

REVIEW SUMMARY

CANCER MICROBIOME

The microbiome and human cancer

Gregory D. Sepich-Poore, Laurence Zitvogel, Ravid Straussman, Jeff Hasty, Jennifer A. Wargo, Rob Knight*

BACKGROUND: Historical accounts linking cancer and microbes date as early as four millennia ago. After establishment of the germ theory of infectious diseases, clinical research of microbial influences on cancer began in 1868, when William Busch reported spontaneous tumor regressions in patients with *Streptococcus pyogenes* infections. Over the next century, poor reproducibility, erroneous microbiological claims, and severe toxicity led many to discount the role of bacteria in carcinogenesis and cancer therapy. However, these studies provided the first crude demonstrations of cancer immunotherapy. Contemporaneously, the viral theory of cancer flourished, spurred by the 1911 discovery of Rous sarcoma virus, which transformed benign tissue into malignant tumors in chickens. The

decades-long search to find viruses behind every human cancer ultimately failed, and many cancers have been linked to somatic mutations. Now the field is encountering intriguing claims of the importance of microbes, including bacteria and fungi, in cancer and cancer therapy. This Review critically evaluates this evidence in light of modern cancer biology and immunology, delineating roles for microbes in cancer by examining advances in proposed mechanisms, diagnostics, and modulation strategies.

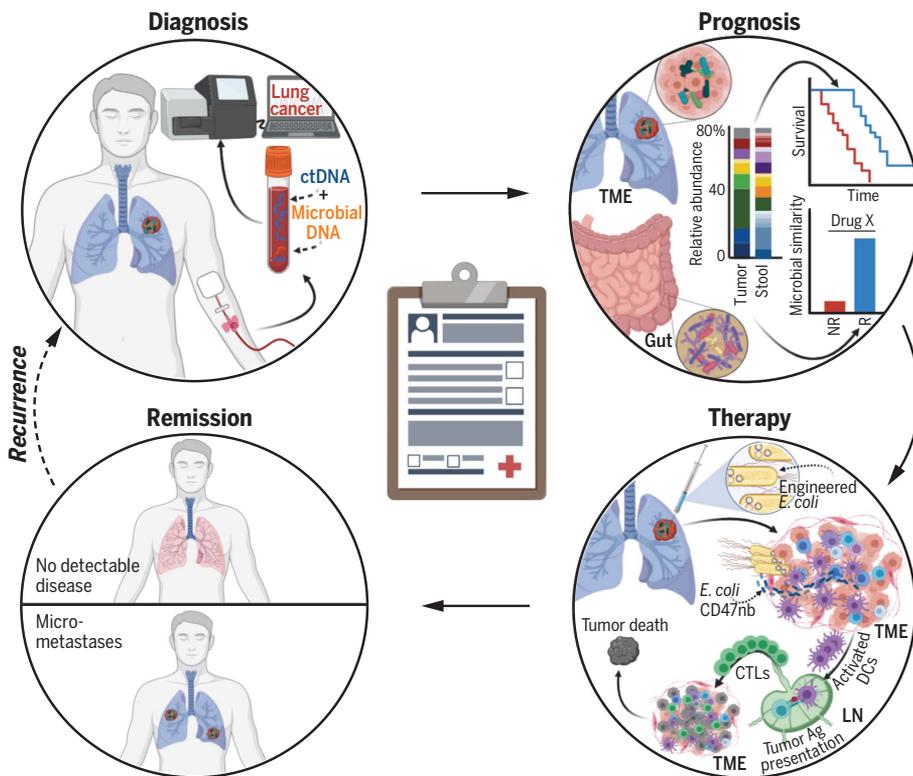
ADVANCES: Few microbes directly cause cancer, but many seem complicit in its growth, often acting through the host's immune system; conversely, several have immunostimulatory properties. Mechanistic analyses of gut

microbiota-immune system interactions reveal powerful effects on antitumor immunity by modulating primary and secondary lymphoid tissue activities. Many of these pathways invoke Toll-like receptor-initiated cytokine signaling, but microbial metabolic effects and antigenic mimicry with cancer cells are also important. In preclinical models, microbial metabolites also regulate phenotypes of tumor somatic mutations and modulate immune checkpoint inhibitor efficacy.

Emerging evidence suggests that intratumoral bacteria exist and are active, with overlapping immunohistochemistry, immunofluorescence, electron microscopy, and sequencing data in ~10 cancer types. Preliminary studies further suggest that fungi and bacteriophages contribute to gastrointestinal cancers. However, the abundance of intratumoral microbial cells is low relative to cancer cells, and knowledge of their functional repertoire and potency remains limited. Further validation of their prevalence and impact is needed in diverse cohorts and therapeutic contexts.

The immunomodulatory effects of host microbiota have reinvigorated efforts to change their composition as a form of immunotherapy. Despite extensive preclinical evidence, translation of microbiota modulation approaches into humans has not yet materialized into commercialized therapies. Synthetic biology approaches are also gaining traction, with engineered bacterial cancer therapies in preclinical and clinical trial settings.

OUTLOOK: A better understanding of the roles of microbes in cancer provides an opportunity to improve each stage of the cancer care cycle, but major challenges remain. Concerted efforts to characterize cancer-associated microbiota among tumor, stool, and blood samples with gold-standard contamination controls would tremendously aid this progress. This would be analogous to The Cancer Genome Atlas's role in characterizing the cancer somatic mutation landscape. Large-scale clinical trials are currently testing the efficacy of microbiota modulation approaches, ranging from dietary modifications to intratumorally injected, engineered bacteria. These bacterial cancer therapies, if safe and effective, could tremendously expand the cancer therapy armamentarium. Altogether, integrating the host-centric and microbial viewpoints of cancer may improve patient outcomes while providing a nuanced understanding of cancer-host-microbial evolution. ■



Opportunities for microbes to affect cancer care. Diagnosis: Cancer-specific, blood-borne microbial DNA may complement cell-free tumor DNA (ctDNA). Prognosis: Gut and intratumoral microbiota may stratify patient outcomes (NR, nonresponder; R, responder; TME, tumor microenvironment). Therapy: Intratumoral injection of CD47 nanobody (CD47nb)-producing *Escherichia coli* may create systemic antitumor immunity by enhancing dendritic cell (DC) phagocytosis, lymph node (LN) antigen (Ag) presentation, and cytotoxic T lymphocyte (CTL) activity.

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Microbial roles in cancer formation, diagnosis, prognosis, and treatment have been disputed for centuries. Recent studies have provocatively claimed that bacteria, viruses, and/or fungi are pervasive among cancers, key actors in cancer immunotherapy, and engineerable to treat metastases. Despite these findings, the number of microbes known to directly cause carcinogenesis remains small. Critically evaluating and building frameworks for such evidence in light of modern cancer biology is an important task. In this Review, we delineate between causal and complicit roles of microbes in cancer and trace common themes of their influence through the host's immune system, herein defined as the immuno-oncology-microbiome axis. We further review evidence for intratumoral microbes and approaches that manipulate the host's gut or tumor microbiome while projecting the next phase of experimental discovery.

The histories of cancer and human microbiota are intimately interwoven. Writings as early as 1550 BCE in the Ebers Papyrus, attributed to the Egyptian physician Imhotep (around 2600 BCE), suggest a crude treatment for tumors (swellings) involving application of a poultice to the site followed by an incision, causing an infection (1, 2). In the 13th century, Peregrine Laziosi described spontaneous regression of his septic, ulcerative tibial bone malignancy that would have required amputation (2), for which he was canonized in 1726. After establishment of the germ theory of infectious disease, Wilhelm Busch and Friedrich Fehleisen independently reported in the late 1800s that *Streptococcus pyogenes* infections were associated with spontaneous tumor regressions in several patients (3, 4). Shortly thereafter, William Coley started testing a highly contentious and sometimes lethal vaccine of live or heat-killed *Streptococcus* and *Serratia* species on terminal cancer patients, which was only later shown to yield >10-year disease-free survival in ~30% of them

(60 of 210 total), representing the first intentional demonstration of immunotherapy (5). Contemporaneously, Thomas Glover and Virginia Livingston-Wheeler claimed, controversially, that bacteria were cultivable from tumors and that bacterial vaccines were effective against tumors, and suggested a universal bacterial origin of cancer (6, 7). These early treatment approaches and theories were fraught with error: Livingston-Wheeler's bacterial "cause" of cancer, *Progenitor cryptocides*, turned out to be the skin commensal *Staphylococcus epidermidis* (a frequent contaminant), and Glover's findings were not reproducible by researchers at the National Institutes of Health (7). With no mechanistic evidence, irreproducible results, and hazardous therapies, the bacterial theory of cancer was dismissed.

