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The Influence of the Gut Microbiome on Cancer, Immunity, and Cancer Immunotherapy

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The microbiome is receiving significant attention given its influence on a host of human diseases including cancer. Its role in response to cancer treatment is becoming increasingly apparent, with evidence suggesting that modulating the gut microbiome may affect responses to numerous forms of cancer therapy. A working knowledge of the microbiome is vital as we move forward in this age of precision medicine, and an understanding of the microbiome's influence on immune responses and cancer is key. It is also important to understand factors influencing the gut microbiome and strategies to manipulate the microbiome to augment therapeutic responses.

Introduction

There is mounting evidence supporting the role of the microbiome in response to cancer therapy, with several recent studies demonstrating the influence of the gut microbiome, specifically on the response to immune checkpoint blockade across cancer types (Chaput et al., 2017; Frankel et al., 2017; Gopalakrishnan et al., 2018; Matson et al., 2018; Routy et al., 2018). How and why this occurs necessitates an understanding of the intricate web of cancer, immunosurveillance, and the factors that influence both host and anti-tumor immunity—including the gut microbiome. Each of these factors, as well as their impact on one another and on responses to immunotherapy, will be discussed herein. Finally, strategies to modulate the gut microbiome and ongoing trials to do so will also be described.

Cancer Immunosurveillance and Response to Cancer Therapy

The immune system has long been recognized as a dominant force in cancer control, with defects in immunity contributing not only to carcinogenesis and cancer progression, but also to poor responses to cancer therapy. Recent evidence substantiates the need for preserved overall systemic immunity in mediating responses to immunotherapy specifically (Chen and Mellman, 2013, 2017; Spitzer et al., 2017).

Tremendous progress has been made in identifying factors contributing to response to cancer therapy—and have largely focused on tumor-centric markers including "foreignness" (including mutational load) (Snyder et al., 2014), aspects of tumor metabolism (such as glucose metabolism), and factors affecting tumor sensitivity to immune effectors (including human leukocyte antigen [HLA] and interferon gamma gene expression) (Blank et al., 2016). However, markers of response to cancer therapy have continued to evolve, and now include factors well beyond established tumor-centric markers—providing a more holistic paradigm that encompasses the multitude of factors that affect therapeutic response. These newer models have been extended to recognize components that contribute to overall immune status (Blank et al., 2016) and the tumor microenvironment, including infiltrating immune cells that can either stimulate (such as $CD8^+$ T cells) or inhibit (such as myeloid derived suppressor cells) an immune response (Sharma et al., 2017; Blank et al., 2016). In addition, recent insights importantly highlight the impact of microbiota (particularly the gut microbiota) on responses across several cancer therapies (Kroemer and Zitvogel, 2018).

The Microbiome and Immunity

The microbiome is defined as the collective genomes of microbes within a community, whereas the term microbiota refers to the microbes themselves in aggregate. Within a human organism, there are trillions of microbes–as numerous as human cells–which interact with the host constantly at numerous sites (including the skin and mucosal surfaces such as the gastrointestinal tract) throughout development. Therefore, it is not surprising that they play such a large role in numerous host functions including immunity (Sender et al., 2016; Morgan and Huttenhower, 2012).

The crosstalk between microbiota and the immune system at the level of the gut is critical, and, not only allows for the tolerance of commensal bacteria and oral food antigens, but also enables the immune system to recognize and attack opportunistic bacteria thereby preventing bacterial invasion and infection. In addition to influencing localized immune responses, these microbiota also have broader effects contributing to innate and adaptive immunity at multiple levels. This concept is supported in pre-clinical models; germ-free (GF) mice that lack intestinal microbiota are noted to have severe defects in immunity, with an absent mucous layer, altered immunoglobulin A (IgA) secretion, and reduced size and functionality of Peyer's patches and draining mesenteric lymph nodes (mLNs)

