

## Microbes and Inflammation in Colorectal Cancer

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### Abstract

Over the past decade, there has been a renaissance in research on physiologic interactions between humans and their resident microbiota, the vast numbers of bacteria, fungi, and viruses that live within and on the body. The burgeoning interest in what constitutes the human microbiome has also focused on the contribution of microbes to carcinogenesis. Given the microbiomes of malignancies arising at mucosal sites, the microbiota may prove as influential as stromal cells and immune cells in the tumor microenvironment. Herein, we focus on the interconnections of microbes and inflammation in colorectal carcinogenesis. *Cancer Immunol Res*; 1(3); 150–7. ©2013 AACR.

### The Intestinal Ecosystem: The Microbiota

Advances in sequencing technology and analysis have fueled a tremendous expansion in microbiome research in recent years. Other driving forces in this field are longstanding interests in genomic-based pathogen discovery in cancers (1, 2), the role of the gut microbiota in chronic diseases with inflammatory components, for example, inflammatory bowel disease (IBD), diabetes, and obesity (3), and a new appreciation for the impact of antibiotic use on the microbiota (4, 5).

The microbiota are necessary for the maintenance of physiologic homeostasis. However, there is significant variability in the density and complexity of the microbiota throughout the body. Within the gastrointestinal tract, there are gradients of bacterial concentration— $10^{8-10}$  colony-forming units (CFU) of bacteria per gram (g) in saliva,  $10^3$  CFU/g in gastric juice,  $10^{2-4}$  CFU/g duodenal and jejunal contents,  $10^{10}$  CFU/g in ileal contents, and  $10^{10-14}$  CFU/g in colonic luminal contents (6). Interestingly, the higher microbial density in the colon relative to the small intestine correlates with the much higher incidence of cancer in the colon relative to the small intestine (7). In 2013, the American Cancer Society estimates that there will be approximately 103,000 new cases of adenocarcinomas in the colon in contrast with 2,900 new cases of small intestinal adenocarcinomas.

### Inflammation and cancer: microbial sensors and responders

Inflammation evolved as a defense strategy against the microbial world, and yet it is also known as a hallmark of cancer. Inflammation can function in all three stages of

tumorigenesis: initiation, promotion, and progression (Fig. 1; refs. 8, 9). Inflammatory mediators can drive sustained tumor growth and metastasis; they can also directly promote malignant cell transformation by inducing chromosomal and microsatellite instability, epigenetic alterations, and posttranslational modifications (10). Chronic exposure to reactive oxygen and nitrogen species produced by inflammatory myeloid cells can result in oxidative damage leading to genetic instability and oncogenic mutations (11, 12). Once a tumor has been initiated, intratumoral immune cells and their secreted growth factors and proteases can contribute to tumor promotion (i.e., dysregulated proliferation) and progression (i.e., malignant transformation, invasion, and metastasis).

### Sensors

Pattern recognition receptors (PRR) bind a broad range of microbial ligands, including: bacterial cell wall components, nucleic acids, and microbial polysaccharides. PRRs are central for both immune protection against pathogens and immune homeostasis with the endogenous microbiota. PRRs include Toll-like receptors (TLR), nucleotide-binding domain leucine-rich repeat proteins (NLR), and C-type lectin receptors (CLR) among others (13–16).

TLRs play a crucial role in the innate immune response by sensing the microbe-associated molecular patterns (MAMP) of bacteria, viruses, or parasites in the extracellular environment or within host cells. TLR expression has been implicated in mouse models of colitis-associated cancer. High levels of expression of TLR4, which recognizes lipopolysaccharide (LPS) of gram-negative bacteria, have been found in inflammatory bowel disease (IBD)-associated colorectal cancer, and deletion of *Tlr4* reduces the number of colonic tumors in mice treated with dextran sulfate sodium (DSS) and azoxymethane (AOM; refs. 17, 18). However, deficiency of TLR2, which recognizes peptidoglycan and lipoteichoic acid produced by bacteria and fungi, results in an increased intestinal tumor load (19).