The viral theory of cancer gained traction after Peyton Rous's 1911 discovery of a transmissible oncogenic virus in chickens (8). The subsequent decades-long search to find a virus behind every cancer linked Epstein-Barr, human papilloma, and hepatitis viruses to carcinogenesis (9) but failed to find a viral cause for most human cancers, and the theory was overtaken by the somatic mutation hypothesis.

Now, after decades of research thoughtfully characterizing the hallmarks of human cancer through somatic mutations and other host-centric perspectives (10, 11), the field is encountering nuanced claims that microbes may play a broad role in cancer diagnosis, pathogenesis, and treatment (12–26). This reappraisal stems from greater appreciation of the number of microbes that inhabit the human body (roughly equal to the number of human cells), their gene count that exceeds the human genome's gene count by ~100-fold and enables diverse metabolic programming, and their effects on host immune system de-

velopment and activity, including antitumor immunosurveillance (27–31). Although most proposed cancer-microbe relationships focus on gut microbiota (30, 32, 33), recent studies also contentiously suggest the existence, metabolic activity, and functional importance of intratumoral microbiota using a combination of imaging, sequencing, and cultivation techniques and genetically engineered and germ-free mouse models (12–14, 18–20, 23, 34). These studies raise many questions about microbes and cancer. How should microbes be viewed in light of known host-centric cancer characteristics? To what extent are microbes causal agents, complicit actors, or passive bystanders? If intratumoral microbes exist, do they have therapeutic implications? What role do microbes play in patient management? With these questions in mind, this Review aims to critically evaluate the known roles of microbes in cancer and to outline the next steps for evaluating their clinical utility.

Overview of the cancer microbiome

Of the estimated ~10¹² distinct microbial species on Earth (35), just 11 are labeled human carcinogens, or "oncomicrobes," by the International Association for Cancer Registries (IACR) (36). These oncomicrobes cause an estimated 2.2 million cases per year (~13% of global cancer cases), and their epidemiology, molecular mechanisms, and clinical studies have been extensively reviewed (36). Strong experimental evidence suggests that additional microbes initiate cancer through genotoxin-mediated mutagenesis; in particular, colibactin (a DNA alkylator), cytolethal distending toxin [CDT; direct deoxyribonuclease (DNase) activity], and *Bacteroides fragilis* toxin (Bft; reactive oxygen species (ROS) producer) cause mutational signatures found in colorectal, head and neck, and urinary tract cancers (22, 37–41). Experimental evidence also implicates several microbes with virulence factors that amplify tumorigenesis through E-cadherin–Wnt–β-catenin signaling, including FadA from *Fusobacterium nucleatum* and AvrA from several *Salmonella* strains (42, 43). A few dozen microbial species can thus directly cause cancer, based on current epidemiological and experimental evidence.

Increasing evidence suggests an important additional category of "complicit" microbes and microbial functions that promote carcinogenesis but are insufficient to cause cancer (18, 20, 25, 38, 44–47). This category encapsulates many immunomodulatory functions of microbiota and their bioactive metabolites in tumor development and may be linked to the immune system's role in solid tumorigenesis; the immune system rarely initiates the incipient lesion but can facilitate progression through tumor-stroma feedback loops, inflammation, or dysfunctional immunosurveillance

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(11). One example is that common p53 mutations are only carcinogenic in the presence of microbially produced gallic acid and are protective otherwise in the gut, both in vivo and in organoids, suggesting a microbiome-functional genomic interaction (44). A second is microbially produced secondary bile acids, which reduce hepatic sinusoidal CXCL16 expression (the sole ligand for CXCR6) and prevent CXCR6⁺ natural killer T cell aggregation and liver cancer immunosurveillance—this carcinogenic effect is eliminated by vancomycin treatment (45). A third comes from the inability of Kras mutation and p53 loss to produce lung cancer in germ-free or antibiotic-treated mice: Commensal lung microbiota promote expansion and activation of $\gamma\delta$ T cells, which drives tumor-promoting inflammation through local interleukin-17 (IL-17) and IL-23 release (18). These examples illustrate how microbes or microbial functions can be complicit in cancer rather than directly causal.

In contrast to the few bona fide oncomicrobes, the many complicit microbes and their functions are broad and understudied. Complicit microbes require mediators to promote tumor development but modulate tumor progression and therapeutic efficacy locally or from a distance. Complicit microbes are also least understood, requiring comprehension and integration of host and microbial biology, so we emphasize them in this Review. Together with known causal mechanisms, the diversity of these “complicit” mechanisms and their relationships to host-centric cancer hallmarks (10, 11) are notable (Fig. 1), but they will require more rigorous experimentation and cross-cohort validation to establish clinical prevalence and utility.

Understanding the distribution of microbes across the body is important for understanding their relationships to cancer. About 4×10^{13} microbial cells spanning $\sim 3 \times 10^3$ species inhabit the human body: About 97% of these cells are bacteria in the colon, ~ 2 to 3% are extracolonic bacteria (proximal gut, skin, lungs, etc.), and ~ 0.1 to 1% are archaea and eukarya (including fungi) (27, 48). Human-infecting virus and phage counts and diversity may be greater (49). The high density of colonic bacteria is thought to drive most of the known microbial immunomodulatory effects in the mammalian intestinal tract, the largest immune organ in the body (50), but organ-specific commensals may exert their own overriding influence (18, 46). Intratumorally, Nejman *et al.* used quantitative polymerase chain reaction (qPCR) of 16S ribosomal RNA (rRNA) to estimate the number of bacteria relative to 40 ng of DNA in melanoma, lung, ovarian, glioblastoma, pancreatic, bone, and breast cancer tissue sections (12). Assuming tissue homogeneity and 8 pg of DNA per cancer cell (based on 2.36 average tumor ploidy from the Pan-Cancer

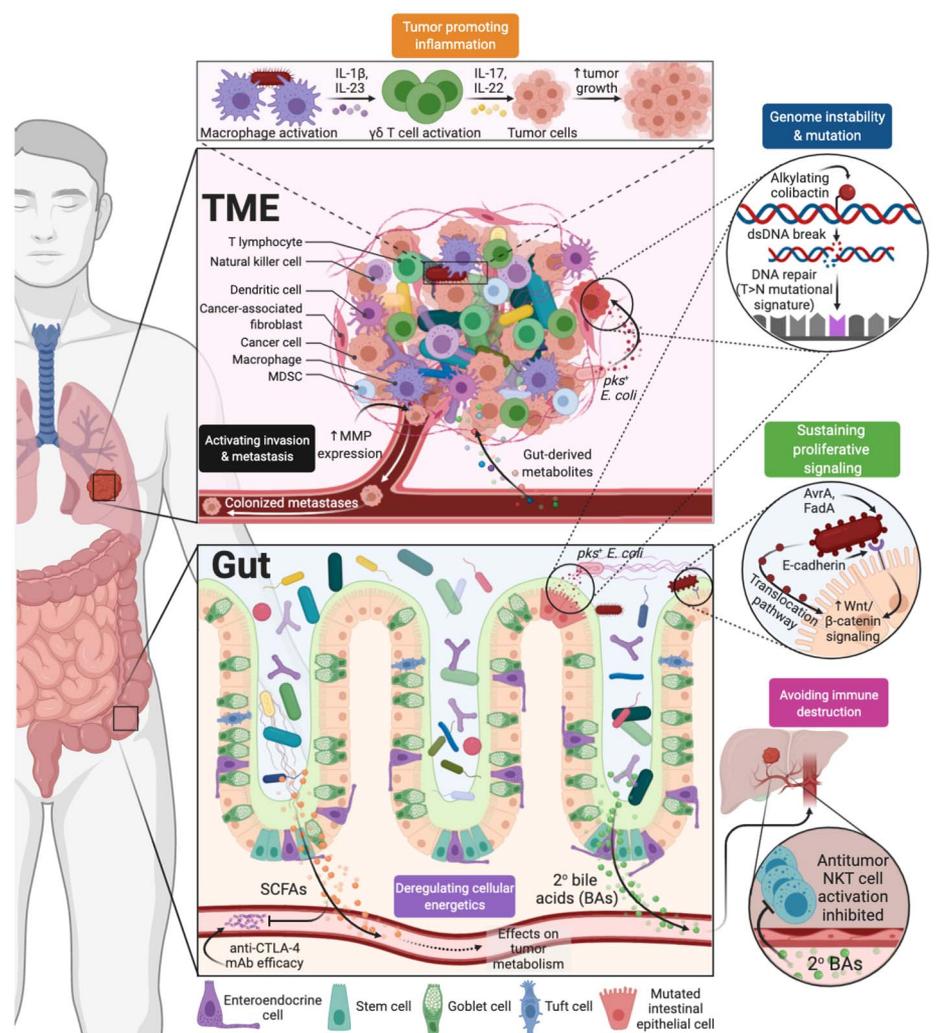


Fig. 1. Examples of the intersection of microbial mechanisms with cancer hallmarks. The cancer hallmarks are described in (10, 11). Microbiota-derived metabolites, genotoxins, and antigens influence host antitumor immunity, inflammation, energetics, cellular signaling, and metastasis. dsDNA, double-stranded DNA; mAb, monoclonal antibody; MDSC, myeloid-derived suppressor cell; MMP, matrix metalloproteinase; NKT cell, natural killer T cell; SCFAs, short-chain fatty acids.