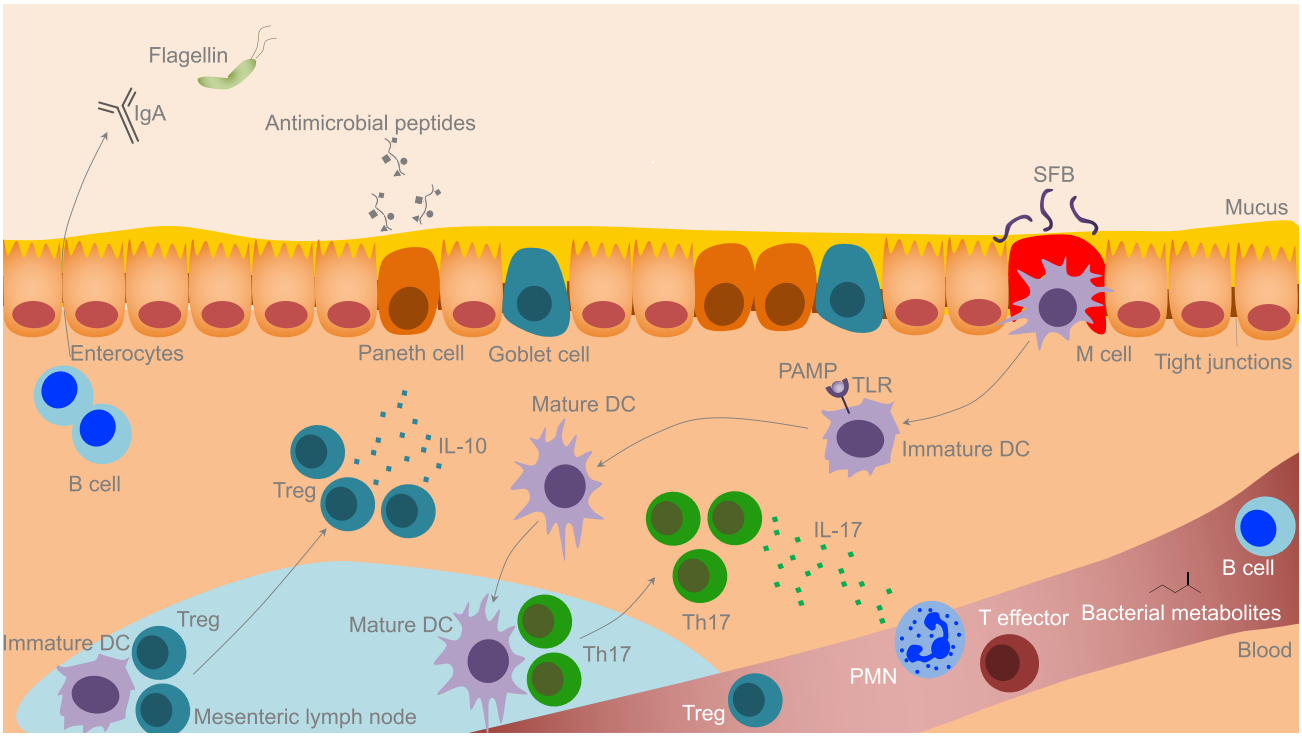

Figure 1. The Microbiome and Immunity

Commensal organisms within the lumen of the gut have profound influences on the immune system at the local level within the gut mucosa, in draining mesenteric lymph nodes, and systemically. The immune system likewise can alter the gut microbiota. Goblet cells create a thick mucus protective layer covering the mucosa; this mucosal layer is largely deficient in GF animals. Plasma cells in the lamina propria secrete IgA into the lumen of the gut. Paneth cells secrete a number of anti-microbial peptides; their activity is amplified in response to signaling from local immune cells in response to the microbiota. Bacterial metabolites or bacteria themselves can activate local DCs which migrate to the draining lymph nodes to activate naive T cells to effector T cells, Tregs, or Th17 cells, which can migrate back to the gut mucosa or enter systemic circulation. Specific metabolites or bacterial byproducts can alter the DC in a fashion that allows them to skew toward a Treg versus Th17 phenotype. Tregs function in secreting IL-10, creating a local anti-inflammatory cytokine milieu. Th17 cells, meanwhile, produce IL-17, which can increase Paneth cell production of anti-microbial peptides and can function in recruiting polymorphonuclear neutrophils (PMNs) from the bloodstream. Some bacterial metabolites can enter the bloodstream directly further altering the systemic immune system.

(Johansson et al., 2015; Spiljar et al., 2017). Overall, there is compelling evidence that the microbiota help to shape the immune system as a whole (Honda and Littman, 2016).

The structure of the gut, which contains a mucosa consisting of a single epithelial cell layer made up of intestinal epithelial cells (IECs) and intraepithelial lymphocytes, facilitates this interaction with the immune system. The IECs contain Paneth cells that secrete anti-microbial peptides and goblet cells that secrete mucus, which in turn coat the epithelial layer. Beneath the mucosal layer lies the lamina propria, a connective tissue layer containing Peyer's patches and a host of other immune cells including antigen-presenting cells and innate lymphoid cells, as well as $CD4^+$ and $CD8^+$ T and B cells. This gut-associated lymphoid tissue represents the largest component of the immune system in the body and influences immune responses both locally and systemically (Figure 1).

Local immunity is promoted via recognition of pathogen-associated molecular patterns (PAMPs) (such as lipopolysaccharide and flagellin) by pattern recognition receptors (such as Toll-like receptors (TLRs) present on IECs as well as innate immune effectors within the gut). Metabolites produced by bacteria may also affect local immunity via production of short-chain fatty acids (SCFAs), which, among a number of key activities, have been shown to augment immunity via IgA production by plasma cells (Pabst, 2012). IgA acts primarily by blocking bacterial adherence to epithelial cells; agglutination, entrapment, and clearance; and also has direct effects on bacterial virulence (Mantis et al., 2011).

Draining lymph nodes for the gut lie within the mesentery of the small bowel and colon, where adaptive immune responses are further shaped by the gut commensals, impacting the differentiation of naive T cells within the mLNs. PAMPs act to induce maturation of antigen-presenting cells, such as dendritic cells (DCs), as they sample antigen from the lumen, either directly via interdigitation of dendrites through the mucosal layer or indirectly after processing and transphagocytosis by specialized IECs called M cells. Once activated, DCs travel to mLNs where they interact with and stimulate naive T cells to form $CD4^+$ T cells (Lathrop et al., 2011), specifically $CD4^+$ T regulatory cells (Tregs) and T helper 17 (Th17) cells, both of which have a tropism for the gut. DCs may also directly stimulate $CD8^+$ T cells.

