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## Uncovering the role of the gut microbiota in immune checkpoint blockade therapy: a mini-review

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### Abstract

In recent years, the microbiota has been implicated as a key factor associated with both response and toxicity from immune checkpoint blockade therapy. Numerous studies have been published that specifically highlight the importance of the microbiome as a distinct influencer of anti-PD-1/PD-L1 and anti-CTLA-4 activity in cancer patients, but a full understanding of mechanisms behind these interactions has yet to be achieved. With greater insight into how the microbiome can modulate immune checkpoint blockade comes the potential to target the microbiome to improve

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response rates and minimize toxicities. This mini-review looks at noteworthy studies that have explored the relationship between the microbiome and immune checkpoint blockade response and toxicity in both pre-clinical and clinical studies, with an emphasis on current hypotheses regarding mechanisms of action and potential microbiome-targeted therapeutic strategies under development.

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## Introduction

Immune checkpoint blockade therapy (ICBT) has revolutionized the treatment of cancer and is now a critically important tool used to combat an increasing number of cancer types. As of the writing of this review, ICBTs are currently approved by the Food and Drug Administration (FDA) to treat melanoma, non-small cell lung cancer (NSCLC), renal cell carcinoma, head and neck squamous cell carcinoma, Hodgkin's lymphoma, urothelial carcinoma, small cell lung cancer, esophageal squamous cell carcinoma, cervical cancer, primary mediastinal large B-cell lymphoma, MSI-H/dMMR colorectal cancer, hepatocellular carcinoma, Merkel cell carcinoma, triple-negative breast cancer, and cutaneous squamous cell carcinoma. Additional indications are being actively investigated in ongoing clinical trials (1).

The most successful ICBT-based strategies have targeted anti-programmed death 1 (PD-1)/programmed death-ligand 1 (PD-L1) or anti-cytotoxic T lymphocyte-associated antigen (CTLA-4), using monoclonal antibodies. These drugs are not thought to be directly tumoricidal, but rather mediate anti-tumor effects indirectly by inhibiting T-cell suppression mechanisms and thus enhancing the body's endogenous immune response against cancer cells (2).

Despite proven overall efficacy for many cancers, individual therapeutic responses vary substantially, as do autoimmune toxicities. Researchers and clinicians have identified biomarkers that can serve as predictors of ICBT response, including unique gene expression patterns, mutational burden, presence of immunogenic tumor antigens either at the cancer site or circulating throughout the body, and expression patterns of ICBT targets (i.e. PD-1/PD-L1/CTLA-4) and their ligands on T-cells, tumor cells and tumor stroma (3). Included among these factors that have shown potential for predicting clinical outcomes is the microbiome. Interest in investigating this aspect of ICBT biology has been high, particularly because unlike most other predictors, the microbiome can be potentially modulated.

The human body harbors trillions of resident microbes that play a variety of roles in human health and disease, many of which are relevant to cancer. These include competitive exclusion of pathogens, education of the host's immune system regarding appropriate responses to self- and non-self antigens, and a variety of additional mechanisms of immunomodulation (4). Pioneering studies have shown that interactions between commensal bacteria and the immune system can impact on tumorigenesis, particularly at mucosal sites where bacteria and epithelial cells interface (5).

There are significant challenges to identify associations between the microbiome and clinical outcomes. These include considerable heterogeneity in the microbiome of patients and healthy individuals, as well as the complexity of the microbiome itself. Despite these, researchers have begun exploring the potential impact of the microbiome on many aspects of cancer treatment, including ICBT. This review will focus on studies examining the impact of the gut microbiome in ICBT response and related toxicities, as well as recent advances that could develop into potential therapeutic strategies.

