRs forming homodimers except for TLR2, which includes a heterodimer with TLR1 or TLR6, allowing intracellular TIRs to interact with different intracellular TIR-containing adapters. There are five TIR-containing adapters, including myeloid differentiation primary response protein 88 (MyD88) and TIR domain-containing adaptor-inducing IFN-β (TRIF; also known as TICAM-1), MyD88 adaptor-like protein (TIRAP/Mal), TRIF-related adaptor molecule (TRAM), Sterile-alpha and Armadillo motif-containing protein. TLR signaling is broadly separated into two different pathways using other adaptor molecules, MyD88 and TRIF. The signaling pathway generated by the recruitment of MyD88 articulators is called the MyD88-dependent pathway, while the pathway induced by the recruitment of TRIF is called the MyD88-independent pathway or the TRIF pathway.

The MyD88-dependent pathway is involved in the early initiation of NF-κB, and all TLRs but TLR3, which only signals via TRIF, have been shown to activate the course. MyD88 contains an N-terminal dead domain (DD), an intermediate domain, and a C-terminal TIR domain; in most cases, MyD88 recruits directly to TLR activation, while TLR2 and TLR4 require TIRAP/Mal involvement. TLR-bound MyD88 recruits and activates other members of the IRAK family, like IRAK-1 and IRAK-2, through their respective DD interactions with IL-1R-associated kinase (IRAK)-4 to form Myddosome. IRAK-1 then undergoes autophosphorylation, which interacts with tumor necrosis factor (TNF)-α receptor-associated factor 6 (TRAF6). IRAK-1 and TRAF6 are subsequently detached from the complex, and TRAF6 functions as an E3 ubiquitin protein ligase, catalyzing the formation of lysine 63 (K63)-linked polyubiquitin chains between TRAF6 itself and the E2 ubiquitin ligase complex consisting of Ubc13 and Uev1A. As a scaffold, this polyubiquitin chain recruits TGF-β-activated kinase 1 (TAK1) with its bound proteins TAB1, TAB2, and TAB3, leading to phosphorylation and activation of IKKα/β. Then, the IKK complex consisting of IKK-α, IKK-β, and the nuclear factor-kappaB (NF-κB) essential modulator (NEMO, also known as IKKγ) phosphorylates the inhibitor of NF-κB (IκB). This phosphorylation leads to the degradation of IκB, and the release of NF-κB into the nucleus, and this phosphorylation results in the IκB degradation. NF-κB is released into the nucleus and later synthesized into pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, which are inflammatory key mediators. In addition, TAK1 affects translation by triggering the phosphorylation of mitogen-activated protein kinases (MAPKs), activating the MAPKs Erk1, Erk2, p38, and Jnk, which in turn activates several transcription factors, including AP-1.

TLR4 is unique among TLRs because it can conduct signals through both the MyD88-dependent pathway and the TRIF pathway; the two pathways have different dynamics, and TLR4 initiates recruitment of TIRAP and MyD88. TLR4 activation of TRIF necessitates the involvement of TRAM. TLR3 and TLR4 activate TRIF, which binds to TRAF3 and TRAF6 via the presence of their N-terminal partial TRAF-binding groups. TRIF activates TAK1 upon interaction with TRAF6 via RIP1, which then starts NF-κB in a way similar to the MyD88-dependent pathway. TRAF3 triggers TANK-binding kinase 1 and IKK-i (also known as IKK-ε), phosphorylates IFN regulatory factor-3 (IRF3) and IRF7, and activates the type I interferon (IFN) signaling pathway (Figure 1).

**TLR and Gut Microbial Homeostasis**

The gastrointestinal tract is microbially colonized shortly after birth, and a simple bacterial community gradually develops into a complex ecosystem that then begins to show a symbiotic relationship with the host. Without perturbations, the gut microbial community oscillates around a stable ecological state, displaying dynamic balance. The gut microbiota is typically highly resilient to disruption, allowing the host to retain key species over time, and the microbiome remains relatively stable in adulthood; however, the composition of the resident microbiota may be altered by environmental factors such as diet and antibiotic use. There are no single species that is universally found in all humans. The gut microbiome provides numerous benefits to the host, including the breakdown of indigestible foods, the provision of energy to colonic epithelial cells, and the provision of a barrier to invading pathogens; they also have significant effects on many host systems, particularly the development of the gut and immune system. The microbiota are the most important source of microbial stimulation in the intestine and are required for developing the intestinal immune system.