MyD88 is an adaptor protein common to several TLRs (with the exception of TLR3) and interleukin 1 (IL-1) and IL-18 receptor signaling. Thus, loss of MyD88 may be expected to affect a wide range of innate immune sensing of the

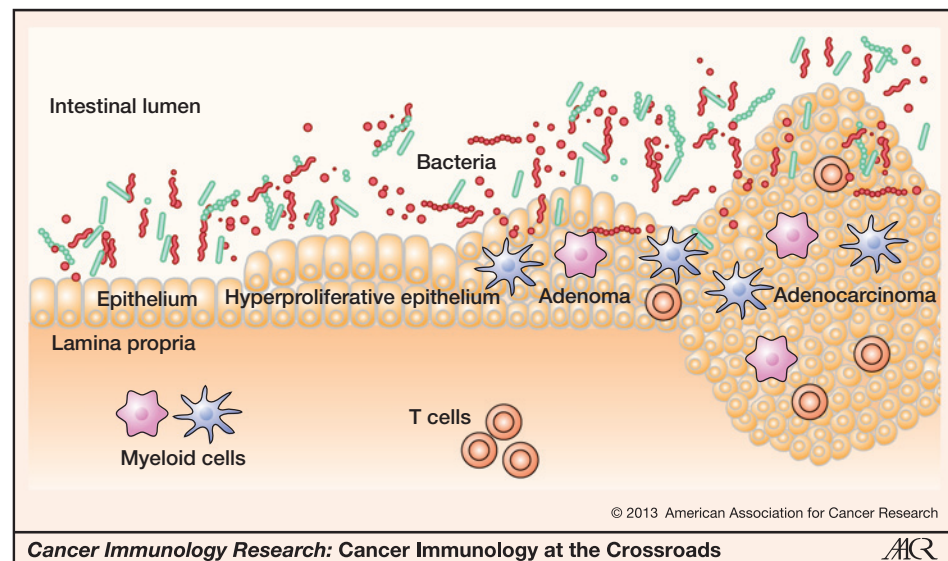
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**Figure 1.** The intestinal ecosystem in tumorigenesis. The colonic lumen is densely colonized with a diverse microbiota. Colonic adenomas and adenocarcinomas also harbor microbiota. Bacterial species, including enterotoxigenic *Bacteroides fragilis*, *Fusobacteria spp.*, and *pks+* *Escherichia coli*, have been associated with intestinal tumors. These bacteria may potentiate tumorigenesis by triggering various immune cell (myeloid cell or T cell) responses, increasing cell proliferation, or inducing DNA damage via toxins. This figure is adapted from Tjalsma and colleagues, 2012 (84).



microbiota. MyD88-deficient mice develop less inflammation and decreased colonic tumorigenesis compared with controls when treated with oxazalone-AOM (20) or DSS-AOM (21). *Apc<sup>Min/+</sup> X MyD88<sup>-/-</sup>* mice have a significantly reduced frequency of small intestinal and colonic tumors relative to *Apc<sup>Min/+</sup>* mice (22), because MyD88 signaling posttranscriptionally stabilizes the c-myc protein through activation of the kinase ERK (23).

Another major class of PRRs is the NLRs. The NLR family member Nod1 has a protective role against colitis-associated cancer. DSS-AOM-treated *Nod1<sup>-/-</sup>* mice develop more colonic tumors than DSS-AOM-treated WT mice, and *Apc<sup>Min/+</sup> X Nod1<sup>-/-</sup>* mice exhibit increased intestinal tumors compared with *Apc<sup>Min/+</sup>* controls (24). The inflammasome is a multiprotein complex (composed of several members of the NLR family, Procaspace-1, and the adaptor protein ASC) that drives an innate immune response against intracellular pathogens (25). NLRP6, an NLR family member that functions in inflammasomes, has a role in suppressing inflammation-induced colonic tumorigenesis. Loss of NLRP6 increased colitis and colonic tumors in mice treated with DSS-AOM (26, 27). Similar phenotypes are seen in mice deficient in NLR family members *Nlrp3* (28, 29) and *Nlrp12* (30, 31). Inflammasome activation results in caspase-1 activation and subsequent caspase-1 proteolytic processing of two proinflammatory cytokines: IL-1 $\beta$  and IL-18 (32). Caspases are cysteine proteases that play wide-ranging roles in apoptosis and inflammation. In keeping with the pivotal role of caspases in inflammasome function, both *Caspase-1*-deficient (33) and *Caspase-12*-deficient (34) mice have increased susceptibility to developing colon tumors following treatment with DSS-AOM.