### Microbiota mediates response to ICBT

**Pre-clinical Mouse Models**—Pre-clinical mouse models have been a critically important tool for studying the potential effects of microbes on ICBT response. A pioneering study utilized mice purchased from different vendors as a convenient method of obtaining different baseline microbiomes. These investigators found that vendor source had a significant impact on the responsiveness of melanoma to anti-PD-L1 treatment, which led the researchers to explore gut microbiome differences as a potential cause. They were able to identify *Bifidobacterium* as a bacteria of interest (6). Mechanistic studies pointed to dendritic cell modulation leading to greater CD8+ T cell priming as a potential mediator. Another instrumental study used mouse models of sarcoma (MCA-205) and melanoma (RET) to show that the use of antibiotics during ICBT resulted in shorter progression-free survival after recognizing similar associations in patient cohorts. The abundances of several bacterial species (i.e. *Akkermansia muciniphila*, *Ruminococcus spp*, *Eubacterium spp*, *Alistipes spp*) were enriched in the stool of patients that responded favorably to ICBT. For clinical relevance, 16S sequencing revealed the loss of *Bacteroidales* and *Burkholderiales* and increase in *Clostridiales* following CTLA-4 antibody treatment in patients. Re-introduction of select species from these groups and adoptive transfer of CD4+ T-cells in mice showed restoration of respective phenotypes (7). Re-introduction of responder/non-responder stool via fecal microbiota transplant (FMT) into the mouse models resulted in the recapitulation of the disease phenotypes seen in patients. In a separate study, an anti-CTLA-4 response that also utilized the MCA-205 sarcoma model in mice, select species of *Bacteroides* (i.e. *B. thetaiotaomicron* and *B. fragilis*) were shown to confer tumoricidal effects upon re-introduction into mice that received an antibiotic cocktail (8). These studies highlight the complex network of relationships that are all in play when it comes to the microbiota and its effects on immune checkpoint blockade therapy. Importantly, recent evidence suggests that specific taxa may be far less important than their functional role, thus focusing on functional relationships rather than taxonomic characterization will likely be more fruitful as such studies evolve.

**Clinical**—These pre-clinical studies laid the foundation for subsequent observational studies in clinical cohorts. Much of the work to elucidate the role of the gut microbiota in immune checkpoint blockade response has been done in the context of metastatic melanoma (MM). In a large cohort study of MM patients that received anti-PD-1 immunotherapy, distinct differences were seen in the microbial composition of responders vs. non-responders following 16S sequencing of stool. A favorable response was predicted by increased abundance of certain families of bacteria (i.e. *Clostridiales/Ruminococcaeae*) and the poor response was shown to be associated with the presence of *Bacteroidales* (9). Similar to

previously mentioned studies, fecal microbiota transplants (FMTs) into germ-free murine melanoma resulted in a transfer of phenotype to responder/non-responder accordingly (9). In a separate study, MM patients treated with ipilimumab (anti-CTLA-4) with high levels of *Firmicutes* at baseline had longer overall survival which was suggested to be related to the lower levels of regulatory T-cells and decreased serum concentrations of IL-6, IL-8, IP-10, and TNF- $\alpha$ , common markers of inflammation. Contrarily, patients showing greater OTUs for *Bacteroides* at baseline were associated with poor response to anti-CTLA-4 (10). These differences in microbial composition between responders and non-responders regarding anti-PD-1 efficacy in metastatic melanoma patients are potentially mediated by increases in CD8<sup>+</sup> cytotoxic effector T-cells and marked decrease in regulatory T-cells, which is consistent with previous findings. And, in a separate cohort of 39 melanoma patients, responders to both combination and single-agent common immunotherapies gave stool samples that were subjected to metagenomic shotgun sequencing and metabolomics. Results showed significant enrichment of *Bacteroides caccae* in all types of ICBT with additional organisms implicated depending on the immune checkpoint blockade agent given. Moreover, Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed high levels of anacardic acid, a plant derivative previously shown to stimulate neutrophils and macrophages, in responders, suggesting metabolic components as a regulator of ICBT response (11). Based on these results, microbial diversity and composition have dramatic roles in ICBT response and the effects can be seen in a short amount of time. Recently, the bacteria that reside within tumor sites have also been implicated in direct modulation of response to different treatment options, including ICBT. In a recent study, researchers showed that long term survivors of pancreatic adenocarcinoma (PDAC) have higher tumor microbial alpha diversity (e.g. the number of species present) and transplantation of fecal contents from patients into murine PDAC tumor models showed direct gut microbiota crosstalk with tumor microbiome (12). These results only begin to explore the potential effects of the tumor microbiome but represent important strides for the field; additional studies looking at the impact of tumor microbiome specifically on ICBT are necessary next steps. Numerous ongoing studies seek to identify more species of interest that may predict or alter the response to ICBT; however, finding consistency amongst colleagues within the field is a challenge.