The microbiota, the persistent presence of gut microbes, that modulate TLR expression and activation, strongly influences TLR expression as an innate immune sensor. Changes in gut microbiota composition may differentially modulate the responsiveness of mucosal TLRs, subverting the immune response dominated by a pro-inflammatory phenotype.
we mentioned above, the location of TLRs is crucial for their function, and the gut microbiota can influence the area of TLRs; it was found that TLR1, TLR2, TLR5 and TLR9 were found to be up-regulated in specific pathogen-free mice compared with germ-free mice, while TLR1, TLR2, TLR4 and TLR5 were most highly expressed in the colon with a high microbial burden.

The symbiotic gut microbiota is essential in host development and inflammation. Luminal microbial populations are determined by a combination of environmental and host genetic factors, particularly the innate immune system. The role of the natural immune system in defense against pathogenic microbial infections is critical, and its importance in regulating the gut microbiota is becoming increasingly recognized.

The TLR is the intestinal epithelial barrier, the interface between the microbiome and the immune system, and cannot be overlooked in shaping the gut microbiome. TLR5 (a cell surface receptor that recognizes bacterial flagellin) expression on IECs, for example, regulates the composition and location of the intestinal microbiota to prevent diseases associated with intestinal inflammation. TLR5 deficiency in mouse intestinal epithelial cells altered the intestinal microbiota, resulting in low inflammation, metabolic syndrome, and colitis. The TLR trigger found in IEC is proposed to maintain immune stress on symbiotic flora, limiting symbiotic and pathogenic bacteria colonization and translocation.

**Gut Microbiota Affects CRC Progression Through TLR-Related Pathways**

TLR’s role in CRC is becoming clear as research into the role of gut microbiota continues. TLR’s effect on cancer progression has not been conclusively established, and continued positive TLR expression throughout the course of CRC lesions suggests that TLR may promote CRC progression, several research studies have also indicated that TLR has a dual role in cancer progression. TLR agonists are widely used in treating infectious diseases and as adjuvants in cancer treatment because of their role in specific and non-specific immunity.

**TLR Mediates Inflammation and Increases Cancer Cell Stemness via NF-κB to Promote CRC Progression**

Inflammation is an essential factor in tumorigenesis; it is not only one of the factors that promote cancer progression, but it is also a significant contributor to tumor formation. Chronic inflammation is well known to contribute to the development of CRC, and the long-term use of anti-inflammatory drugs dramatically lowers the risk of CRC. TLR senses and recognizes the gut microbiota and plays a vital role in maintaining...
gut homeostasis, controlling immune responses, and shaping the microbiome; dysfunctional expression and function are linked to the onset of inflammation and CRC.71 TLRs activate the NF-κB signaling pathway when stimulated by endogenous ligands released by oncogenic microbes and cells killed during cancer treatment.72,73 TLR activation in tumor cells upregulates the manifestation of several inflammatory cytokines, IL-1β, TNFα, and IL-6. These cytokines are released into the tumor microenvironment, where they activate NF-κB, resulting in sustained inflammatory cell responses and the development of chronic inflammation.74 Enhancement of the NF-κB signaling pathway is a primary pro-tumor function of TLR. NF-κB triggers the transcription of many pro-inflammatory genes and is a vital mediator of both acute and chronic inflammatory responses. Chronic inflammation modifies the tumor microenvironment, which serves as a scaffold and barrier for tumor growth and promotes tumor formation and development.23 The inflammatory microenvironment can cause CRC by increasing the mutation rate and allowing mutant cells to proliferate. Inflammatory cells that are activated can produce reactive oxygen species (ROS) and reactive nitrogen intermediates, which can cause DNA damage and genomic instability.66

Furthermore, NF-κB-mediated inflammation increased the stemness of cancer cells, and cancer stem cells (CSCs) constitutively showed elevated NF-κB activation, which, in turn, enhanced their stemness. These interactions result in a positive feedback loop that magnifies cancer cell inflammation and stemness, increasing the number of CSCs in the tumor. As a result of their role in cancer transfer, tolerance to treatment, and recurrence after treatment, these cells may contribute to malignancy and reduce the outcome of successful cancer therapy27 (Figure 2).

