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Microbiota and Immune Responses in Colon Cancer:

More to Learn

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Abstract

Increasing knowledge about the gut microbiota composition together with a resurgence in attention to the impact of the host immune system on tumor development triggered our interest in exploring how the interplay of the microbiota and the immune system represents an emerging area of interest. Determining how the immune system may alter gut microbiota composition, or the converse, and whether these interactions increase or reduce cancer risk may be relevant to generate more effective colon cancer preventive strategies.

Keywords

Microbiome; immune system; colorectal cancer

The human colon yields high rates of cancer globally[1,2] and is home to one of the most densely populated microbial ecosystems. Colon epithelial-microbiota interactions are thought to be important to colon carcinogenesis. The mammalian gut contains around 10^{13} bacteria,[3] and most belong to the phyla Bacteroidetes or Firmicutes.[4] Coevolution with bacteria drives development of immune function within the gastrointestinal tract as well as, in part, systemic immune function.[5,6] In the absence of microbes, abnormal physiologic process and host defense develop. The human intestinal microbiome contains 500 to 1000 species. If we assume a mean genome size of 5 million base pairs (bp) and 4000 genes per genome, the 2.5 billion– to 5 billion–bp intestinal microbiome might contain 2 to 4 million genes, exceeding the human genome by at least 100-fold.[4] Multiple studies have demonstrated a role for the gut microbiota in the development of colorectal cancer (CRC), and we review contributions on this association in the first section of this article. In particular, *Streptococcus gallolyticus*, *Enterococcus faecalis*, *Escherichia coli*, enterotoxigenic *Bacteroides fragilis* (ETBF), and *Fusobacterium nucleatum* are discussed. In the second section, we focus on reviewing human studies that have reported a role of the immune system in colon cancer development. Finally, we discuss the few reports in which the interplay between the immune system, the gut microbiome, and cancer development has been studied.

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THE GUT MICROBIOTA AND COLORECTAL CANCER

It takes decades for CRCs to develop, and microbes may play primary and/or secondary roles. Their relative contributions may be further influenced by environmental factors such as diet or exposure to pharmacologic or nonpharmacologic mutagens. In 1965, Austin Bradford Hill, a British medical epidemiologist and statistician, provided insight into the minimal conditions required to establish a causal relationship between 2 items. Hill's criteria[8] form the basis of modern epidemiological research, which aims to scientifically establish valid causal relationships between potential responsible agents and human diseases. Whereas microbes have been postulated as causal to the development of CRC, the application, to date, of the Hill criteria (Table 1)[8] and subsequent modifications[9,10] remain notably incomplete.

Traditionally, attempts to associate individual microbes with CRC have involved bacteria.[11,12] Several studies support the idea that a microbial leader recruits a consortium of disease-facilitating microbes to initiate the biologic events causing CRC.[13–15] Engineered murine gene knockouts yield colon carcinogenesis that is decreased in germ-free animals or sometimes by a vivarium change, which is presumed to represent the acquisition of a new microbiota. Whereas these animal models support the hypothesis of the microbiota contributing to colon carcinogenesis, they poorly imitate human disease development, highlighting the need and importance of human studies.

Most microbes, predominantly bacteria, within the colon microbial community are "non-cultivable".[16] Thus, most of the associations made between the microbiota and human CRC are based on approaches to broadly define the composition or function of the colon microbiome. The current primary analytical strategy consists of 16S ribosomal RNA (rRNA) gene sequencing that has been complemented in some studies by polymerase chain reaction–based detection of specific bacteria, whole genome (metagenome) sequencing, RNA library sequencing, and fluorescent in situ hybridization).[17] The bacterial community composition in colon adenoma or patients with CRC patients, both in mucosal samples and feces, is different from control samples but no consistent associations have been yet established.