Several recent studies have shown that the use of antibiotics, specifically broad-spectrum antibiotics, drastically disrupt gut microbes, reduce response to ICBT, and decrease pro-inflammatory cytokine levels (i.e. IL-6, IFN $\gamma$ , etc) which are necessary for an efficacious immune response. Multiple reports have observed detrimental effects of antibiotic treatment, including reduced response, earlier tumor progression, and reduced overall survival, supporting the concept that intact gut microbiome is important for ICBT response (7, 8). A meta-analysis of published clinical data showed that antibiotic use reduced overall survival and progression-free survival in a majority of the profiled cases (13). This combined analysis included many major cancer types treated with ICBT, both as a monotherapy and in combination. Notably, however, these associations seem to vary depending on the timing of antibiotics. One study showed that previous antibiotic use, but not concurrent use, was associated with reduced response to ICBT (14). Collectively, these studies show the potentially deleterious impact of antibiotics on response to ICBT; though important

questions remain as these studies did not include longitudinal microbiome characterization before and during therapy (both ICBT and antibiotics)- hence the direct relationship between antibiotic use and impact on the microbiome and associated immunity/anti-tumor immunity remains unknown. Further studies are needed to determine the exact mechanisms of action behind ICBT response and antibiotic use.

### Microbiota modulation of ICBT Toxicity

One of the challenges that detract from ICBT efficacy is the number of off-target effects they introduce due to the hyperactivation of effector T-cells. Immune-related adverse events (irAEs) result from harmful activation of T-cells by self-antigens and categorical subsets include gastrointestinal, dermatological, endocrine, pulmonary, and neurological toxicities. The risk and severity associated with these toxicities can vary substantially from patient to patient and can be exacerbated by numerous environmental cues, including the gut microbiome (15). Many irAEs manifest similarly to common autoimmune disorders, sometimes with effects so severe that they require clinical cessation of ICBT, potentially compromising the cancer care of the patient. The prevalence of at least one irAE occurring during treatment is extremely high and the heterogeneity surrounding irAEs continues to be a major problem with ICBT.

Numerous reports highlight patient cohorts that have experienced varying types and degrees of irAEs. In one study, patients treated with either ipilimumab or tremelimumab (i.e. anti-CTLA-4 mAb) who developed colitis were assessed endoscopically and investigators found that an increase in T-cell proliferation and decrease in regulatory T-cells following anti-CTLA-4 treatment may be the main cause of symptoms (16). This is consistent with what is seen in patients that experience chronic inflammatory bowel disease (IBD). It has also been reported that the severity of irAEs often dictates how long a patient can continue treatment. In one example one-third of select cancer patients (melanoma, NSCLC) that resumed immune checkpoint therapy following temporary cessation after irAEs had mild recurrent colitis/diarrhea; this happened more frequently for those using anti-CTLA-4 therapy (17).

**Pre-clinical Mouse Models**—Because irAEs mimic certain autoimmune disease phenotypes, researchers are exploring specific similarities between the two phenomena to combat T-cell autoreactivity. Inflammation serves as a major physiological cue in the human body and has been shown to shape microbial composition in the body but mechanisms are still being explored. It is thought that the microbiota can be manipulated to help combat some of the irAEs associated with ICBT. Select mouse models of irAEs have been used as powerful tools to better understand the basic mechanisms that may be contributing to disease. In a prime example, it was shown that ICBT-associated colitis is exacerbated with the use of antibiotics that target gram-positive organisms (i.e. vancomycin) and common immunopathology is eliminated with the re-introduction of a common probiotic species *Lactobacillus reuteri* in a mouse DSS-colitis/B16 melanoma model in combination with combined ICBT. Increased amounts of Innate lymphoid cells (ILCs) were shown to mediate some pathology in ICBT associated colitis and *L. reuteri* was able to decrease ILC numbers and IL-23 and IL-17 cytokine levels (18). In another model, anti-CTLA-4 was paired with the dextran sulfate sodium (DSS) treatment to exacerbate colitis in mice; when the mice

were treated with vancomycin, weight loss and survival were significantly reduced. Researchers introduced probiotic *Bifidobacterium* to the mice and toxicity was mitigated (19). These results collectively propose ways to modulate the microbiome to eliminate toxicity in ICBT, which continues to be a potentially fatal challenge to overcome.