After education in the mLNs, T cells can influence immunity at a number of different sites. They play a critical role in gut homeostasis, highlighted by mucosal tolerance induced by Tregs, and via production of immunosuppressive cytokines, such as interleukin-10 (IL-10). Importantly, there is ongoing crosstalk

between gut commensals and mucosal T cells (such as Tregs), as maintenance of these cells at the level of the gut is facilitated by bacterial metabolites such as SCFAs; the fuction of SCFAs is dependent on their ability to inhibit histone deacetylase activity suggesting epigenetic regulation (Rooks and Garrett, 2016). In addition, some bacterial species have been shown to drive Treg development via alternative pathways dependent on polysaccharide A and TLR signaling to DCs (Round and Mazmanian, 2010; Shen et al., 2012).

A specific subset of $CD4^+$ cells proven to be important in gut microbiota interactions are Th17 cells. These cells are prominent within the lamina propria of the small and large intestine, and are critical in protecting against bacterial and fungal infections. Th17 cells also function in mucosal immunity as cytokine secretion from Th17 cells stimulates IECs to form tight junctions and to secrete anti-microbial proteins (Weaver et al., 2013). Th17 cells are markedly depleted in GF animals and can be induced by specific bacterial subsets such as segmented filamentous bacteria (Ivanov et al., 2008, 2009). IL-17 can cause further release of additional inflammatory cytokines and recruit neutrophils from the circulation to the gut microenvironment. In addition to influencing local immunity within the gut mucosa, microbiota can also shape systemic immune responses via immune cell priming. DCs primed by commensals typically do not pass into the circulation or travel to distant lymph nodes but can do so in certain settings. TLR signaling from microbial peptides to DCs and other innate immune effectors generates cytokines and interferons that act in both a paracrine and endocrine manner at distant sites; it is thought that this signaling in the gut creates immune system "tone." That is, the systemic immune system is primed (potentially at epigenetic or transcriptional level) to enact a robust response in the setting of pathogens, and, in the absence of threat, to maintain a non-inflammatory state (Abt et al., 2012) Furthermore, B and T cells, including Tregs and Th17 cells, can, upon being primed by DCs presenting antigen derived from commensal organisms in draining mLNs of the gut, circulate systemically to facilitate immune responses at distant sites against the same organism or against other antigens by cross-reaction to similar epitopes (Stary et al., 2015). Interestingly, Th17 cells that emigrate can have significant plasticity in function, changing their cytokine output based on the existing local inflammatory or non-inflammatory state (Hirota et al., 2011).

Disruption of the delicate balance of commensal bacteria is seen in the setting of dysbiosis, which is characterized by a less-diverse and less-stable microbiota, with potential enrichment of opportunistic pathogenic bacteria (Frosali et al., 2015). Dysbiosis can lead to impaired local, locoregional, and systemic immune responses with breakdown of mucosal barriers, translocation of gut bacteria to the mLNs and into the peripheral circulation, alteration of the cytokine milieu within the gut mucosa and draining mLNs towards an inflammatory phenotype, and activation of Th17 cells and effector T cells, causing an influx of neutrophils and inciting a profound inflammatory state both locally and systemically (Levy et al., 2017).

Exemplification of the importance of eubiosis in preserving immunity is seen in response to vaccination. A highly diverse microbiota has been associated with improved adaptive immune responses to a variety of vaccines in infants (Huda et al., 2014). Specific components of the microbiota can prime the immune system by activating TLR signaling pathways serving as a natural vaccine adjuvant (Oh et al., 2014). Thus, it is increasingly clear that the gut microbiota may affect not only local immunity but also systemic immune responses.

The Gut Microbiota in Response and Toxicity to Immunotherapy

Given the role of the gut microbiota in modulating host immunity, it is fairly intuitive that it could significantly influence response and toxicity to various forms of cancer therapy. Although early studies primarily used murine models to assess these interactions, there is now mounting data from human cohorts suggesting that the gut microbiota is a dominant force in mediating both response and toxicity to these therapeutic strategies. Bacterial taxa implicated in response as well as toxicity to immunotherapy in human studies (Figure 2) and in murine models will be discussed herein.

The Gut Microbiota and Stem Cell Transplantation

Perhaps one of the earliest demonstrations of the role of the gut microbiota in response and toxicity to cancer therapy was in the setting of allogeneic stem cell transplant (allo-HSCT) for hematologic malignancies. Dysbiosis and impaired systemic immunity is fairly common in these patients, as they are often treated with concurrent therapies that significantly alter immunity and composition of the gut microbiota—including immunosuppressants, broad-spectrum antibiotics, and even total body irradiation (Routy et al., 2017; Shono et al., 2016). Several investigators hypothesized that dysbiosis could be associated with altered responses and potentially with toxicity to therapy. Analyses of longitudinal fecal samples demonstrated a disruption of the existing state of equilibrium of the gut microbiota post-HSCT, with a loss of bacterial diversity and stability, and dominance of *Enterococcus*, *Streptococcus*, and various *Proteobacteria* (Holler et al., 2014; Taur et al., 2012). Importantly, health-promoting bacteria such as *Faecalibacterium* and *Ruminococcus* were reduced (Biagi et al., 2015).