**TLR Promotes CRC Progression by Mediating Aberrant Cell Proliferation and Anti-apoptotic Effects**

Cancer is distinguished by resistance to cell death and the activation of invasion and metastasis,78 TLR signaling is tightly linked to abnormal cell multiplication and death resistance, as well as increased tumor cell invasion and metastasis via matrix metalloproteinases and integrin regulation.79 Pro-inflammatory signals typically reduce the components of the adaptive immune response. TLR influences inflammatory homeostasis and suppresses antitumor immunity, causing this reaction to shifting from antitumor to pro-tumor.23 NF-κB is closely associated with anti-apoptotic pathways that control the anti-apoptotic gene expressed and also limits the activities of pro-apoptotic pathways such as JNK.80 In CRC, TLR-induced
NF-κB stimulation was shown to promote tumor cell survival. Tumor-derived factors such as IL-6, IL-10, VEGF, and TGF-β may bias immune response induction and lead to tumor resistance in the tumor microenvironment. TLR promotes the release of these cytokines. TLR4 was found to be overexpressed in inflammation-associated colorectal tumors in humans and mice. In contrast, TLR4-deficient mice were significantly colorectal cancer-protected, indicating that TLRs on tumor cells either directly or indirectly contribute to tumor development. Furthermore, a study in the MC26 mouse colon cancer cell line discovered that TLR4 stimulation decreased both tumor cell sensitivity to CTL attack and the function of T cells and natural killer (NK) cells and that blocking TLR4 signaling delayed tumor growth and prolonged the survival of tumor-bearing mice.

**TLR-Mediated Antitumor Effects**

Because TLRs function as signaling agents in immune surveillance, the use of TLRs against tumors is becoming more researched. TLRs influence tumor progression via multiple pathways and numerous studies have revealed that TLRs and their ligands have antitumor effects by inducing apoptosis/necrosis in tumor cells or activating immune cells. TLR-activated DCs can mediate antitumor responses through antigen presentation and effects on T cells and direct cytotoxic effects on tumor cells. TLR agonists can also target CD4+ and CD8+ cells, improve their survival, increase cytokine secretion, and enhance their cytotoxic and antitumor effects. TLR ligands can also be used as immunostimulatory molecules to boost the immune system's anti-cancer therapy, and TLRs have been used in numerous preclinical and clinical studies in this regard. TLR agonists promote dendritic cell maturation and the production of pro-inflammatory cytokines such as IFN, which stimulates the adaptive immune system.

**Involvement of TLRs in CRC**

**TLR2/4**

TLR2 and TLR4 have received the most attention. TLR2 is the primary signaling receptor for Gram-positive bacteria’s cell wall components peptidoglycan and lipoprotein acid, whereas TLR4 is the primary signaling receptor for Gram-negative bacteria’s cell wall component LPS. TLR2 and TLR4 have been linked to infections, inflammation, allergic diseases, and carcinogenesis. TLRs are strongly linked to microbiota-induced colitis-associated carcinoma (CAC), and...
severe chronic colitis may be linked to the direct progression of CRC from adenoma to invasive carcinoma. There is no uniform conclusion on the function of TLR2 and TLR4 in CRC.

References: Kawai and Akira; Basith et al; Botos et al; Chang; Ding et al; Kumar and Barrett; Medvedev; Ohto; Sameer and Nissar; Sandor and Buc.

TLR2, which is highly expressed in CAC, has a standard function in tumor proliferation in CAC and sCRC, according to a study based on TLR2 knockout mice, and TLR2 knockdown inhibits CAC and sCRC growth, reduces tumor severity, and decreases tumor proliferation. TLR2-deficient mice developed more extensive and more colonic tumors, implying that TLR2 protects against CRC and that TLR2 deficiency causes CAC and early intestinal tumors. Another study found that TLR2 expression was significantly upregulated at the protein and mRNA levels in CRC patients. Still, there was no correlation between TLR2 expression and CRC staging, which is consistent with previous findings. However, TLR2 promotes CRC multiplication, migration, and invasion, and TLR2 inhibition directly inhibits CRC growth.