**Clinical**—One study of 34 patients with metastatic melanoma examined the association of colitis with the microbiota at baseline before start of ICBT. They found that the presence of *Bacteroides* and select metabolic pathway regulation were associated with a reduced risk for colitis (20). Finding ways to combat toxicity is of great interest to overall patient progress and well-being. For ICBT-mediated colitis that is unable to be treated with steroids, the use of fecal microbiota transplant (FMT) from healthy donors has been shown to ameliorate colitis symptoms in a small case study. Use of FMT derived from a healthy donor abrogated immune checkpoint associated colitis in patients that had received immune checkpoint therapy treatment (21).

### Potential Mechanisms of Action

The effector cells of the immune system require stimulation from foreign molecules to fully activate and carry out their effector functions. Microbes can be a natural source of these foreign molecules. Given that individuals must coexist with a commensal microbiome without developing excessive inflammation, powerful immune regulatory mechanisms have been developed to help maintain T cell ignorance or tolerance to molecules derived from commensal organisms. It seems plausible that mechanisms modulating this immune balance at mucosal interfaces could also tip the balance between tolerance and immune activation in the cancer microenvironment. Many of the mechanisms underlying the precise immune-microbial interactions that affect immunotherapy response and toxicity have yet to be fully determined, but major advances in the last several years have created a foundation for a better understanding of this phenomenon.

**Antigen-independent Immune Responses**—General immune responses that act independently of foreign antigens are among the most common and well-understood known mechanisms. These include Th1 and Th17 T cell subsets responses which act as cytotoxic mechanisms of targeting and eliminating pathogens invading the host. These responses are potentially complicated by the fact that commensal organisms also play substantial roles in selecting for effector T cells (22). Previous studies identified a role for gram-positive pathogens for the efficacy of chemotherapeutic agent cyclophosphamide in a manner that was dependent on the bacterial stimulation of pathogenic Th17 cells (23). Specifically, the innate immune system and toll-like receptor (TLR) signaling pathways play important roles in the distinction of commensal microbes from pathogenic organisms. TLR5-dependent signaling by commensal bacteria is at least partially responsible for malignant tumor (UPK10/ID8-ovarian cancer cell line) progression through an increase in IL-6, triggering a signaling cascade that dampens anti-tumor immunity (24). Genetic polymorphisms have also been shown to affect microbiome composition and modulate immunity. A PTPN22 mutation was shown to decrease the amount of butyrate-producing microbes and these changes affected colitis onset and severity through IL-18 regulation (25); genetic associations like this may be used to predict the risk of irAEs following ICBT in the future.

**A role for antigen mimicry**—An interesting potential mechanism for microbial modulation of the immune environment lies in a process termed “antigen mimicry”, where certain bacterial proteins may contain antigenic epitopes similar to those expressed by the host, either in tumor cells or normal tissues. There are numerous examples of this phenomenon in the context of host-pathogen interactions but an exploration into its potential implications in ICBT is just beginning. A study in non-obese diabetic mice showed activation of CD8+ T-cells in Type 1 diabetes through the association of islet-specific glucose-6-phosphate catalytic subunit-related protein (IGRP) with immune signaling adapter protein MyD88 (26). Importantly, IGRP shares homology with select microbial peptides present on *Fusobacteria spp.* and *Leptotrichia goodfellowii*; researchers were able to show that the modulation of MyD88 by these peptides and a synthetic mimic can control diabetes development and progression. Loss of gut barrier integrity facilitates an increase in these islet reactive T-cells and bacterial translocation may play a role in stimulating immune cells through exposure to bacterial antigens. In a separate study, using a transgenic mouse model of spontaneous autoimmune myocarditis, it was also shown that Th17 cells stimulated by commensal gut microbes drive the progression of lethal disease (27). Activation of effector T cells by microbial peptides that share significant similarities to self-antigens may contribute to ICBT effectiveness and potential toxicity. While there is not much information regarding the relationship between the autoreactive antigens/autoimmunity produced by commensal microbes and ICBT, there is evidence suggesting that focusing on this area to elucidate new mechanisms may lead to an emergence of effective new strategies. Additional examples of microbe-based mechanisms of action with potential relevance to ICBT can be seen in Table 1.