A long proposed contributor to the pathogenesis of human CRC is *S. gallolyticus*.[18,19] *S. gallolyticus* DNA has been detected in approximately 20% to 50% of colon tumor hosts compared with less than 5% of control tissue[19] and is proposed to contribute to colon tumor growth.[18] The mechanism for this bacterium to induce tumor growth may involve enhancement of inflammatory signals including cyclooxygenase-2.[13,20]

E. faecalis has also been long suggested as a bacterium with a role in CRC pathogenesis. The proposed mechanism involves induction of mucosal macrophages to produce clastogens that cause DNA damage through a bystander effect.[21,22] Furthermore, superoxide-producing *E. faecalis* induces distal colitis, DNA damage, and cancer in germ-free *IL10*−/− mice, whereas superoxide-deficient *E. faecalis* induces inflammation but not tumors.[23] A prospective cohort study found that 40% of human stool samples from adults presenting for colonoscopy contained superoxide-producing enterococci, but no association was

established between colonization with these bacteria and risk for colorectal adenomas or cancer.[24] Stool cultures were performed for follow-up on the same patients a year later and showed significant changes in the enterococcal flora. From the patients that initially had superoxide-producing enterococcal strains, 11%were no longer colonized by the bacteria whereas 14% had acquired a superoxide-producing strain. Changes in colonic flora over time would obviously render single point-in-time measurements of intestinal contents inadequate for the determination of long-term risk.[25] Further work is needed to establish a direct link between bacterial-induced mucosal oxidative stress and the formation of adenomatous polyps and CRC.

One of the first molecular studies performed to examine the microbiota of colorectal tumors identified *E. coli* as disproportionately associated with CRC tumor samples. *E. coli* was recovered from 81% of 16 colon tumor (adenoma or cancer) samples examined compared to none of 25 control biopsies.[26] Specifically, the phylogenetic group B2 *E. coli* induces double-strand DNA breaks through the polyketide synthase (*pks*) island containing the toxin named colibactin.[27,28] Furthermore, deletion of the *pks* island reduced DNA damage, tumor numbers, and bacterial invasion, but not inf lammation, in a murine colonic oncogenesis model (*IL10*−/− mice treated with AOM).[29] In this model, both inflammation and a specific bacterial virulence factor were required for carcinogenesis. Limited human studies showed a higher prevalence of B2 phylogroup *E. coli* harboring the colibactin-producing genes in tissues of patients with CRC (55%–67%) than in those of patients with other conditions such as diverticulosis (approximately 20%).[30,31] Although these *E. coli* strains have been identified in infant fecal samples sometimes with persistent colonization,[30,31] little else is known about the epidemiology of *E. coli* (*pks* island) strains.

A human colonic bacterium, ETBF, was originally suggested as associated with CRC based on the direct effects of its recognized virulence factor, *B. fragilis* toxin on the epithelium.[32] Using the multiple intestinal neoplasia Min*Apc*$^{716+/-}$ mice (expressing a mutant gene encoding an adenomatous polyposis coli protein truncated at amino acid 716), Wu et al[33] showed that persistent colonization with ETBF markedly and rapidly increased colon adenoma formation. The protumorigenic effect was mediated by Stat3 activation and a mucosal IL-17 response, constituting the first evidence of the contribution of helper T cell (T_H)17 adaptive immune responses to carcinogenesis. Furthermore, a study from Zitomersky et al[34] reported that 40% of healthy adults are colonized by ETBF. Consistent with ETBF-induced murine carcinogenesis, a Turkish study published in 2006 examined the prevalence of ETBF in stools of a CRC population versus hospitalized controls, and ETBF was detected significantly more often in the stools of CRC (38%) compared to concurrent hospital-based, age- and sex-matched patients without CRC (12%).[35]

Recently, a group compared CRC mucosa with histologically normal colon control tissues from the same cancer-bearing host and identified enrichment of *Fusobacterium* species (in some, specifically, *F. nucleatum*) associated with CRC mucosa.[36,37] Subsequent studies showed enrichment of *Fusobacterium* species in fecal samples from CRC hosts compared to healthy controls and established a procarcinogenic effect in a mouse model.[38,39] In addition, Rubenstein et al suggested that the invasive and carcinogenic properties of *F. nucleatum* were mediated by the activated complex of the FadA adhesin of *F. nucleatum*.[40] In vitro

colon carcinoma cell line studies and tumor xenograft models revealed that FadA adhesin binds to a select extracellular domain of E-cadherin, triggering invasion of the organism as well as activation of β-catenin/Wnt signaling with stimulation of cell proliferation or tumor growth, respectively. Evaluations of tumor tissues from patients with adenoma and those with adenocarcinoma compared to colon tissue from healthy individuals revealed elevated *fadA* gene copy number in tumor tissues. The highest *fadA* gene copy numbers were found in cancer tissues and were associated with increased *fadA* transcripts in parallel with increased expression of *Wnt* and nuclear factor kappa B (*NF-kB*) genes, which is consistent with the in vitro results.