Dysbiosis in the setting of HSCT has also been associated with differences in long-term survival, with patients having a lower diversity of microbiota in their gut at the time of HSCT having shortened overall survival and higher mortality rates (specifically transplant-related mortality), compared with those with a high diversity of gut microbiota (Taur et al., 2014). Further confirmation of this finding was reported when low levels of 3-indoxyl sulfate in the urine, a by-product of L-tryptophan metabolism by commensal microbiota and a marker for bacterial dysbiosis, was found to be associated with worse overall survival following allo-HSCT (Weber et al., 2015). In addition to diversity, compositional differences in the gut microbiota have also been studied in response and survival after HSCT, with a higher abundance of bacteria within the genus *Blautia* associated with improved overall survival (Jenq et al., 2015) and a higher abundance of *Eubacterium limosum* associated with a reduced risk of relapse (Peled et al., 2017).

In addition to the relationship to response and survival, the influence of the gut microbiota has also been studied in the context of toxicity to HSCT therapies—particularly with regard to graft-versus-host disease (GVHD). GVHD is characterized by the vigorous activation of immune-competent donor immune cells (mostly T cells) and causes significant damage to a variety of organs including the skin, liver, and gut, as well as sites of

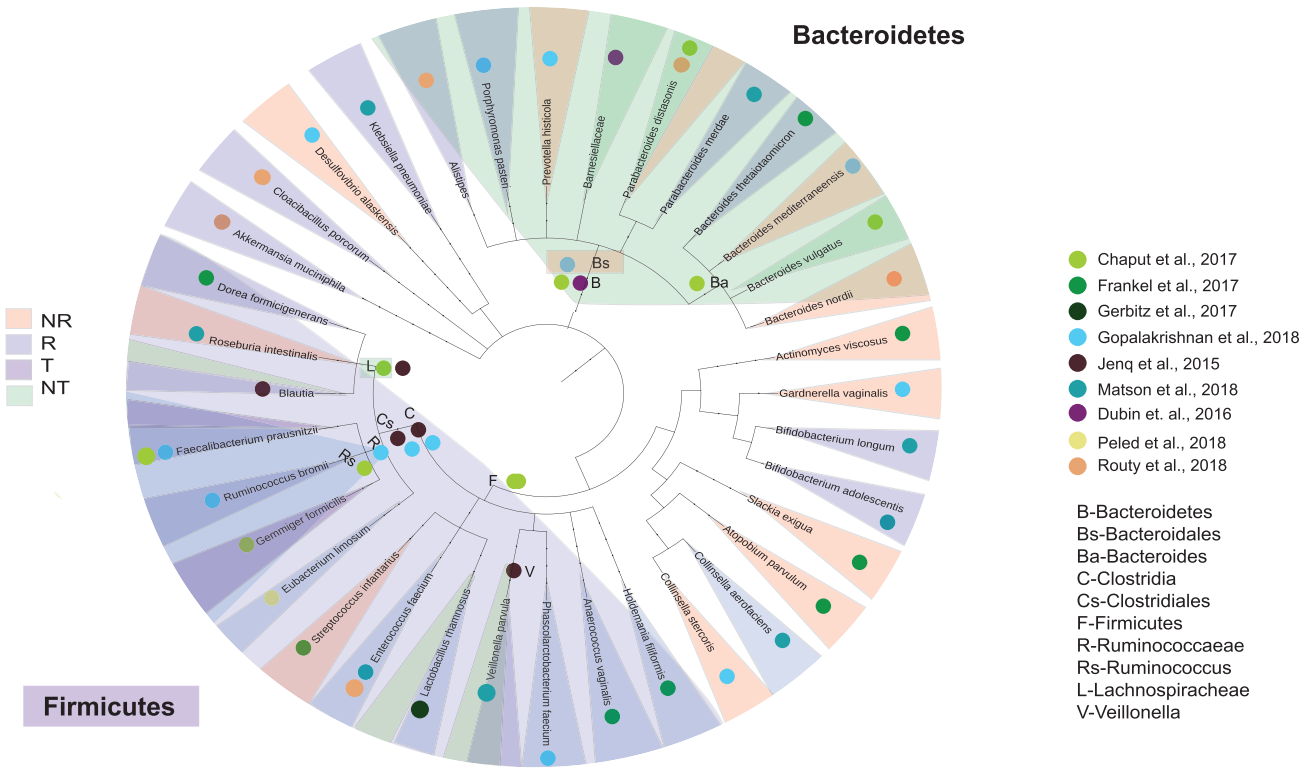

Figure 2. Phylogenetic Tree Summarizing Previously Established Links between the Gut Microbiome, Treatment Outcomes, and Toxicities in Cancer Patient Populations

A phylogenetic tree was constructed using evolutionary distances with the phyloT software (Letunic and Bork, 2016) to depict phylogenetic similarity (or lack thereof) of all bacterial taxa reported to be associated with response or toxicity to immune checkpoint blockade in human studies, moving from broader (kingdom) to more specific (species) taxonomies from inside-out. Bacterial taxa are labeled according to publication (colored dots) of origin and shaded according to the phenotype(s) of association in the various studies included. R, treatment response (light blue); NR, treatment non-response (light red); T, toxicity (light green); and NT, non-toxicity (light purple).

hematopoiesis (Jacobsohn and Vogelsang, 2007). GVHD susceptibility varies with the type and extent of conditioning regimen, the degree of HLA mismatch, and the activation status of donor cells. Severe acute GVHD has a long-term survival probability of less than 5% (Cahn et al., 2005) and chronic GVHD is also associated with significant morbidity and mortality.