### Ongoing efforts and Future Directions

Research in the field is rapidly expanding to include novel methods for microbiome manipulation to better modulate immune checkpoint blockade efficiency, toxicity, and response. Because select targets are constantly being identified from recent clinical and pre-clinical models, some researchers have chosen to focus on specific targets as novel probiotics. In one example, *Lactobacillus* was shown to be depleted in ICBT treated melanoma mouse models but, with the introduction of common probiotic *Lactobacillus reuteri*, ICBT-mediated toxicity is ameliorated without affecting the ability to diminish tumors (18). Administration of common probiotic *Bifidobacterium infantis* to colorectal cancer (CRC) model rats attenuates chemo-induced intestinal inflammation via suppression of Th1 and Th17 responses (28). However there are also clear examples of deleterious impact of orally-administered probiotics in cancer therapy, as published studies suggest that administration of commercially-available probiotics is associated with increased tumor penetrance, multiplicity, and adenocarcinoma invasion in pre-clinical models of colorectal cancer (29). Thus the use of probiotics needs to be carefully assessed in the context of clinical trials, and off-protocol use of these agents in patients with cancer is discouraged.

As treatment with ICBT is being investigated and approved in various cancer types, there is a growing interest in investigating the effects of microbiota (and potential microbiome modulation via FMT) in these cancers. In a recent search of the US. National Library of Medicine Clinical Trial database, eight clinical trials of FMT in patients receiving ICBT was

identified, with relevant features summarized in Table 2. Of these, six studies are aimed at using FMT as a strategy to improve response to ICBT, while two are targeting irAE.

A big limitation for microbiome studies lies in the inability to properly culture all the species that may be relevant to ICBT to study *in vitro*. Culturable microbes only represent about one percent of the species that can be identified in the gut with 16S sequencing and, although the gut represents the site in which most microbes reside, it is not the only area of interest. This also does not consider any crosstalk between sites that may also influence ICBT response in patients. With the development of novel methods for bacterial isolation and culturing, researchers can begin to better study the bacterial cell and genetic components that modulate the host environment (30). Additionally, the safety of human FMT has come into question recently when two patients received FMT to treat infection but ultimately responded negatively and died as a result of their treatment. Because FMT is a consortium of a myriad of organisms that vary dramatically based on the donor, it is challenging to reliably predict how a patient will respond.

## Conclusion

This mini-review covered influential studies from the field that highlight the importance of the gut microbiome in the context of immune checkpoint blockade therapy efficacy and potential toxicity. Combining the use of pre-clinical mouse models and clinical patient data, researchers have been able to discover new organisms of interest and mechanisms of action that help to inform how patients can be treated. Future strategies focusing on additional species identification, direct microbial impact on ICBT *in vivo*, contributions from the host environment, and microbial by products will uncover additional mechanisms of action and allow for more precise treatment of cancer patients.