Although TLR and NLR pathways are similarly involved in microbial detection and innate immune response, TLR/MyD88 activity seems to promote the development of colorectal cancer, whereas NLR and inflammasome activation seem to protect the host from colorectal cancer. This difference might

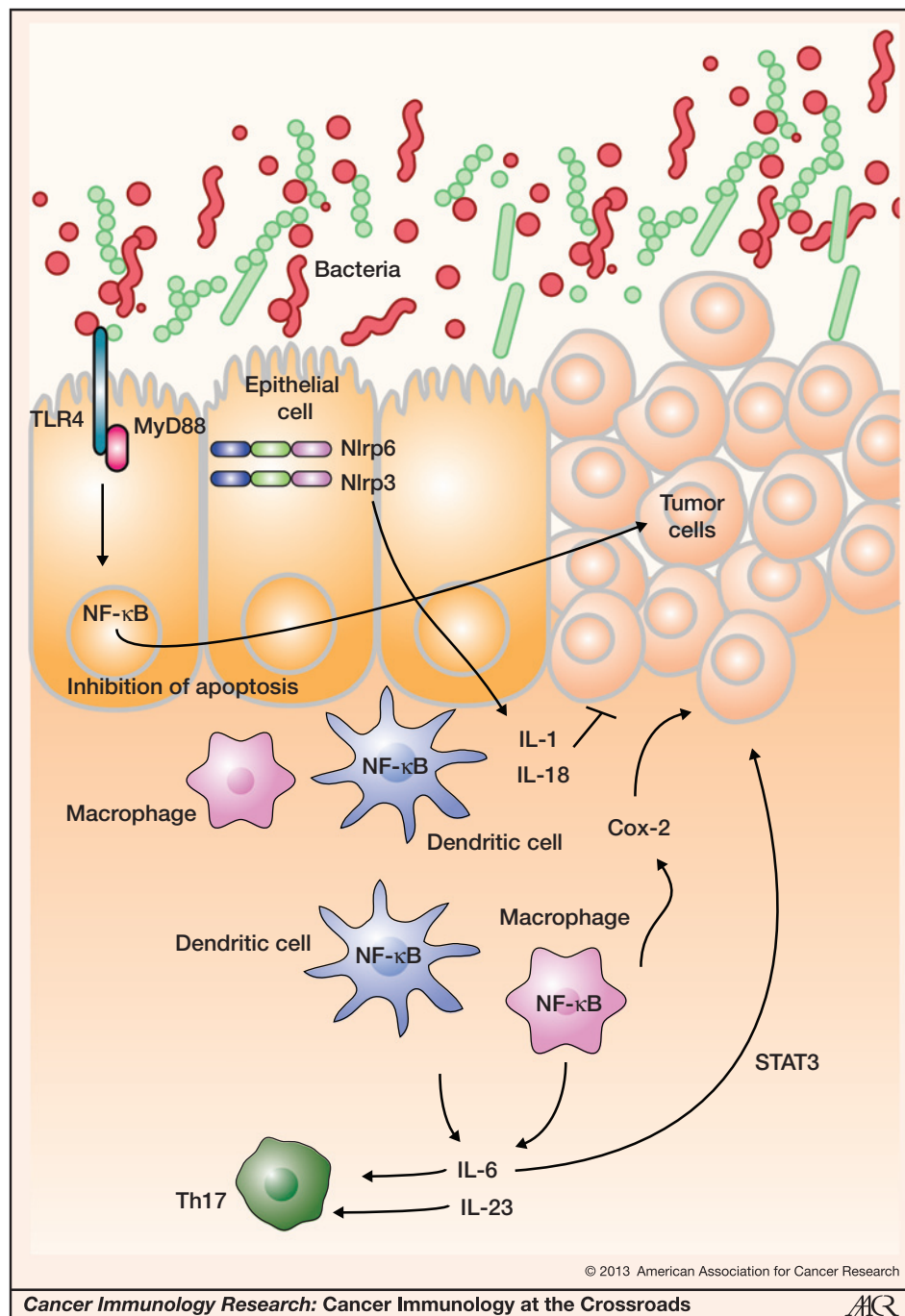
be conferred by the motif that the PRR recognizes or the cellular location of the PRR (ref. 7; see Fig. 2).