Analysis of Whole Genomes project) (51), the Nejman *et al.* data suggest an average pan-cancer percent bacteria composition at 0.68% bacterial (bootstrapped 95% confidence interval of mean: [0.52%, 0.87%], 1000 iterations), with individual tumors ranging from no bacteria to nearly 70% bacterial by cell count (12). Applying this percent bacterial composition to three-dimensional and planar contexts equates to $\sim 10^5$ to 10^6 bacteria per palpable 1-cm³ tumor (52) or ~ 34 bacteria/mm² [assuming 5000 cells/mm² (53)], the latter of which is comparable to the average PD1⁺ T lymphocyte tumor core density of ~ 21 cells/mm² from a recent pan-cancer cohort (54). Importantly, these bacterial composition estimates remain to be confirmed in other cohorts and cancer types and validated with orthogonal methods. Furthermore, which of these microbial

taxa and functions can affect the host despite their low abundances remains unknown, as does the proportion that are merely passengers in a nutrient-rich and immunosuppressed space.

Mechanisms and interactions between the gut and tumor microbiome

Gut microbiota can regulate many functions of the tumor-bearing meta-organism, typically through immunomodulation, and putative intratumoral microbes may also be important (31, 55). Known microbial mechanisms can manipulate nonhematopoietic and hematopoietic components of the gut epithelial barrier, modulate primary and secondary lymphoid organ activities, and regulate immune tone of the tumor microenvironment (TME). We define these immune-mediated interactions

and collective feedback loops as the immunology-microbiome (IOM) axis (Fig. 2). Gut-TME cross-talk, especially in nongastrointestinal cancers, remains a key area of discovery.

The effects of gut microbiota on primary lymphoid organs

After allogeneic hematopoietic stem cell transplantation (HSCT), robust immune reconstitution governs both relapse and transplant-related patient mortality (56, 57). A recent multicenter, multinational clinical trial demonstrated that higher diversity of intestinal microbiota is significantly associated with lower patient mortality after allogeneic HSCT (58). Moreover, in an allogeneic HSCT trial that coanalyzed daily changes in patient differential blood counts with >10,000 longitudinal fecal samples, immune-reconstitution dynamics were closely linked to gut microbiota composition

(59). Links between gut microbiota, nutrition, post-transplant bone marrow (BM) and thymic cellularity, and lympho- and myelopoiesis have also been demonstrated in mouse models (57). Gut microbe depletion impairs systemic infection clearance after BM transplant and sensitizes mice to semilethal doses of radiation. Microbiota-derived compounds can protect against irradiation-induced hematopoietic injury (60–62) through production of propionate and tryptophan metabolites (63) or by releasing microorganism-associated molecular patterns (MAMPs) known to maintain BM-derived myeloid cells and neutrophil function (64, 65). This effect may be explained in part by the delivery of endogenous ligands for RIG-I (such as 3pRNA and RNA derived from viruses, phages, or bacteria) that can induce protective type I interferon (IFN-I) signaling in enterocytes and intestinal barrier repair (66). Post-transplant lymphopoiesis also depends

on energy harvest from the diet and potentially on genera whose genomic repertoires encode carbohydrate-active enzymes (57).

The effects of gut microbiota on adaptive immunity

The gut microbiota has broad effects that contribute to host immune tone at steady state and during tumorigenesis (30, 67). Anticancer therapies have demonstrated strong links between distinct commensals and protective anti-tumor T cell responses: (i) Cyclophosphamide enables *Enterococcus hirae* to translocate and stimulate pathogenic helper T cell 17 (T_H17) responses and IFN-producing CD8⁺ T cell effectors that check tumor growth in sarcoma and lung adenocarcinoma models (68, 69); (ii) in some patients with melanoma, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) blockade allows fecal relative enrichment of *Bacteroides thetaiotaomicron* and *B. fragilis*

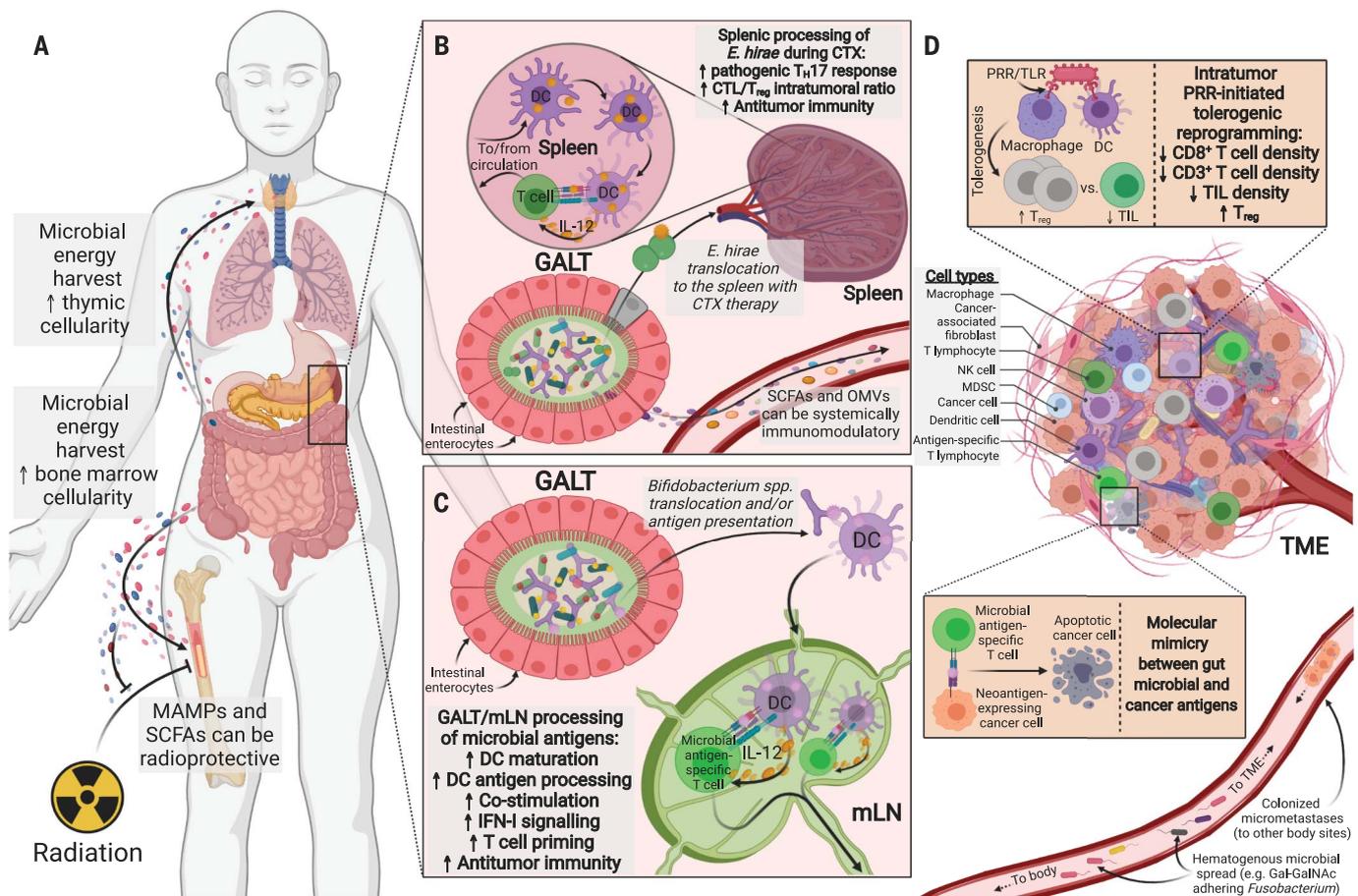


Fig. 2. Defining the IOM axis. Gut and TME microbiota regulate host metabolism and immunity, which ultimately influence antitumor immunity. (A) Gut microbial metabolites and by-products influence host lympho- and myelopoiesis, including during allogeneic HSCT and radiotherapy (59, 63). (B) Cyclophosphamide (CTX)-derived gut epithelial damage enables *E. hirae* translocation and antitumor immunity (68, 69). (C) Gut translocation of *Bifidobacterium* species or its antigens can increase IFN-I signaling and antitumor immunity (73, 76). (D) Microbes within the TME can be either immunosuppressive (often PRR-mediated) or immunogenic,

including shaping the response to immunotherapy (12, 23). Cancer (neo)antigens may share epitopes with microbes through molecular mimicry (73, 83). Microbial hematogenous spread (117, 172, 173) or colonized micrometastases (19) may complete this feedback loop that originated in the gut. CTL, cytotoxic T lymphocyte; DC, dendritic cell; GALT, gut-associated lymphoid tissue; MAMPs, microbe-associated molecular patterns; mLN, mesenteric lymph node; NK cell, natural killer cell; OMVs, bacterial outer membrane vesicles; SCFAs, short-chain fatty acids; TIL, tumor-infiltrating lymphocyte.

that mediates Toll-like receptor 4 (TLR4)- and IL-12-dependent T_H1 responses and therapeutic efficacy (70); (iii) inhibition of programmed cell death protein 1 and/or its ligand [PD-(L)1] leads to T cell priming against melanoma and is more effective when hosts harbor *Bifidobacteria* species in their microbiome (21, 71); (iv) adoptive T cell transfer efficacy against melanoma after total body irradiation depends on the composition of the microbiota, the translocation of gut bacteria, and host TLR4 signaling (72–74); (v) oxaliplatin-induced cell death of ileal enterocytes inversely governs the immunogenic *Erysipelotrichaceae* and tolerogenic *Fusobacteriaceae* proportions in the ileum, dictating the balance between antitumor follicular T helper cells and deleterious T_H17 responses in colon cancer (75).