The onset of acute GVHD is associated with significant shifts in the composition of the microbiota, with a loss of overall diversity and reduction of health-promoting obligate anaerobes such as *Faecalibacterium, Ruminococcus, Lactobacillus*, and *Blautia,* and an enrichment of *Enterococcus* and *Clostridiales* (Jenq et al., 2012; Heimesaat et al., 2010; Gerbitz et al., 2004; Biagi et al., 2015). A high abundance of the genus *Blautia* of the Clostridia class was found to be associated with reduced GVHD lethality in two independent cohorts of patients undergoing treatment with allo-HSCT (Jenq et al., 2015). The gut microbiota may also be affected by antibiotic treatment for infectious complications during HSCT, with differences in GVHD-associated mortality seen with different antibiotic regimens (Shono et al., 2016).

Modulation of the gut microbiota to abrogate toxicity has been studied in pre-clinical models with mixed results. The administration of the probiotic *Lactobacillus rhamnosus* GG alone or in combination with Ciprofloxacin before and during transplantation in mice was associated with reduced rates of GVHD and improved overall survival (Gerbitz et al., 2004). In this work, the authors hypothesized that probiotic supplementation contributed to the preservation of gut mucosal integrity, as surveying the mLNs revealed an absence of enteric pathogens (Gerbitz et al., 2004). However, results in human cohorts have been more heterogeneous, with early studies demonstrating reduced rates of GVHD in patients treated with broad-spectrum antibiotics prior to HSCT (van Bekkum et al., 1974) and more recent studies demonstrating detrimental effects of antibiotic use with higher rates of GVHD (Routy et al., 2017; Shono et al., 2016). More refined strategies to modulate the gut microbiota to reduce the risk of GVHD are now being tested in clinical trials including dietary modifications and fecal microbiome transplant (FMT) (NCT03359980, NCT03148743, NCT03214289, and NCT02763033). These trials are based on the central hypothesis that re-shaping the intestinal microbiota to its pre-treatment eubiotic state would lessen the risk of subsequent GVHD development, and are primarily exploratory in nature, seeking to assess the safety and feasibility of such modalities. Nevertheless, these trials will serve as a foundation, and additional trials will be implemented based on insights gained.

Gut Microbiota and Immunotherapy

Although stem cell transplant may be considered one of the earliest effective forms of cancer immunotherapy, there are

now a host of novel immunotherapeutic approaches, and, not unsurprisingly, these are similarly affected by the gut microbiota. Important initial insights came from murine models (Sivan et al., 2015; Vetizou et al., 2015), and many of these findings have now been validated in patient cohorts treated with immunotherapy, specifically immune checkpoint blockade.

Over a decade ago, investigators from the National Cancer Institute demonstrated that administration of antibiotics significantly abrogated anti-tumor activity in a murine model of adoptive cell therapy for melanoma. The authors posed that the proliferation of transferred T cells in the tumor was augmented by the translocation of the gut microbiota to the mLNs associated with total body irradiation, which was used as a preparative regimen. They surmised that the translocation of gut bacteria helped to prime an immune response via TLR4 signaling (Paulos et al., 2007). This notion is supported by studies demonstrating impaired responses to intra-tumoral injection of TLR agonists in GF or antibiotic-treated mice. In this setting, tumor-associated myeloid cells are primed by commensal gut bacteria (via TLR4 signaling) for the production of tumor necrosis factor and other inflammatory cytokines that mediate the anti-tumor effect of these agents (Iida et al., 2013).

Although not traditionally considered immunotherapy, effective treatment with conventional chemotherapy is also dependent on intact immune responses, thus substantiating the notion that the gut microbiota could shape responses to these forms of therapy as well. This has certainly been demonstrated in platinum-based chemotherapies and cyclophosphamide therapy. During treatment with cyclophosphamide, translocation of commensal bacteria (specifically Gram-positive organisms such as *Lactobacillus johnsonii* and *Enterococcus hirae*) into mLNs can potentially facilitate robust Th17 responses in the spleen and the induction of memory Th1 responses. Immune responses to cyclophosphamide have also been shown to be dependent on MyD88 and TLR signaling—suggesting that commensal microorganisms may play a role. Indeed, the effects of cyclophosphamide and other chemotherapy regimens were abrogated in GF or antibiotic-treated mice and were differentially affected by the presence of particular bacterial species (Viaud et al., 2013; Iida et al., 2013).

Importantly, the impact of the gut microbiota has also been studied in the setting of treatment with immune checkpoint inhibitors, which target immunomodulatory molecules on the surface of T cells (or their ligands) to enhance anti-tumor immune responses. Despite the enthusiasm for treatment with these agents, a significant proportion of patients do not experience objective responses and, when responses do occur, may not be durable. Tremendous efforts have focused on identifying predictors of the response to immune checkpoint blockade as well as strategies to overcome therapeutic resistance (Cogdill et al., 2017; Sharma et al., 2017). Emerging evidence suggests that the gut microbiota may play a significant role in modulating responses to these therapies.