Specific bacteria or communities of bacteria may contribute to human CRC pathogenesis in several ways. First, bacteria, which breach the mucus layer and persistently adhere to colonic mucosa, may initiate oncogenic signaling in epithelial cells via the delivery of specific virulence proteins or molecules to the colon. As such, bacteria may induce DNA damage and/or interfere with DNA repair processes that might be critical for colorectal tumor initiation. Second, bacteria may trigger procarcinogenic colon epithelial cell signaling such as excessive Wnt or Stat3 signaling as well as a procarcinogenic inflammatory environment(s) involving, for example, IL-17 production and/or myeloid cell recruitment.

However, our knowledge about the community microbial associations in CRC and putative molecular mechanisms of carcinogenesis remains quite limited. For example, if we consider the points in Table 1 about causality, exposure must antecede disease expression. To discern if shifts in the colon microbiome happen prior or coincidentally with the development of CRC, prospective longitudinal studies of individuals at high risk for CRC development are required.

HUMAN IMMUNE RESPONSES AND COLORECTAL CANCER

Tumors interact with their surroundings to grow, invade, and metastasize. The tumor microenvironment is composed of a diverse array of cells including fibroblasts, endothelial cells, and immune cells. Studies have suggested that immune infiltrates in CRC may be clinically relevant.

The immune system is able to control and shape cancer through a process known as immunoediting. This process has 3 phases: elimination, equilibrium, and escape.[41] Experimental evidence supports the idea that inflammation promotes tumor development,[42] but the role of the adaptive immune reaction is still comparatively unclear based on multiple murine and human studies. Although the presence of high density of tumor-infiltrating lymphocytes (TIL) has been mostly associated with a better prognosis in CRC, the prognostic value of the regulatory T cells (Tregs) recruitment has been controversial.[43] In this section, we will focus mainly on recent human data linking changes in the adaptive immune system with CRC.

A study by Pages and Galon[44] showed that human CRCs with a higher density of infiltrating memory T cells were less likely to disseminate to lymph nodes and to perineural structures. They later investigated the relationship between type, density, and localization of the immune cells within the same cohort of patients, using immunostaining and genomic

analyses, and identified a dominant cluster of genes comodulated for T_H1 adaptive immunity [T-box transcription factor 21, interferon regulatory factor 1, IFN-γ, CD3-ζ, CD8, granulysin, and granzyme B]. When they classified the patients based on the expression levels of genes from this cluster, it revealed an inverse association with tumor recurrence, suggesting that T_H1 immunity correlates with better clinical outcomes.[45] Consistent with this report, Halama et al looked at TIL in the margins of liver metastases from CRC and found an association of high-density TIL with longer progression-free survival under chemotherapy.[46]

With respect to the prognostic value of Tregs, a study published by Sinicrope et al[47] looked at the ratio between intraepithelial CD3+ and Foxp3+ cells in stage II and stage III CRC and found an association between a low $CD3^+/FoxP3^+$ cell ratio and shorter survival. Another study[48] looked at Treg density in normal versus tumor tissue and found that high Treg density in normal mucosa was associated with worse prognosis, whereas high Tregs density in tumor tissue was associated with improved survival, in contrast to the report of Sinicrope et al[47] and to data from several other cancer types.[49,50]

With regard to the contribution of human T_H17-mediated immune responses, consistent with the murine data reported by Wu et al,[33] a subsequent clinical study by Tosolini et al[51] analyzed a panel of immune-related genes in +100 frozen colorectal tumors and by cluster analysis determined that the T_H17 cluster was associated with poor prognosis, whereas patients with a T_H1 cluster had prolonged disease-free survival.