In most of these models, dendritic cells (DCs) from the gut-associated lymphoid tissue (GALT), spleen, or tumor draining lymph node (LN) sense various commensals (*Bifidobacterium* spp., *B. fragilis*, *Akkermansia muciniphila*, *Bacteroides rodentium*, *Bacteroidales* S24-7), catalyzing immune responses through IFN-I- and IL-12-mediated pathways (17, 70–72, 75–77). Apart from providing DC adjuvants, the gut microbiota represent an antigen source that can elicit commensal-specific T cell responses systemically (55, 78). In the context of homologous self-antigens, these commensal-specific immune responses can be deleterious or protective for the host, depending on the involved peptide(s). For instance, Gil-Cruz and colleagues demonstrated how homology between *B. thetaiotaomicron*-derived β -galactosidase and host cardiac myosin heavy chain 6 could drive lethal autoimmune inflammatory cardiomyopathy (79); conversely, Nanjundappa and others reported how cross-reactivity between *Bacteroides* species-derived integrase and host islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) could hijack autoreactive $CD8^+$ T cells to instead suppress colitis (80). Recent studies have further expanded this cross-reactive homolog list to include exogenous dietary antigens, notably between gliadin epitopes and gut *Pseudomonas fluorescens*-derived succinyl-glutamate desuccinylase in the context of HLA-DQ2.5-mediated celiac disease (81). Molecular mimicry between cancer and microbial antigens has also been hinted (82) and recently studied in depth (83). H-2Kb-restricted T cell immune responses against a phage that infects distinct strains of enterococci (*E. hirae*) cross-reacted with an oncogenic driver (PSMB4). Oral administration of *E. hirae* strains containing this phage then boosted phage-specific T cell responses effective against extraintestinal tumors overexpressing PSMB4 during therapy with cyclophosphamide or anti-PD-1 antibodies (83). Similarly, T cells targeting an epitope, SVYRYGL (SVY), expressed in the commensal bac-

terium *Bifidobacterium breve*, cross-reacted with a model neoantigen, SIYRYGL (SIY), expressed by mouse melanoma B16-SIY (73). Moreover, some human T cells specific for naturally processed melanoma epitopes were found to recognize microbial peptides (83), suggesting clinical importance. However, mechanisms outside of molecular mimicry that boost antitumor immunity must also exist. For example, Tanoue and colleagues identified an 11-bacteria cocktail that increased tumor antigen-specific $CD8^+$ IFN- γ^+ T cells in the context of immune checkpoint blockade that were not cross-reactive with microbial antigens and did not originate from the colon (84).

Gut-derived metabolites can also modulate immune responses. Radiotherapy of tumor lesions was more effective when vancomycin eliminated *Clostridiales*-derived immunosuppressive metabolites (butyrate and propionate) putatively by increasing DC antigen presentation and concomitant $CD8^+$ T cell priming (72); conversely, gut microbial-derived propionate and tryptophan pathway metabolites (1H -indole-3-carboxaldehyde, kynurenic acid) were shown to provide long-term radioprotection in vivo (63). High blood butyrate and propionate levels were also associated with resistance to CTLA-4 blockade in mice and melanoma patients, with concomitantly increased regulatory T cell (T_{reg}) proportions, reduced DC and effector T cell activation, and lower responses to IL-2 (85), although they were also found to be associated with longer progression-free survival during anti-PD-1 treatment (86). Moreover, increased ex vivo outgrowth of *A. muciniphila* by prebiotic mucin decreased growth kinetics of aggressive melanoma in a gut microbiota- and T cell-dependent manner, reducing serum levels of proinflammatory and immunosuppressive IL-6, IL-1 α , IL-10, IL-17A, and IL-23 cytokines (87); notably, prebiotic inulin operated through a different mode of action, facilitating the dominance of *Bifidobacteria* species in the intestines, boosting splenic cytotoxic T lymphocyte functions, and overcoming melanoma resistance to MEK inhibitors (87).

The effects of gut microbiota on the TME

The intestinal ecosystem can influence both local and distant neoplasia by affecting their immune context, influx of myeloid and lymphoid cells, and inflammatory and metabolic patterns. Secretory components of gut microbiota can be important: For example, outer membrane vesicles (OMVs) can reprogram the TME toward a pro- T_H1 pattern (CXCL10, IFN- γ) (88), or metabolites, including butyrate and niacin, can mediate Gpr109a-dependent induction of IL-18 in colonic epithelium and suppress colitis and colon cancer (89).

Tumor-associated, NOX2-mediated, myeloid cell ROS production is reduced by antibiotic

administration or germ-free status, reducing oxaliplatin's capacity to mediate early tumor genotoxicity (90). Similarly, commensal microbiota primed tumor-associated innate myeloid cells for tumor necrosis factor- α (TNF α) (IL-1 β , IL-12, and Cxcl10) production in response to anti-IL-10R/CpG-ODN treatment, and antibiotics, germ-free, or TLR4 $^{-/-}$ status attenuated this response and the TNF-dependent early tumor necrosis (90). Supporting the adjuvant role of commensals against developing cancers, pasteurized *A. muciniphila* or its pili-like TLR2 agonist blunted azoxymethane-induced colitis and colon carcinogenesis by inducing TNF α -producing cytotoxic T lymphocytes in mesenteric lymph nodes (mLNs) that eventually reached the colonic mucosa (91). Spontaneous gut bacterial translocation in *Tet2* $^{-/-}$ mice also drove preleukemic myeloproliferation (PMP), which leads to leukemia if unchecked, in an IL-6-dependent manner (47). PMP was reversible with antibiotics and abolished in germ-free mice, suggesting new clinical management opportunities. However, an intact gut microbiome was later shown to be necessary to prevent leukemia progression in genetically predisposed mice (92).

Nonhematopoietic components of the intestinal mucosa are also linked to the TME (77). Gene-deficient mice and BM chimeras identified a role for RNF5, an E3 ubiquitin ligase, in immunosurveillance of severe melanoma. *Rnf5* $^{-/-}$ mice exhibited decreased secretion of antimicrobial peptides and increased cell death in the ileal crypts, causing changes in intestinal microbiota community composition. This bowel injury amplified mobilization of CCR7-expressing DCs to Peyer's patches, mLNs, and melanoma-draining LNs, increasing IFN- γ -producing T lymphocyte tumor infiltration. Confirming a *Rnf5* $^{-/-}$ -specific microbial effect, cohousing *Rnf5* $^{-/-}$ mice with wild-type mice, or administering antibiotics, restored tumor aggressiveness, whereas oral gavage with 11 species overrepresented in *Rnf5* $^{-/-}$ animals (*Bacteroides* and *Parabacteroides* spp.) into germ-free wild-type mice recapitulated tumor immunosurveillance (77). In another study, oxaliplatin-induced caspase 3/7-dependent ileal apoptosis of crypts coincided with immunogenic bacteria dominance in the ileal mucosa (75). These commensals regulated follicular T helper cell priming in mLNs, culminating in B cell activation, immunoglobulin production, and infiltration of colon cancers with tumor-infiltrating lymphocytes (TILs) in mice and patients. Anti-CTLA-4-induced gut barrier dysfunction was also critical for systemic translocation of *Bifidobacterium*-derived inosine, in turn promoting T_H1 activation and antitumor immunity by agonizing T cell-specific adenosine 2A receptor ($A_{2A}R$) signaling in the context of DC costimulation (93). These examples illustrate that barrier injury is accompanied by

a deviation of the local microbiome or translocation of microbial metabolites that, in turn, mobilizes DCs to and outside the GALT and contributes to tumor bed infiltration by activated helper or cytotoxic T cells.