The impact of the gut microbiota on response to immune checkpoint blockade was first studied in mouse models, with landmark publications in *Science* in 2015 demonstrating that the composition of the gut microbiota could influence the response to immune checkpoint inhibitors targeting the cytotoxic T lymphocyte antigen 4 (CTLA-4) and the programmed death receptor 1 (PD-1) (Vetizou et al., 2015; Sivan et al., 2015). In the case of CTLA-4 blockade, notable changes in the abundance of gut microbiota in mice were seen following anti-CTLA-4 therapy, with a relative increase in *Bacteroidales* and *Burkholderiales* and a decrease in *Clostridiales*. The efficacy of anti-CTLA-4 therapy was markedly reduced in GF mice and SPF mice with broad-spectrum antibiotics. Furthermore, oral feeding with *Bacteroides fragilis* in combination with either *Bacteroides thetaiotaomicron* or *Burkholderia cepacia* augmented the action of anti-CTLA-4 therapy by eliciting a Th1 response in the lymph nodes and facilitating the maturation of intra-tumoral DCs. The translational impact of these findings was demonstrated when FMT from patients having dominant *Bacteroides* species in their gut resulted in improved tumor control compared with FMT from patients with distinct *Bacteroides* or *Prevotella* species (Vetizou et al., 2015). These findings were complemented by parallel studies in the context of treatment with PD-1 blockade, demonstrating significant differences in tumor outgrowth in genetically similar mice with differing gut microbiomes purchased from two separate vendors. Therapeutic responses also differed in these mice, and beneficial effects from mice with a more "favorable" microbiota could be transplanted to other mice using FMT or co-housing. Profiling of the gut microbiome revealed an over-representation of *Bifidobacterium* species in mice with delayed tumor outgrowth and favorable responses to PD-1-based therapy. Furthermore, supplementation with an oral probiotic containing *Bifidobacterium* restored anti-tumor efficacy of PD-L1-blockade in mice with an "unfavorable" gut microbiota, which primarily occurred through enhanced DC maturation resulting in increased tumor-specific $CD8^+$ T cell activity (Sivan et al., 2015).

These studies were further supplemented by multiple studies published in the past several months demonstrating a role for the gut microbiota in patients on immune checkpoint blockade (Chaput et al., 2017; Frankel et al., 2017; Gopalakrishnan et al., 2018; Matson et al., 2018; Routy et al., 2018). Several provocative findings were reported substantiating the role of the gut microbiota in shaping responses to therapy. First, the impact of antibiotic use on response to immune checkpoint blockade was shown in a large cohort of patients with non-small-cell lung cancer, renal cell carcinoma, or urothelial cancer. Patients treated with antibiotics for routine indications shortly before, during, or shortly after treatment with anti-PD-1/PD-L1 mAB had significantly lower progression-free survival and overall survival rates compared with patients who had not received antibiotics. This suggests that disrupting the gut microbiota (via antibiotic use) could potentially impair anti-tumor immune responses as well as response to immune checkpoint blockade (Routy et al., 2018). The group also studied the gut microbiota directly by performing whole metagenomic sequencing in fecal samples from these patients, demonstrating that responders to PD-1 blockade had differential composition of gut bacteria, including specific genera highlighted by the group as being enriched in responding patients (*Akkermansia* and *Alistipes*). FMT was performed in GF and SPF mice using a stool sample from either responder (R) or non-responder (NR) patients prior to treatment with PD-1 blockade, demonstrating enhanced responses in the setting of R-FMT. In these studies, the efficacy of anti-PD-1 in GF mice receiving NR-FMT could be restored by administration of

Akkermansia muciniphila alone or in combination with *E. hirae*, where administration of *A. muciniphilia* was associated with increased intra-tumoral immune infiltrates, mediated by the recruitment of $CCR9^+CXCR3^+CD4^+$ T cells into the tumor bed and an increased ratio of $CD4^+$ T cells to $CD4^+FoxP3^+$ T cells (Tregs) in response to PD-1 blockade (Routy et al., 2018). These findings were corroborated in two additional papers published in the same issue of *Science* describing the impact of gut microbiota on responses to anti-PD-1 therapy in patients with metastatic melanoma.

The study by Gopalakrishnan et al. revealed that patients who responded to anti-PD-1 therapy had a significantly higher diversity of bacteria in their gut microbiota as well as a higher relative abundance of *Clostridiales*, *Ruminococcaceae*, and *Faecalibacterium* (Gopalakrishnan et al., 2018; Matson et al., 2018). In contrast, NR had significantly lower diversity of gut bacteria and higher abundance of Bacteroidales. Importantly, comparing the composition of bacteria in the gut with immune profiling in the tumor microenvironment revealed that patients with a favorable gut microbiota had increased expression of cytolytic T cell markers and antigen processing and presentation compared with patients with unfavorable gut microbiota. Mechanistic studies were performed in GF mice with FMT from R versus NR, recapitulating findings in parallel published studies that mice receiving FMT from R had significantly delayed tumor outgrowth and enhanced responses to treatment with immune checkpoint blockade (Gopalakrishnan et al., 2018). Another cohort of patients with metastatic melanoma studied by Matson et al. (2018) also demonstrated significant differences in response to treatment with immune checkpoint blockade based on profiles within the gut microbiota. Specifically, the group found that patients who responded to anti-PD-1 therapy had enrichment of *Bifidobacterium longum, Collinsella aerofaciens*, and *Enterococcus faecium* in baseline fecal samples. Transfer of stool samples from patients to GF mice in this study also successfully recapitulated the phenotype, with mice that received R stool growing tumors at a slower rate and having markedly improved efficacy to anti-PD-L1 immunotherapy compared with mice that received NR stool. These effects were mediated by increased densities of $CD8^+$ T cells and reduced $FoxP3^+CD4^+$ Tregs in the tumor microenvironment (Matson et al., 2018).