TLR4 recognizes a variety of pathogens, and its expression varies in different intestinal regions and is heavily influenced by the region’s microbial composition. TLR4 promotes the development of colorectal tumors in chronic colitis, and microorganisms abundant in the intestine may activate TLR4 via their antigens (e.g., antigenic lipopolysaccharides of Gram-negative bacteria), thereby inducing the growth of CRCs. TLR4 is thought to be expressed and functionally active on human CRCs, and it may help CRCs escape the immune system by activating immunosuppressive factors and apoptosis resistance. TLR4/MyD88 signaling promotes CRC progression by promoting hepatic metastasis. Silencing TLR4/MyD88 signaling in tumor cells reduces tumor development. It can increase mouse survival time after subcutaneous tumor injection. TLR4 and MyD88 immunohistochemistry in the normal colonic mucosa and adenomas. CRC revealed that elevated levels of TLR4 and MyD88 expression are associated with liver metastasis and are independent predictors of poor prognosis in CRC patients. TLR4/MyD88 signals were found to be responsible for CRC tumorigenesis in colitis-associated cancers and sporadic CRC. TLR4 mRNA expression in CRC tissues has not been shown to be significantly different from that in normal tissues, and TLR4 expression may not be involved.

TLR3

The TLR3 gene is found on the long arm of chromosome 4, and intestinal epithelial cells of normal mucosa express TLR3 constitutively, which recognizes live bacteria’s double-stranded RNA and is a crucial component of the innate immune system. TLR3 is protective against intestinal injury. It was found that upon TLR3 activation, it initiates a series of signals that trigger the production of interferon regulatory factor 3 and other cytokines and ultimately activate NF-κB, which stimulates the adaptive immune system. According to a large case-control study, TLR3 significantly interacts with colorectal cancer. Genetic variants in TLR3 may influence the development of colon cancer and the impact on survival after colon cancer diagnosis. Previous research has shown that TLR3 agonists activate tumor-specific immune responses in mice and patients. Research into the safety and therapeutic potential of TLR3 agonists is ongoing, and combination therapy with TLR3 agonists may be a treatment option for colorectal cancer.

TLR9

The TLR9 gene is found on chromosome 3 and comprises two exons that encode 1032 amino acids. It is mainly found in intracellular vesicles (e.g., endoplasmic reticulum, lysosomes, endosomes, and lysosomes), and detects unmethylated CpG groups in bacterial DNA, activating the TLR9 signaling pathway, which initiates a type 1T helper cell immune response and promotes B cell proliferation, protecting the host from invading outside microbes. TLR9 signaling is involved in colon carcinogenesis, but the precise mechanisms by which it does so are unknown. It was found that TLR9 expression levels were upregulated with increasing severity of colorectal lesions and that TLR9 interacted with NF-κB, suggesting that TLR9 expression might be consistently activated during colitis-CRC progression and that it may promote CAC through NF-κB signaling. Furthermore, CpG oligodeoxynucleotide (CpG ODN) stimulation of cell surface TLR9 promotes CRC cell proliferation, and these CpG-ODN TLR9 agonists reduce the cytotoxicity of the anticancer drug adriamycin. Another study discovered that TLR-9 mRNA expression was lower in hyperplastic and adenomatous patient cases compared with normal subjects, implying that lower TLR9 expression may promote the progression of polyps into CRC and malignancy. Furthermore, as previously stated, TLR9 agonist therapy is now in clinical trials. More research into the role and specific mechanism of TLR9 in CRC is required.

Conclusion

Colorectal cancer is one of the most common human tumors, but its treatment and outcome are unsatisfactory. The presence of a large number of microbiota in the intestine is involved in establishing intestinal homeostasis and disease development, which is linked to the pathogenesis of colorectal cancer, and microbiota dysbiosis may trigger the malignant transformation of colon cells. TLR plays an essential role in developing inflammatory diseases and CRC by regulating intestinal barrier permeability and maintaining intestinal microbial homeostasis. In contrast, as a source of TLR ligands, the intestinal microbiota plays an essential role in TLR ligand activation and distribution. A better understanding of the relationship between gut microbiota, TLR, and CRC will lead to new directions in CRC prevention and treatment.
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Yongkun Fang, Cheng Yan, Qi Zhao contributed equally to this article and participated in writing it. Bin Zhao, Yiqun Liao, Yuji Chen, participated in drawing the pictures in this article and gave guidance, Daorong Wang, Dong Tang gave constructive comments on the structure of the whole article. All authors read and approved the final.

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