The TME comprises not only stromal, tumor, endothelial cells, and hematopoietic progenitor-derived immune components but also a dense network of adrenergic nerve fibers that influence oncogenesis in brain and non-brain cancers (94–97). Interestingly, enteric nervous system neurons are both affected by the gut microbiota and functionally tuned according to their location in the gut. A subset of microbiota-responsive neurons could influence metabolic control independent of the central nervous system (98). These findings suggest intimate relationships between mucosal or tumoral commensals and tumor innervation that need further study.

Gut microbiota-mediated effects on anticancer drugs

Gut microbes are intimately involved in the biotransformation of xenobiotics, including cancer drugs, with unintended consequences for clinical cancer control (99). For example, in prostate cancer, abiraterone acetate (AA) was used as an energy source by *A. muciniphila* and inhibited *Corynebacterium* species relying on AA-inhibited androgens for growth (100). Because *A. muciniphila* is anti-inflammatory and *Corynebacterium* species are proinflammatory, this change in their relative abundances increased the pharmacologic efficacy of AA therapy. *A. muciniphila*'s immunomodulatory effects (78), including association with responders during PD-1 blockade (17), has prompted speculation that increased *A. muciniphila* may explain the efficacy of AA in androgen-independent prostate cancer (100), although this remains to be tested in large patient cohorts. These types of bidirectional drug-microbiota feedback loops warrant further study.

Intratumor microbiota effects on the TME

Mechanistic studies of live microbiota within diverse tumor types have been limited, particularly outside the aerodigestive tract, but many of their effects on the TME appear to suppress local antitumor immunity (15, 23, 34, 46, 75, 101, 102). Additionally, intratumor microbes have been reported to have cancer-specific effects on (i) gastrointestinal and urinary tract mutagenesis through secreted genotoxins, most notably *pks*⁺ *Escherichia coli*-derived colibactin and *B. fragilis*-derived toxin (22, 37, 38, 40, 41); (ii) CagA-mediated or IL-17-producing $\gamma\delta$ T cell-mediated inflammation in stomach and lung cancers, respectively (18, 103); (iii) chemoresistance through direct microbial metabolism (cytidine deaminase degradation of gemcitabine) in pancreatic cancer or indirect

amplification of cancer cell autophagy in colorectal cancer (14, 104); (iv) tumor proliferation through fungal activation of the host's C3 complement cascade in pancreatic cancer (20); and (v) metastasis through up-regulation of tumor matrix metalloproteinases in breast cancer or reduction of tumor immunosurveillance in lung cancer (34, 46). Immunologically, intratumor microbes often create tolerogenic programming through pattern recognition receptor (PRR) ligation with lower proportions of TILs, including CD8⁺ T cells, and occasionally more CD4⁺CD25⁺FoxP3⁺ T_{regs}, as observed in colorectal, pancreatic, breast, and lung cancers (18, 23, 34, 46, 75, 101, 102). However, in certain cases, injection of intratumoral bacteria or their antigens may conversely provide immunostimulatory effects, as demonstrated by Coley's toxins and recent developments in bacterial cancer therapy (5, 105, 106). In breast cancer, experiments comparing SCID-beige and C57BL/6 mice with intratumor *Fusobacterium* suggested lymphoid-lineage cells as key mediators of intratumor microbiota-derived effects on tumor immunosurveillance (34). There are also associations between intratumor microbiota and immunogenicity, including differential melanoma immunotherapy response and triple-negative breast cancer associations, but their underlying mechanisms remain uncharacterized (12).

Extraintestinal barriers and cancer microbiota

Given that the intestinal barrier offers the largest host-microbial interface and greatest microbial diversity, investigations on the potential impact of the microbiota in oncogenesis or cancer prognosis have primarily focused on this barrier (107). These studies could unveil cause-effect relationships between gut microbial composition changes and compromised tumor immunosurveillance, even in extraintestinal malignancies. However, it is noteworthy that extraintestinal cancers can develop within tissues that harbor their own microbiome and may play a role in the exacerbation of neoplasia (12, 18, 25, 46).

For instance, the lungs' surface approximates 1 m² per kg of body weight and is not sterile (108). Experimental evidence in oncogene-driven autochthonous lung cancer models in mice unveiled that local commensals may be perturbed by carcinogenesis, triggering an inflammatory cross-talk between alveolar macrophages and IL-17-producing lung resident $\gamma\delta$ T cells contributing to tumor progression (18). The clinical importance of this observation has been recently brought up in 83 lung cancer patients (25). Tsay *et al.* highlighted that microaspiration of supraglottic commensals in lung cancer patients can affect response to therapies and overall survival, owing to a T_H17-mediated exacerbated in-

flammation corollary to immune checkpoint inhibition (25).

Skin is also recognized as our largest and outermost organ, maintaining host homeostasis through tight interconnections between its resident microbes, keratinocytes, and skin immune components through metabolic, innate, and cognate immune responses (109). Compositional shifts in the skin microbiota appear to influence nonmelanoma skin carcinogenesis (110). Similarly, cervical cancer caused by persistent high-risk human papillomavirus infection is often associated with a deviated cervical microflora (111, 112). The intertwined and/or interkingdom relationships between commensals and virus-associated cancers and their synergistic effects on tumorigenesis need further study, and exploration of cancer-microbe interactions at other extraintestinal barriers is warranted.

Cancer microbiome diagnostics

Variation in human microbiome composition among body sites (113) contrasts with stable human genetics that exhibit only minor variation resulting from somatic mosaicism and clonal hematopoiesis (114). Because both host tissues and microbiota are affected by carcinogenesis, the genetic heterogeneity of microbes may provide an opportunity to diagnose and locate disease. For example, a blood-derived TP53 mutation can indicate host cancer status but implicates >25 cancer types (115); conversely, *Streptococcus gallolyticus* (formerly *S. bovis*) bacteremia can reflect host cancer status and type (colon cancer) based on its gastrointestinal origin (116, 117). Many challenges exist for microbial-based diagnostics, including low biomass relative to host and confounding from reagent or environmental contaminants. Many questions about their specificity, prevalence, and stability during cancer treatment or utility during antibiotic administration remain to be answered and must be addressed before clinical deployment.

Nearly all microbial-based cancer diagnostics are sequencing-based and have focused on tumors within the aerodigestive tract (31), such as colorectal (118–121), pancreatic (122, 123), and lung cancer (124–126). It was only recently suggested that cancer types outside of the aerodigestive tract, such as breast or brain cancer, may also harbor microbiota with distinctive compositions. Nejman *et al.* (12) and Poore *et al.* (13) suggest distinct intratumoral microbiomes among >30 cancer types (Fig. 3), proposing their applicability to blood-based diagnostics and providing imaging evidence of these microbes' intratumoral spatial distribution and intracellular localization in seven cancer types, although imaging evidence remains lacking for most cancer types.