A variety of chemokines and cytokines produced by the tumor cells, stromal cells, and/or infiltrating immune cells play an important role in TIL recruitment. Given the complexity involved, Mlecnik et al[52] studied cytokines and chemokines from a systems perspective using data integration and a biomolecular network reconstruction approach, which revealed that chemoattraction and adhesion play key roles in determining the density of the intratumoral immune cells. Moreover, the presence of specific chemokines (CX3CL1, CXCL10, and CXCL9) and adhesion molecules (ICAM1, VCAM1, and MADCAM1) correlated with high densities of T-cell subpopulations and with better outcomes. Using a more specific approach, Baker et al[53] showed that tumor insensitivity to transforming growth factor β (TGF-β) or increased TGF-β secretion were independent predictors of elevated TILs, suggesting that the TGF-B signaling pathway plays a key role in the recruitment and retention of TILs in CRC.[53]

Most studies to date evaluated tumor tissues, but Qiu et al[54] analyzed blood from patients with CRC and found that higher numbers of T-lymphocytes as well as natural killer cells in the peripheral blood were independent prognostic indicators of overall survival, suggesting that measurement of cellular immunity in the blood of patients with CRC may also allow us to predict clinical outcomes.

In the context of cancer therapy, a study by Morris et al[55] looked at the value of TIL infiltration as a predictor of response to chemotherapy with 5-fluorouracil and reported that the patients with higher TIL density in their tumors gained survival benefit from chemotherapy, suggesting a potential interaction between the immune system and

chemotherapy. Thus, the host immune response likely affects the development of CRC, and knowledge of this would aid more effective cancer prevention and therapy.

WHAT DO WE KNOW ABOUT THE INTERPLAY BETWEEN IMMUNE RESPONSES AND GUT MICROBIOTA IN COLORECTAL CANCER?

It should be stated that what constitutes a “normal” immune repertoire in the healthy colon is not clearly defined. One would estimate that this repetoire may change considerably according to diet and environment, with subsequent impact on the composition of the gut microbiota. For instance, we would expect that individuals living in regions with high exposures to fecally contaminated water and foods, and/or with parasites, will have mucosal immune systems very different from those without these exposures and that these differences may determine differential risk for colon cancer development.

Human genetic-based models of colorectal carcinogenesis involve molecular alterations in multiple genes influencing the development of a hyperplastic epithelium with progression onto adenoma and adenocarcinoma.[56] Mutations in human genes influencing adenoma and adenocarcinoma development may alter the growth rate of colonic epithelial cells, reduce their sensitivity to cell death, and provide them with abilities to suppress the immune system and further promote growth and invasion. In the same way, microbes may affect epithelial cell and/or cancer genomic stability and immune responsiveness.

No study has yet prospectively analyzed the microbiome composition and the parallel associated tumor or normal mucosal immunologic profile. Similarly, detection of proposed procarcinogenic bacteria, alone or together, in cancer or noncancer hosts, and their impact on the human mucosal immune environment is also unknown. Sequence analysis using whole-genome (metagenomic) sequencing, bacterial 16S rRNA gene DNA sequencing[37] or RNA-Seq analysis[36] revealed enrichment of *Fusobacterium* species in CRC, but the influence on tumor development and its mechanisms were not clear. Recent rigorous experimental studies were conducted in Min mice that are heterozygous for the *Apc* and a classic model for CRC pathogenesis due to the presence of *Apc* mutations in most human CRC. Despite an absence of histologic inflammation in the Min mouse colonized with *F. nucleatum*, the results support a tumor-inducing role for *F. nucleatum* through expansion of myeloid-derived immune cells and inflammatory genes in both small intestinal and colon tumors.[39] These results were further supported by expression of myeloid-associated and NF-kB–driven inflammatory genes as well as NF-kB p65 nuclear translocation that correlated with *Fusobacterium* abundance on human CRCs, whereas the abundance of other bacterial genera did not show similar correlations. Additional work to correlate the microbiota, mucosal immune responses, and carcinogenesis is needed.