### Responders

**NF- $\kappa$ B activation and the IL-6-STAT3 axis.** Inflammatory processes lead to the production of cytokines and growth factors that prevent malignant cells from undergoing apoptosis (35, 36). NF- $\kappa$ B activation in myeloid cells regulates the expression of multiple inflammatory and tumor-promoting cytokines, including TNF- $\alpha$  and IL-1 $\beta$  (37, 38), which in turn can activate NF- $\kappa$ B in epithelial and malignant cells (39). Reducing NF- $\kappa$ B activity in myeloid cells in mice reduces both colon tumor size and multiplicity (40). Genetic ablation of components of the NF- $\kappa$ B pathway in epithelial cells alters the expression of antiapoptotic genes including *Bcl2L1* (*Bcl-xL*) and *Bcl2*, resulting in increased levels of apoptosis and decreased tumor load (40–42).

NF- $\kappa$ B activation in myeloid cells also leads to the production of IL-6, which is a key cytokine in colitis-associated colorectal cancer. IL-6 enhances the proliferation of colonic carcinoma cells *in vitro* and contributes to the development of colitis-associated colorectal cancer *in vivo*; interference with IL-6 signaling in the late stages of colitis-associated colorectal cancer slows tumor growth (42–44). In addition, IL-6 drives STAT3 activation in epithelial cells, which leads to the upregulation of antiapoptotic genes (*Bcl2L1* and *Bcl2*), cell-cycle regulators (*cyclin D1/CCND1*, *myc/c-myc*), and angiogenic factors (*bFGF*, *VEGFA*) through the activation of the Ras–Erk and phosphoinositide 3-kinase (PI3K)–Akt pathways (36, 45). Epithelial inactivation of STAT3 reduces cell survival and proliferation, and results in decreased colitis-associated colorectal cancer tumor growth and multiplicity (41, 42).

**IL-23.** IL-23 is a member of the IL-12 cytokine family. IL-23 expression is upregulated in many cancers including colorectal cancer (46, 47), and IL-23 receptor (IL-23R) blockade reduces intestinal inflammation and tumor growth in *Apc<sup>Min/+</sup>* mice colonized with enterotoxigenic *Bacteroides fragilis* (48),



**Figure 2.** Microbial sensors and responders in colorectal cancer. Intestinal epithelial cells and myeloid cells sense microbe-associated molecular patterns (MAMP) through TLR and NLR PRRs. Interaction of MAMPs with cell surface-bound TLRs leads to activation of transcription factor NF-κB via the adaptor molecule MyD88. NF-κB signaling in epithelial cells alters the expression of antiapoptotic genes including *Bcl2L1* and *Bcl2*, contributing to the transformation of epithelial cells. NF-κB activation in the myeloid cells upregulates levels of IL-6 and IL-23, which increase Th17 cells that play a critical role in colitis-associated colorectal cancer. IL-6 can directly contribute to the development of colon cancer through the activation of STAT3. PTGS2 (COX-2) is a key inflammatory mediator in colorectal cancer. IL-6 can directly contribute to the development of colon cancer through the activation of STAT3. PTGS2 (COX-2) is a key inflammatory mediator in colorectal cancer. Nlrp3 and Nlrp6, members of NLR family sense microbial and non-microbial patterns in the cytosolic compartment and are components of inflammasomes, which function in protection from colorectal cancer.

discussed below. IL-23 may be a key factor in driving intestinal tumorigenesis that results from defects of the epithelial barrier and infiltration of luminal bacteria into dysplastic regions (49). IL-23 functions, in part, by upregulating the  $T_H17$  response, and IL-23R blockade reduces IL-17A production (48). Therefore, it is plausible that IL-23 exerts its influence by modulating expression of *IL-6*, *IL-22*, and *IL-17A*, and it may not act on cancer cells directly (39).