Combining multiregion 16S rRNA amplicon sequencing, qPCR, immunohistochemistry

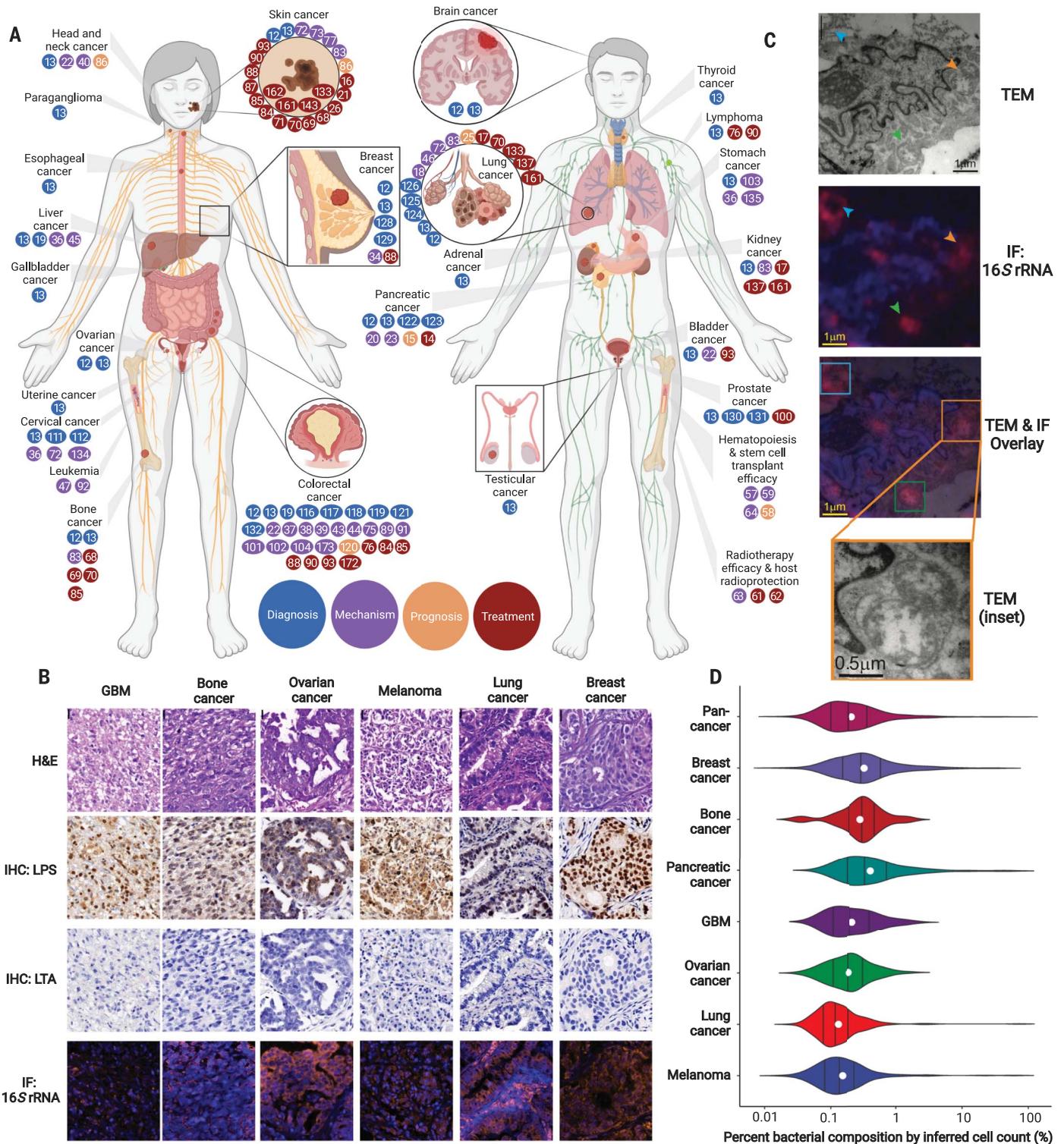


Fig. 3. Current landscape of the cancer microbiome. (A) Body diagram of all cancers currently linked to microbiota, where the colored dots reflect reference numbers and are colored according to the major theme of the referenced paper (diagnosis, mechanism, prognosis, or treatment). Dots are included based on existing preclinical and clinical data. (B) Representative histology, immunohistochemistry (IHC) for lipopolysaccharide (LPS) and lipoteichoic acid (LTA), and immunofluorescence (IF) for bacterial 16S rRNA in six cancers. H&E,

hematoxylin and eosin; GBM, glioblastoma. (C) Representative transmission electron microscopy (TEM) images with overlaid 16S rRNA immunofluorescence of intracellular bacteria (arrows) in breast cancer. (D) Estimation of tumor percent bacterial composition across seven cancer types, assuming tissue homogeneity and 8 pg of DNA per cancer cell. Black lines depict distributional quantiles (25%-50%-75%); white dots reflect averages. Images in (B) to (D) are adapted from Nejman *et al.* (12).

[lipopolysaccharide (LPS), lipoteichoic acid (LTA)], immunofluorescence (16S rRNA), cultivation, and electron microscopy, Nejman *et al.* (12) surveyed 1010 tumors for bacteria across melanoma, lung, ovarian, glioblastoma, pancreas, bone, and breast cancers. They included 811 experimental controls, covering DNA extraction, PCR amplification, and paraffin embedding, which identified and removed 94.3% of bacteria as contaminants. Examining the residual 528 bacterial species revealed substantial differences in composition, diversity, and inferred metabolic functionality between cancer types. Histologic imaging revealed heterogeneous microbial spatial distributions (Fig. 3B) and their frequent intracellular localization in cancer and immune cells (Fig. 3C). As described above in the “Overview of the cancer microbiome” section, qPCR estimated the number of bacteria per tissue section, which we have graphically depicted as percent bacterial composition per cancer type, assuming tissue homogeneity and 8 pg of DNA per cancer cell (Fig. 3D). Applying their pipeline to a melanoma immunotherapy cohort suggested microbiome differences between responders and nonresponders, but not yet a mechanism. Because bacteria were cultured from only five human breast tumors, the widespread viability of intratumoral bacteria from this study was unclear, particularly in cancers with reportedly fewer bacteria. However, other studies have indeed shown cultivable bacteria in breast (127–129), lung (18), prostate (130, 131), pancreas (14, 15), and colon cancers (19, 132), suggesting broad microbial viability. Still, basic questions remain about the functional impacts of these intratumoral microbiota and whether they are parasitic, symbiotic, or passive passengers, and a biopsy specimen is required for analysis, limiting its diagnostic utility.

Poore *et al.* (13) took a different approach by harvesting all treatment-naïve whole-genome and transcriptome studies from The Cancer Genome Atlas (TCGA) ($n = 18,116$ samples; 33 cancer types) to study bacterial, viral, and archaeal nucleic acids. Because no experimental controls were available, they filtered out historically known reagent contaminants and inferred other contaminants using per-sample DNA and RNA concentrations; these steps removed up to 91.3% of microbial taxa. Machine learning revealed intratumor, cancer-specific microbial signatures. Because colon cancer is epidemiologically linked to clinical bacteremia (116, 117), they explored TCGA blood-derived normal samples ($n = 1866$ samples) for cancer-specific microbial DNA and reported highly accurate cancer discrimination. They validated this blood-based diagnostic approach by comparing plasma-derived cell-free microbial DNA from 100 patients with lung, prostate, or melanoma cancers to those from 69 HIV-negative, healthy patients while implementing neces-

sary experimental contamination controls. Although closer to a practical diagnostic approach, the absence of experimental controls in TCGA, sole reliance on deep sequencing data without orthogonal approaches, and current lack of explainable mechanism(s) by which microbial DNA enters into and survives circulation limits these findings. We speculate that the intracellular bacteria in cancer and immune cells identified by Nejman *et al.* (12) may provide one source, though this remains to be demonstrated. A rigorous evaluation alongside blood samples from patients with non-lethal bodily infections, septic patients, and

patients receiving antibiotics during cancer care are necessary preconditions to broad clinical utility.

Modulation of the cancer microbiome

The associations between certain gastrointestinal microbiota and the activity of systemic lymphoid tissues have stimulated interest in microbial modulation as a powerful immunotherapeutic modality. If intratumoral microbiota are eventually verified to be prevalent and immunologically active across most patients, as preliminary data suggest (12, 13), such interventions must account for microbial