Although immune checkpoint blockade agents have been a qualified success in the treatment of various malignancies resulting in sustained responses, a significant proportion of patients continue to experience treatment-limiting toxicity with anti-PD-1 (16%), anti-CTLA-4 (27%), and combination therapies (65%) (Larkin et al., 2015). Approximately one-third of all patients undergoing anti-CTLA-4 therapy develop intestinal inflammation due to mucosal immune dysregulation (Berman et al., 2010; Weber et al., 2013). Efforts to characterize gut microbiota that contribute to toxicity to immune checkpoint blockade are underway. Pre-clinical models have demonstrated an improvement in toxicity scores in anti-CTLA-4-treated mice with oral gavage of *B. fragilis* and *B. cepacia* (Vetizou et al., 2015). The influence of the gut microbiota on toxicity has also been studied in human cohorts (Chaput et al., 2017; Dubin et al., 2016; Frankel et al., 2017) (Figure 2). Taxonomical and functional differences have been reported in anti-CTLA-4-treated melanoma patients who were colitis-free (with enrichment of Bacteroidetes and abundance of genetic pathways involved in polyamine transport and B vitamin synthesis) as opposed to those who developed colitis (Dubin et al., 2016). This may be related to the known influence of these bacteria in Treg differentiation (Round and Mazmanian, 2010; Faith et al., 2014). Additional cohorts have also been studied, showing that patients with a higher abundance of *Faecalibacterium prausnitzii* and other related Firmicutes and low abundance of Bacteroidetes had a higher risk of colitis on anti-CTLA-4 therapy (Chaput et al., 2017; Frankel et al., 2017). The group also reported that patients with colitis had increased expression of ICOS on the surface of effector $CD4^+$ T cells and low levels of Tregs and systemic inflammatory proteins such as IL-6, IL-8, and sCD25 in the blood at baseline, which may be related to the compositional differences in the microbiome (Chaput et al., 2017).

Based on the available literature, there are clearly bacterial taxa that are associated with response and toxicity—with some overlap in the bacterial signatures across the studies (Figure 2). Bacterial taxa within the Ruminococcaceae family of the Firmicutes phylum (such as *F. prausnitzii*) have been associated with both response and toxicity to immune checkpoint blockade across studies (Chaput et al., 2017; Frankel et al., 2017; Gopalakrishnan et al., 2018) (Figure 2). Conversely, bacterial taxa within the Bacteroidales order of the Bacteroidetes phylum have been associated with a lack of response to immune checkpoint blockade, while a higher abundance of these taxa within the gut are also generally associated with a lower incidence of toxicity (Chaput et al., 2017; Dubin et al., 2016; Frankel et al., 2017; Gopalakrishnan et al., 2018). However, at lower levels of taxonomy, these generalizations do not apply, as some taxa within Firmicutes have been associated with a lack of response (*Roseburia, Streptococcus*) (Frankel et al., 2017; Matson et al., 2018) and some taxa within Bacteroidetes have been associated with response (*Alistipes, Porphyromonas pasteri*, and *C. aerofaciens*) (Gopalakrishnan et al., 2018; Matson et al., 2018; Routy et al., 2018). Importantly, taxa outside of Firmicutes/Bacteroidetes have also been associated with response (such as *A. muciniphila, B. longum, Bifidobacterium adolescentis*, and *C. aerofaciens*) and non-response (such as *Actinomyces viscosus* and *Garnderella vaginalis*) (Frankel et al., 2017; Gopalakrishnan et al., 2018; Matson et al., 2018; Routy et al., 2018). Overall, there is not a great deal of overlap between specific bacterial taxa associated with response across these published studies, although several taxa that are implicated with either response or toxicity are phylogenetically related (such as members of the Ruminococcaceae and Lachnospiraceae families and the Bacteroidales order) (Figure 2). Importantly, differences may be related to several different factors—including differences in techniques used to analyze samples and reference databases used for analysis, which varied widely across the studies (Chaput et al., 2017; Dubin et al., 2016; Frankel et al., 2017; Gopalakrishnan et al., 2018; Jenq et al., 2015; Matson et al., 2018; Peled et al., 2017; Routy et al., 2018)—suggesting the importance of developing standardized approaches for microbiome analysis. Geographical influences also may exist, as these studies were performed in centers at different locations around the world. In line with this, dietary and lifestyle factors may also account for some of the differences observed. Nevertheless, the impact of the gut microbiota on