**COX-2.** COX-2 (PTGS2) is an enzyme that converts arachidonic acid to prostaglandins, key mediators of inflamma-

tion (50). Unlike *COX-1* (*PTGS1*), which is involved in producing prostaglandins by several cell types under homeostatic conditions, *PTGS2* is not expressed physiologically by most cell types; however, its expression can be induced by a variety of growth factors and proinflammatory cytokines (51). Approximately 85% of human colorectal carcinomas and 50% of colonic adenomas exhibited elevated *PTGS2* expression (52–54), and *PTGS2* is upregulated in intestinal adenomas from *Apc<sup>Min/+</sup>* mice (55). A daily dose of aspirin or other nonsteroidal anti-inflammatory drugs, which block *PTGS2* activity, over the



course of 10 to 15 years can decrease the relative risk of developing colorectal cancer by up to 50% (56–59) and reduces colonic adenoma size and number in patients with familial adenomatous polyposis (60, 61).

### The Microbiota in Colon Cancer

Early experimental evidence that the microbiota may have a role in colorectal cancer came from gnotobiotic experiments in which rats reared in germ-free conditions showed a higher incidence of colorectal cancer than those reared conventionally (62). Subsequently, a number of studies confirmed that, under germ-free conditions, genetically engineered mice predisposed to colorectal cancer showed a lower incidence of colorectal cancer. Furthermore, under germ-free conditions, T-cell receptor  $\beta$ -chain and p53 double-knockout (*Tcr $\beta$ <sup>-/-</sup> X p53<sup>-/-</sup>*) mice had a 0% incidence of intestinal adenocarcinoma, whereas ileocecal and cecal adenocarcinomas were detected in 70% of the mice housed under conventional conditions (63). Similarly, *Tgf- $\beta$ 1<sup>-/-</sup>* mice develop colorectal cancer with normal housing, but show no signs of intestinal inflammation, hyperplasia, or carcinoma when reared in germ-free conditions. Introduction of a single species such as *Helicobacter hepaticus*, is sufficient for intestinal lesions to reappear (64). *Rag2<sup>-/-</sup>* mice, which lack an adaptive immune system, are also free from inflammation and all signs of hyperplasia under germ-free conditions, but they develop inflammation and intestinal carcinoma in the presence of *Helicobacter hepaticus* (65). When reared conventionally, mice deficient in both *Tbet* and *Rag2* develop colitis and colorectal cancer (66, 67). The administration of broad-spectrum antibiotics is sufficient to prevent these mice from developing colorectal cancer (67), similar to *Tgf- $\beta$ 1<sup>-/-</sup>* and *Tbet<sup>-/-</sup> Rag2<sup>-/-</sup>* mice that do not develop intestinal inflammation when reared in germ-free conditions (68). *Apc<sup>Min/+</sup>* mice have reduced numbers of small intestinal and colonic tumors under germ-free versus conventional conditions and the gut microbiota have been shown to trigger the c-Jun, JNK, and STAT3 signaling pathways to accelerate tumor growth in this mouse model (69).

Gut microbiota contribute to chronic colitis and predisposition to colorectal cancer in IL-10-deficient mice (70). When colonized only with *Enterococcus faecalis*, *Il10<sup>-/-</sup>* mice developed colitis and colonic adenocarcinoma, but when reared in germ-free conditions or mono-associated with a number of strains of bacteria, *Il10<sup>-/-</sup>* mice did not develop carcinoma (71). *Il10<sup>-/-</sup>* mice treated with the carcinogen AOM develop colitis and colorectal cancer under conventional conditions, but do not develop intestinal inflammation or dysplasia under germ-free conditions (72). Results from two related studies indicate that colonization by *Helicobacter hepaticus*, a common symbiont of the mouse gut microbiota, can drive AOM-induced colon tumors in *Il10<sup>-/-</sup>* mice (73, 74). These results suggest that the microbiota is required for the development of colonic tumors in a number of different mouse models and, therefore, raise the question: what specific microbes and microbial factors are responsible for contributing to tumorigenesis?