Two recent murine studies examined the influence of gut microbiota and immune responses on cancer therapy efficacy. The first study showed that the maximum anticancer effects of oxaliplatin, a drug commonly used to treat CRC, depends on the colon microbiota-immune system interactions.[57] By using mice exposed to antibiotics to alter the murine gut microbiota and germ-free mice, they found that the gut microbiota influences the expression of multiple enzymes that are required by myeloid immune cells to make reactive oxygen

species, which are also required for optimal chemotherapy activity. The second study reported that cyclophosphamide, a chemotherapy drug used for the treatment of many tumors, causes injury in the small intestine and induces the generation of a T_H17 pathogenic population as well as a T_H1 antitumor response, making the tumors resistant to cyclophosphamide.[58] More studies are needed to address how the gut microbiome could be tuned to both mitigate adverse effects and optimize tumor responsiveness to chemotherapies. This is particularly relevant for cancer patients, as they often require multiple antibiotics for recurrent infections over the course of their care while they are receiving chemotherapy. Greater knowledge of microbial and host factors that optimize immune responses to tumors may improve conventional chemotherapy outcomes for CRC.

The early CEC barrier changes associated with CRC[59] likely enhance the uptake of bacterial molecules contributing to inflammatory signals and colon carcinogenesis. Grivennikov et al investigated the mechanisms responsible for inflammation-related tumorigenesis in a murine model of CRC and, besides showing that IL-23 signaling promotes tumor growth and progression, they reported defective expression of several barrier proteins in early and late colorectal neoplasms. Based on these findings, they proposed that barrier deterioration induced by colorectal tumor initiation results in adenoma invasion by bacteria with induction of tumor-promoting inflammation and tumor growth.[60] In the same line, Bongers et al recently showed that host-specific microbiome was associated with colon polyps and that alterations of the microbiota induced by antibiotic treatment inhibited the development of polyps in the cecum with the mechanism postulated to be a local decrease in epithelial barrier function.[61]

Despite the fact that multiple murine models have provided extraordinary strong evidence about the interaction between the immune system and the intestinal microbiota, further studies have started to show that many conclusions drawn from mouse studies may not necessarily translate to humans. As an example of this, recent work focused on T_H17 responses suggested that unlike the murine microbiota, highly populated by segmented filamentous bacteria (SFB), microbiota found in human donor feces is not sufficient to drive immune gene expression in the small intestine of germ-free mice.[62] This example illustrates the need for carefully designed human studies to address the role of the human microbiome in CRC pathogenesis. Prospective human studies focused on the analysis of the immune response together with molecular analysis of the gut microbiota may prove very useful.

CONCLUSIONS

In the past decade, we have gained tremendous knowledge about the gut microbiome composition and about the immune responses to CRC. By integrating data on the gut microbiome, immune responses and cancer outcomes, we will begin to obtain the necessary scientific knowledge to optimize microbiota-immune response interactions for effective cancer prevention and therapy.

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TABLE 1

Viewpoints on Disease Association or Causation: The Bradford-Hill[8] Criteria (1965)

1	Strength of association Both strong and slight associations are relevant. Slight associations do not mitigate causation. For example, the low invasive potential of meningococcus in the colonized host does not negate the conclusion that meningococcus causes meningitis.
2	Consistency Has the observed association been repeatedly observed by different persons, in different places, circumstances, and times?
3	Specificity Interpretation must be cautious, as diseases may have more than one cause.
4	Temporality Which is the cart and which is the horse? This criterion is particularly relevant with diseases of slow development.
5	Biological gradient Defining a dose-response curve between exposure and disease outcome should be sought, although it can be difficult to secure a satisfactory quantitative measure of the associated environmental factor.
6	Biologically plausible This criterion is dependent on the biologic knowledge of the day, and the observed association may be new to science and medicine.
7	Coherence The cause-and-effect interpretation should not significantly conflict with known principles of science or the natural history of disease. However, the failure to replicate disease in an experimental model cannot nullify human epidemiologic observations. For example, John Snow's observations on transmission of cholera by water were not invalidated by lack of contemporaneous isolation of the inciting *Vibrio cholerae* that awaited Robert Koch's work 30 years later.
8	Experiment Experimental evidence, when available, may provide the strongest support for disease causation.
9	Analogy It is reasonable to consider prior examples in which an exposure was linked to disease causation in judging a new putative exposure associated with disease.
10	Test of significance Formal tests of significance serve as a reminder of the effects of chance on observations but contribute nothing to the "proof " of the hypothesis incorporating the integrated analyses (above) required for determination of disease association or causation.