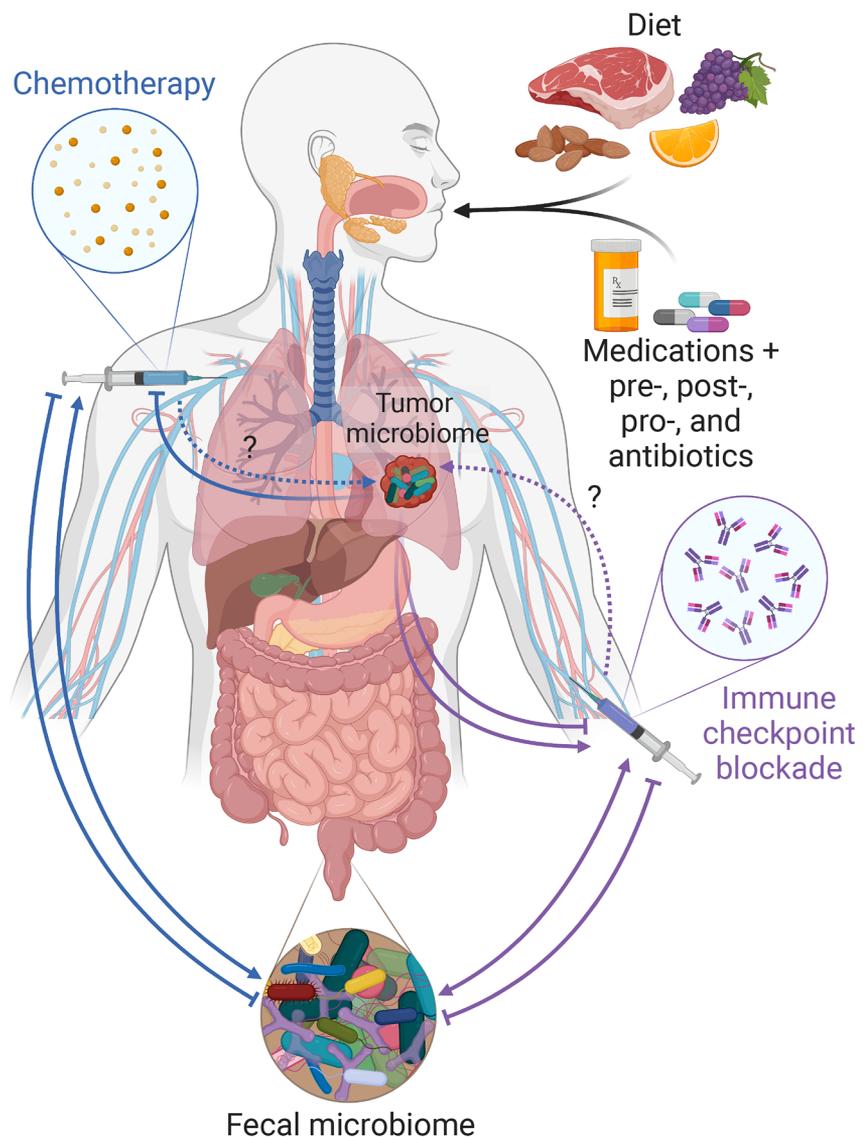


Fig. 4. Considerations when modulating the endogenous cancer microbiome. Diet, medications, and prebiotics, probiotics, and antibiotics all have the capacity to modify the gut and tumor microbiomes. Bidirectional influences may exist between these microbiomes and cancer therapies (chemotherapy and immunotherapy). For instance, chemotherapy can cause compositional changes in the gut microbiome, which in turn enhance treatment efficacy (75); in other cases, chemotherapy may be degraded by microbes (14). Thus, modification of the gut and/or tumor microbiomes may be advantageous for one modality of therapy while disadvantageous for another. Dotted arrows denote gaps in the literature.

niches and their cross-talk (Fig. 4). These dynamics sometimes appear related; for example, modulation of gut microbiota influences the composition of the intratumoral microbiome in pancreatic cancer, presumably through pancreatic duct communication (15, 20, 23). However, in other cases, these changes are incongruent; for instance, antibiotics appear to abrogate immunotherapy response by inhibiting the gut microbiome (133), but paradoxically they improve immunotherapy efficacy by upregulating PD-1 expression when eliminating the pancreatic intratumoral microbiome (23). These complexities necessitate more in-depth mechanistic studies of modulation approaches and better clinical understanding before applying prebiotics, probiotics, postbiotics, and antibiotics in the setting of cancer.

Antibiotics and the cancer microbiome

The use of antimicrobial therapy in cancer is limited to addressing or preventing known microbial carcinogens. This includes treating *Helicobacter pylori*-derived gastric lymphomas with triple or quadruple antibiotic therapy, administering direct-acting antivirals against active hepatitis C virus, and vaccinating against major human papillomavirus serotypes and hepatitis B virus to prevent urogenital, cervical, head and neck, and liver cancers (36, 134, 135). Excluding antibiotic-derived chemotherapies (e.g., doxorubicin), there is circumstantial and conflicting evidence for the use of antibiotics in solid tumors. Several studies in lung, colon, and pancreatic cancer suggest that eliminating intratumoral microbiota can check tumor-promoting inflammatory processes, reduce cellular proliferation, or convert a tolerogenic TME to an immunogenic one (18, 19, 23, 46). However, increasing clinical evidence suggests that systemic antibiotics abolish immune checkpoint blockade efficacy and decrease patient survival (133, 136, 137). In hematologic malignancies, preclinical evidence suggests a careful balance, where either antibiotics or gut bacterial translocation can trigger leukemic progression in genetically predisposed hosts (47, 92).

Prebiotics, postbiotics, and dietary interventions to modify the microbiome are also promising. Dietary effects on cancer were recently reviewed in detail, with many epidemiological associations but few causal mechanisms (138). Difficulties in dietary data collection have impeded strong conclusions, but metabolomic data that can reveal dietary intake and concomitant small-molecule effectors may help in the future. Prebiotics (molecules that promote growth of beneficial microbes) such as resistant starch, inulin, and mucin are promising in preclinical models, improving antitumor immunity and therapy response in melanoma and colon cancer (87), and are in clinical trials

(e.g., NCT03870607, NCT03950635). Experimental evidence of postbiotic compounds (microbial-derived molecules) is limited in cancer, but they may provide advantages through defined composition and manufacturing reproducibility (139).

Gut microbiota can also be modulated in cancer through fecal microbiota transplantation (FMT), administration of defined microbial consortia, and commercial probiotics. FMT treats *Clostridium difficile* (now *Clostridioides difficile*) colitis effectively (140), with some efficacy in the treatment of immunotherapy-associated colitis (141). The long-term efficacy

and stability of FMT remain unknown (142). Targeting gut microbes clinically is complicated by factors such as antibiotic preconditioning, administration route, frequency of modulation, and dietary recommendations (142). Ongoing clinical trials suggest that FMT from donors responsive to immunotherapy may enhance antitumor immune and potentially clinical responses [NCT03353402 (143)]. Additional clinical trials are evaluating the impact of transferring microbial consortia, ranging in complexity from monoclonal bacterial strains to multiplexed consortia. Few commercially available probiotic formulations have been

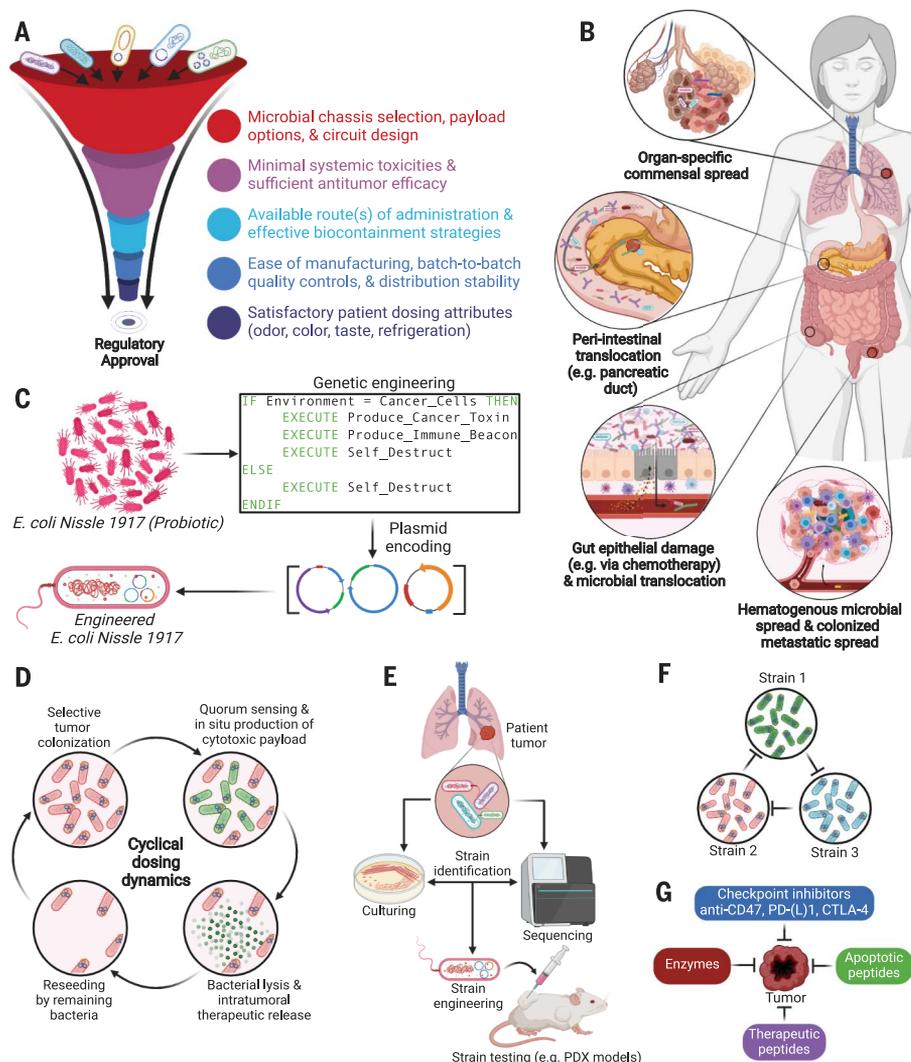


Fig. 5. Synthetic biology for exogenous cancer therapeutics. (A) Regulatory considerations for engineering bacteria against cancer (151). (B) Diverse sources of intratumoral bacteria include organ-specific commensals (18, 19, 25, 46, 112), gut communication (15, 20, 23), hematogenous spread (172, 173), and intrametastatic spread (19). (C) Some probiotics, such as *E. coli* Nissle 1917, possess strong safety records (174), have been shown to naturally migrate to solid tumors in animal models, and can be programmed to produce and deliver therapies from within solid tumors (155). (D) Complex population dynamics can be engineered to generate the cyclical delivery of therapeutics (155, 156, 175). (E) Future efforts will likely center on engineering and testing strains that are found naturally in patient-specific tumors. PDX, patient-derived xenograft. (F) Engineered ecologies can be designed to create tailored, tumor-specific therapeutic cocktails (176, 177). (G) Multiple drug payloads can be encoded by one or more engineered strains against tumors.

tested for impacts on antitumor and systemic immunity, with certain formulations actually increasing tumorigenesis (144). In critically ill patients, commercial probiotic use may even cause bacteremia (145). Therefore, indiscriminate administration of commercially available probiotics in cancer patients should be discouraged.