### Bacterial species

***Helicobacter pylori*.** The best-characterized association between a single bacterial species and cancer is that of *Helicobacter pylori* with gastritis, gastric ulcers, and gastric cancer, a discovery that resulted in a Nobel Prize for the co-discoverers Barry J. Marshall and J. Robin Warren (75). *H. pylori* is the most common etiologic agent in bacterial infection-related cancer, and accounts for 5.5% of all cancers globally (76). Most individuals that harbor *H. pylori*, approximately 50% of the world's population (77), do not develop peptic ulcers or gastric cancer, but there is a significant amount of data supporting a causal relationship between *H. pylori* and gastric cancer based largely on epidemiology and case-control studies (78). In addition, *H. pylori* is involved in the early stages of gastric carcinogenesis, causing chronic active gastritis and atrophic gastritis (Correa hypothesis; refs. 1, 2). Bacterial toxins (CagA and VacA) secreted from *H. pylori* are associated with an increased risk of gastric cancer. CagA binds the cellular tyrosine phosphatase, SHP2, leading to actin cytoskeletal changes and promotes cell survival by hijacking the tumor suppressor p53 (2, 79). VacA induces the formation of many vesicles within the endolysosomal compartment of epithelial cells, together with promoting oncogenic transformation (78).

***Streptococcus gallolyticus*.** There are long-standing clinical observations linking *Streptococcus bovis* (now known as *Streptococcus gallolyticus*) bacteremia and endocarditis with colorectal cancer (80). In clinical practice, patients with *S. gallolyticus* endocarditis or septicemia routinely undergo colonoscopy, because upwards of 60% of patients infected with *S. gallolyticus* are found to have a concomitant adenoma or carcinoma (81). The underlying pathophysiology of this strong association is not understood (82–84), but it may be the result of decreased epithelial barrier function at the site of colonic adenoma or carcinoma that enables streptococcal species to translocate into the systemic circulation (49).

***Pks<sup>+</sup> adherent-invasive Escherichia coli*.** Adherent-invasive strains of *Escherichia coli* play a role in colitis-associated colorectal cancer. Some strains of *E. coli* carry the polyketide synthase (*pks*) pathogenicity island that encodes the polyketide-polypeptide genotoxin, colibactin (85). *E. coli* harboring colibactin induced phosphorylated H2AX foci, signs of breakage-fusion-bridge cycles, and chromosomal instability in intestinal epithelial cells (IEC; ref. 86). A recent study showed that monocolonizing germ-free, AOM-treated, *Il10<sup>-/-</sup>* mice with a *pks*-containing *E. coli* strain resulted in enhanced tumor multiplicity compared with control mice. Remarkably, deletion of the *pks* island in the *E. coli* strain abolished intestinal tumorigenesis (87). Although investigators have observed an association between *E. coli* and colorectal cancer (88) and Crohn disease (89), *pks*-positive *E. coli* were found to be enriched in both IBD and colorectal cancer cohorts (87), suggesting that this DNA-damaging bacterium may be a clinically relevant agent in human colorectal cancer.

***Enterotoxigenic Bacteroides fragilis*.** Enterotoxigenic *Bacteroides fragilis* (ETBF) is a *B. fragilis* strain that secretes the metalloprotease toxin (BFT), and it has been found at a higher prevalence in patients with colorectal cancer compared with healthy individuals in a Turkish cohort (90). This finding,

**Table 1.** Summary of bacterial species, mediators, and potential oncogenic mechanisms

Bacterial species	Mediators	Potential oncogenic mechanisms
<i>Helicobacter pylori</i>	CagA	Binds cellular SHP2, remodels cytoskeleton; co-opts p53 to promote survival of infected cells (2, 79).
	VacA	Internalizes and causes severe vesiculation in the host endolysosomal system; also affects mitochondrial function (78).
<i>Streptococcus gallolyticus (or bovis)</i>	Unknown	Unknown
<i>Escherichia coli</i>	Colibactin (from <i>pks</i> island)	Induces DNA damage and chromosomal instability in epithelial cells (85, 86).
<i>Enterotoxigenic Bacteroides fragilis</i>	BFT	Cleaves E-cadherin, activating $\beta$ -catenin/WNT signaling (91); induces T <sub>H</sub> 17-driven inflammation (48) and spermine oxidase activity (93) leading to generation of ROS and DNA damage in colonic epithelial cells, reviewed in ref. (99).
<i>Fusobacterium nucleatum</i>	Unknown	Recruits tumor-permissive myeloid cells to accelerate neoplastic the increased progression (95–98).