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**Table 1:**

Mechanisms of action that may contribute to microbial effects on immune checkpoint blockade therapy

Proposed Mechanism	Antigen independent/ Antigen specific?	Example organism(s) of interest	Phenotypic manifestation(s)	Reference
Autoreactivity of effector T-cells	Antigen Independent Antigen Specific	<i>Campylobacter jejuni</i> , <i>Citrobacter rodentium</i> , <i>Helicobacter hepaticus</i> Gut commensal microbes	Systemic inflammation, Enterocolitis, colorectal cancer (CRC) Activation of retina specific T-cells to cause Uveitis	(31–33)
Activation of TLR signaling pathways	Antigen specific	<i>Engineered Salmonella typhimurium</i> expressing <i>FlaB</i> from <i>Vibrio vulnificus</i>	Tumor suppression in CRC mouse model in a TLR4 and TLR5 dependent manner	(34)
Genetic variants	Antigen independent	<i>Varies</i>	Protein tyrosine phosphate non-receptor type22 (PTPN22) variant prevents select autoimmunity and shapes microbial composition	(25)
Molecular Antigen Mimicry	Antigen specific	<i>Bacteroides fragilis</i> ; <i>Fusobacterium spp.</i> , <i>Leptotrichia goodfellowii</i> Gut commensal microbes	Activation of protein MyD88 leads to Type I diabetes in NOD mice Progression to inflammatory myocarditis driven by microbial peptide stimulated Th17 cells	(26, 27, 35)
Bacterial translocation from the gut	Antigen independent	<i>Enterococcus gallinarum</i>	Systemic lupus erythematosus (SLE), chronic autoimmunity	(36)

Select studies highlighting potential drivers of external immune stimulation and autoimmunity in humans and mice that may be implicated or exacerbated in the context of immune checkpoint blockade.

**Table 2:**

Clinical trials of gut microbiome modulation via FMT in various cancer types

Patient population	Patients (n)	Intervention	FMT Donor (Healthy vs ICB responder)	FMT Modality (Route of Administration)	Primary endpoint	Secondary Endpoints	Enrollment Status	Sites	NCT Number	Study Phase
Metastatic melanoma	30	FMT + anti-PD1	NS	Capsule	Safety	Microbiome change ORR Immune profile change	Recruiting	MD Anderson Cancer Center, USA	NCT03817125	Ib
Anti-PD-1 refractory stage III/IV melanoma	40	FMT + anti-PD1	Responder	Colonoscopy and Capsule	Safety Engraftment	Immune profile change	Recruiting	Sheba Med Center, Tel HaShomer, Israel	NCT03353402	I
Metastatic melanoma	20	FMT + anti-PD1	Healthy	Capsule	Safety of combination therapy	Effect on the gut microbiome Immune profile change Metabolomics	Recruiting	Western University, Ontario Canada	NCT03772899	I
Anti-PD1-refractory luminal GI cancer	5	FMT + anti-PD1	NS	Capsule	Safety ORR	Effect on the gut microbiome Immune profile change	Not yet recruiting	Peking University, Beijing, China	NCT04130763	I
Anti-PD-1 refractory stage III/IV melanoma	20	FMT + anti-PD1	Responder	Colonoscopy	ORR	Effect on the gut microbiome Immune profile change	Recruiting	University of Pittsburgh, Pennsylvania, USA	NCT03341143	2
Metastatic mesothelioma	1	FMT+ anti-PD1	Healthy	Colonoscopy	Progression free survival	None	Completed	Progenabio, CA USA	NCT040560261	1
Metastatic castrate-resistant prostate cancer	32	FMT + anti-PD1 + Enzalutamide + Androgen deprivation	Responder	Colonoscopy	PSA change	Radiographic response rate Progression free survival Overall Survival	Not yet recruiting	VA Portal HealthCare System, Oregon USA	NCT04116775	2
Gut microbiome modulation via FMT directed at ICB Toxicity										
Patients with melanoma or GU cancer who develop ICB-related colitis	100	FMT	Healthy	Colonoscopy	Safety	Change in stool microbiome	Not yet recruiting	MD Anderson Cancer Center, USA	NCT03819296	I
Patients with GU cancer who develop ICB-related colitis	40	FMT + Loperamide	Healthy	Colonoscopy	Safety Clinical resolution of colitis	Colitis recurrence at 3 months Endoscopic and histologic remission	Not yet recruiting	MD Anderson Cancer Center, USA	NCT04038619	I

ORR=objective response rate, NS=not specified, GU=genitourinary, ICB=immune checkpoint blockade, FMT=fecal microbiota transplant

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