Cancer therapy using exogenous microbiota

Major strides have been made toward engineering exogenous bacterial and viral agents for cancer therapy, particularly as powerful immunotherapy options or neoadjuvants. Two such agents have U.S. Food and Drug Administration approval: oncolytic viral therapy for advanced melanoma using talimogene laherparepvec (T-VEC) (146) and bacterial cancer therapy for high-risk, nonmuscle invasive bladder cancer using live-attenuated *Mycobacterium bovis* (BCG vaccine) (147). Because oncolytic viruses are noncommensals and have been reviewed elsewhere in detail (148–150), we focus our attention on bacterial cancer therapies (BCTs). Though historically contentious, BCT is regaining attention through synthetic biology techniques that programmatically limit systemic toxicities while enhancing regional antitumor immunity (105, 106). Regulatory challenges for BCT agents are considerable (Fig. 5A), and despite ongoing clinical trials (e.g., NCT04167137), they have yet to be commercially surmounted (151).

Engineered microbes as cancer drugs

Natural bacterial mechanisms for tumor tropism are numerous (Fig. 5B), with intratumoral or intravenous injection often leading to ~10,000-fold accumulation in tumors relative to matched liver, spleen, and lung tissues (152, 153). This affinity for tumor tissue provides a creative drug chassis and natural bridge from synthetic biology to cancer therapies, whereby cytotoxic payloads can be encoded for programmed delivery by tumor-homing bacteria (Figs. 5, C to G). Thus far, genetically attenuated, auxotrophic, and inducible versions of *Escherichia*, *Bifidobacterium*, *Listeria*, *Shigella*, *Clostridium*, *Lactococcus*, *Vibrio*, and *Salmonella* species have been engineered and have shown antitumor efficacy in preclinical models with intravenous, intratumor, and oral delivery routes (147). Although some approaches are based on intracellular delivery of drugs through phagocytic uptake of bacteria, others program bacteria to act as “intratumoral bio-reactors” that continuously produce and release payloads extracellularly as part of colonization. An interesting general approach implements engineered bacterial lysis, which enables anti-tumor protein production or release only when a predefined population density of bacteria is reached (154–157). This dramatically reduces bacterial colony size and prevents systemic

toxicities. Din and colleagues were the first to demonstrate how nonpathogenic *E. coli* and *Salmonella* could be engineered to lyse at a threshold population density, releasing a chemokine, hemolysin, or pro-apoptotic protein, or all three, into the TME at desired periodic intervals (155). The drugs are delivered cyclically as the bacterial population is programmed to generate growth-death-regrowth cycles. Chowdhury *et al.* then used this design to produce and release an antibody-fragment nanobody against CD47, which tumors can overexpress to inhibit DC phagocytosis (156). Intriguingly, this stimulated a tumor antigen-specific CD8⁺ T cell response that prevented metastasis and mediated an abscopal effect, which regressed distal noninjected tumors as well. This approach further precluded host anemias and thrombocytopenias that are usually seen with systemic CD47 antagonism, suggesting a clinical opportunity. If intratumoral bacteria prove to be prevalent across various cancer types, lysis circuit designs may also provide an opportunity to flexibly engineer patient-specific, tumor-specific commensal strains (Fig. 5E) or several strains in feedback with each other (Fig. 5F) to regulate payload release. Given the many encodable cytotoxic payloads (Figs. 5G), a clear demonstration of BCT clinical efficacy with minimal systemic toxicities could considerably increase the cancer therapy armamentarium.

Outlook for the cancer microbiome

The past 15 years of microbiome research provide intriguing, though still controversial, evidence of the relationships between microbes and cancer and the nuances of these relationships. Few microbes directly cause cancer, but many more seem complicit, and, perhaps

counterintuitively, several promote host anti-tumor immunity. This complexity may reflect shared evolutionary dynamics between the host's immune system, its commensal microbiota, and tumorigenic processes that we are just beginning to uncover (158–160).

A substantial literature gap still separates clinical observations and clinical interventions targeted at microbiota in cancer. Although gut microbiota modulation in murine immunotherapy models provides tantalizing results, they have not yet translated to commercial therapeutic interventions in humans. Moreover, observations in humans and mice of gut microbes that stratify therapy response, particularly immunotherapy (16, 17, 21, 26), have not uncommonly shown varying taxonomic differences that persist despite uniform bioinformatic reanalyses, although there is greater concordance when examining functional profiles (161, 162). Thus, many of the key problems that plagued researchers in the early 20th century—contamination, irreproducibility, patient toxicities—remain challenges today for microbially based cancer diagnostics, prognostics, and exogenous microbial therapeutics. Additional cohorts with carefully curated samples to limit and mitigate potential contamination are needed to help characterize and understand the impact of intratumoral microbes on carcinogenesis, cancer progression, and therapy response. Other efforts are needed to examine nonbacterial relationships with cancer, gastrointestinally and intratumorally, and their functions, particularly in relationship with known bacterial functions. Further consortium-level efforts are necessary to assess the quantitative impact of technical variables (e.g., DNA extraction, sample handling, bioinformatic protocols) on cancer microbiome

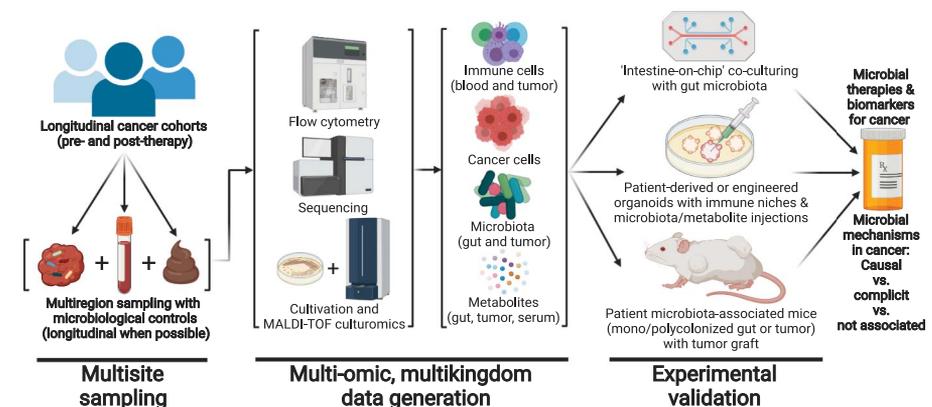


Fig. 6. Study design for characterizing cancer-associated microbiota and their functional impacts.

Opportunities exist to perform large-scale identification of the presence and function of cancer-associated microbiota, beginning with longitudinal cohorts and multiregion sampling. Existing tools can be used to gather multi-omic information on host immune cells, cancer cells, microbiota, and metabolites (51, 178, 179). In vitro and in vivo disease models of a patient's tumor and intestine can then be used to verify or rebut the predicted functional impact and mechanism(s) of a given microbe (or its metabolites) and its causality in carcinogenesis (160, 171). MALDI-TOF, matrix-assisted laser desorption ionization–time-of-flight mass spectrometry.

data and guide the selection of “gold-standard” pipelines, analogous to the Microbiome Quality Control consortium’s analysis of fecal amplicon sequencing among 15 laboratories and nine bioinformatic protocols (163).

Many of these challenges would be aided by a multicenter, longitudinal, concerted effort to study microbiota in cancer, analogous to TCGA’s role in elucidating the somatic mutation landscape, with joint tumor, blood, and stool collection; multi-omic data generation; and incorporation of experimental contamination controls (Fig. 6) (164). Concurrent meta-analyses of existent cancer datasets with uniform in silico host depletion, decontamination, taxonomy calling, and functional profiling may be able to identify global microbial drivers in cancer pathogenesis and treatment despite technical variation between individual studies (13, 118, 119, 165–167). Completion of microbiota modulation trials are additionally crucial for guiding clinical applications and increasing the cancer therapy armamentarium (142), with new evidence demonstrating that modulation of the gut microbiota using FMT in immunotherapy-refractory melanoma patients is associated with clinical responses and changes in the gut and tumor immune microenvironment (143, 168). In-depth functional analyses at community and per-microbe scales are likely necessary to elucidate microbial-immune-cancer cell mechanistic interactions, and emerging spatial multi-omic tools may prove invaluable here (169, 170). Engineered organoids with immune and microbiota niches or metabolites may further help validate or refute microbial causality or complicity in carcinogenesis, as recently demonstrated by colibactin mutagenesis studies (22, 171). Although many challenges remain, building a better understanding of the roles of microbes in cancer may enable a powerful new toolkit for improving patient care.

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