as well as observations that ETBF induces epithelial cell proliferation by triggering cleavage of E-cadherin and subsequently activating  $\beta$ -catenin/WNT signaling (91) and causes colitis in mice (92), raised the possibility that ETBF could have a causal role in intestinal tumorigenesis. In a seminal study, the laboratory of Cynthia Sears and colleagues showed that *Apc*<sup>Min/+</sup> mice colonized with ETBF developed severe colitis and a significantly increased colonic tumor load compared with control mice colonized with a non-BFT-expressing strain of *B. fragilis* (48). The colonic lamina propria of ETBF-colonized mice were enriched for Th17 and  $\gamma\delta$  T lymphocytes, and antibody-based neutralization of IL-17 ameliorated colonic tumorigenesis in this model (48). These studies suggest that ETBF drives carcinogenesis as a result of BFT-induced chronic inflammation. In addition to this inflammatory mechanism, ETBF was found to induce spermine oxidase, a polyamine catabolic enzyme (93). Increased spermine oxidase activity can lead to the generation of reactive oxygen intermediates and subsequent DNA damage, while inhibition of spermine oxidase reduced ETBF-mediated inflammation in *Apc*<sup>Min/+</sup> mice (93). It has been proposed that ETBF, perhaps like *pks*-containing *E. coli*, is an "alpha-bug;" capable of introducing direct DNA damage, promoting IEC proliferation, driving tumor-promoting inflammation, and, perhaps, influencing the microbiota as a whole to drive colonic tumorigenesis (94).

**Fusobacterium nucleatum.** Three independent deep-sequencing metagenomic analyses in colorectal cancer have shown an enrichment of *Fusobacterium* species in colorectal cancers (95–97). *Fusobacterium* species were found to be the most significantly enriched taxon in tumor relative to noninvolved adjacent colonic tissues by three different methods: whole-genome sequencing, transcriptome sequencing, and 16S rDNA sequencing. The enrichment of *Fusobacterium* in colorectal tumors is striking, and a recent study lends support to

the notion that *Fusobacterium nucleatum* may play a direct role in colorectal carcinogenesis. Kostic and colleagues report that *F. nucleatum* accelerates tumorigenesis in both the small and large intestine in *Apc*<sup>Min/+</sup> mice and that *F. nucleatum* exposure resulted in increased intratumoral myeloid cell infiltration (98). Furthermore, by examining human data sets and using the *Apc*<sup>Min/+</sup> model, Kostic and colleagues found that fusobacteria were associated with a distinctive proinflammatory signature shared between humans and mice. Clearly, more studies are needed to define how this bacterium and others directly contribute to the tumorigenic process.

### Summary and Future Directions

Over the past decade, new insight has been gained into the associations and potential contributions of single bacterial species and microbial sensing and response pathways in colorectal carcinogenesis (see summary in Table 1). However, additional studies are needed to determine why some microbes are associated with colorectal cancer and how they can be precisely targeted. Critical questions center on unraveling the fitness and survival advantages that some bacteria have within the tumor microenvironment and how their presence alters the functions of tumor cells, intratumoral immune cells, and stromal cells. Beyond research focused on single bacterial species that can incite inflammation or modulate host pathways that influence transformation, further investigation into how the microbiota may contribute to carcinogenesis via generation of metabolites from foods, medications, and other environmental exposures will be of great import. More data on the microbiome and microbial metabolites from healthy individuals and patients across the neoplastic spectrum of premalignant colorectal tumors to metastatic colorectal cancer will be key for understanding the role of the microbiota in carcinogenesis.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors' Contributions**

**Conception and design:** W. Garrett, A.D. Kostic

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** W. Garrett, A.D. Kostic

**Writing, review, and/or revision of the manuscript:** W. Garrett, A.D. Kostic, E. Chun, and M. Meyerson

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** W. Garrett, A.D. Kostic

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