Table 1. Manipulation of the Gut Microbiome to Enhance Responses to Cancer Immunotherapy

Trial Number	Patient Population	Intervention	Outcome(s)	Status
NCT02843425	all cancer patients treated at MDACC	addition of ½ cup beans per day to regular diet in a crossover design	**primary:** change in fecal microbiome profile from baseline (via 16S profiling)	open and recruiting (MDACC)
NCT02079662	stages II and III breast cancer patients treated at MDACC ages 18+	randomized intensive lifestyle change (diet, exercise, psychosocial)	**primary:** disease-free survival (DFS) **secondary:** change in fecal and oral microbiome (via 16S profiling)	open and recruiting (MDACC)
NCT01895530	CRC patients ages 18+ undergoing elective CRC resection	randomized probiotic (*S. Boulardii*) administration	**primary:** cytokine expression in colonic mucosa (via qPCR) **secondary:** post-operative complications	completed (Consoli et al., 2016)
NCT03072641	CRC patients ages 18+	randomized probiotic (ProBion Clinica *B. lactis* BI-04, *L. acidophilus* NCFM + Inulin) administration	**primary:** change in fecal and tumor microbiota from baseline **secondary:** changes in epigenetic patterns of tumor tissue from baseline	completed (Hibberd et al., 2017)
NCT03358511	post-menopausal breast cancer patients stages I–III	single-arm probiotic (Primal Defense Ultra multi-strain probiotic formula) administration	**primary:** change in mean number of CD8+ cells from baseline	open and recruiting (Mayo Clinic)
NCT02928523	acute myeloid leukemia patients ages 18–65 treated with intensive chemo and antibiotics	single-arm autologous FMT (frozen inoculum)	**primary:** diversity of the gut microbiome, multi-drug-resistant bacteria eradication **secondary:** signature of dysbiosis of gut microbiome	ongoing, closed to recruiting (France)
NCT03353402	metastatic melanoma patients ages 18+ who previously failed standard therapies	single-arm FMT (colonoscopy or gastroscopy) from patient donors who responded to immunotherapy	**primary:** safety (AEs associated with FMT), engraftment of FMT **secondary:** changes in immune cell populations and activity, objective response rate	open and recruiting (Israel)

therapeutic response is uncontested, and these data provide strong evidence that the gut microbiota can modulate anti-tumor immune responses and responses to immune checkpoint blockade.

Manipulating the Gut Microbiota to Improve Responses to Therapy

Given this growing body of literature, it is becoming increasingly clear that modulation of the gut microbiota may represent a novel and important adjunct to current anti-cancer therapeutic modalities. Although studies to further dissect the molecular interactions underlying the effects of the microbiota on cancer development and anti-tumor immune responses are underway, several ongoing and planned clinical trials will investigate the therapeutic potential of manipulation of the gut microbiota directly in cancer patients (Table 1).

Diet

A major function of the gut microbiota is to aid in host food digestion and harvesting of key nutrients that the host is incapable of metabolizing without the help of microbes (Backhed et al., 2005). However, dietary intake can also promote differential composition of the microbiome, with evidence that profound and intensive changes in dietary regimens can significantly alter the gut microbiota in a relatively short amount of time (David et al., 2014). The current body of literature in this area has mapped the responsiveness of specific bacterial groups and their downstream metabolites to a variety of nutrients and immune parameters, and provides preliminary insight into how dietary modulation could be used as a strategy to enrich the gut microbiome and immune health (Ma et al., 2018; Shortt et al., 2018), with parallels that can be drawn in the context of cancer treatment. Dietary fiber is one component that has been shown to have a profound influence on the composition of the gut microbiome, with a decrease in the abundance of immune-promoting *F. prausnitzii* (Benus et al., 2010) and the SCFA-butyrate in stool samples after a reduction in dietary fiber intake in healthy human subjects. Other studies have focused on supplementation of the diet with plant polysaccharide inulin prebiotics, demonstrating significant increases of both *Faecalibacterium* and *Bifidobacterium* species with this dietary intervention (Ramirez-Farias et al., 2009). Conversely, elimination of animal fats in the human diet was associated with a decrease in detrimental Bacteroidales bacteria (Turnbaugh et al., 2007). Given the importance of these bacteria in cancer therapy, it is possible that diet could serve as a possible strategy to improve outcomes through modulation of the microbiome.

The favorable safety profile, cost, and accessibility of dietary interventions could provide a simple and safe opportunity for assessing the implications of microbiota and downstream immune manipulation in cancer patient populations. Indeed, some groups have already begun to explore the dietary impact on the gut microbiota in cancer patients (Table 1). The "BE GONE" trial (NCT02843425) is designed to investigate fiber supplementation in cancer patients, through the addition of a half cup of beans per day into the normal diets of cancer patients to measure shifts in bacterial populations. Meanwhile, "The Role of

Lifestyle Factors in Breast Cancer-Related Outcomes" (NCT02079662) trial utilizes a comprehensive lifestyle overhaul, providing dietary counseling and meal delivery along with exercise and psychosocial services with a randomized design in patients with stage III breast cancer initiating radiation therapy. This study hypothesizes that women in the lifestyle intervention arm will have improved outcomes compared with those in the control arm. Although this trial is primarily powered to detect differences in recurrence rates, longitudinal gut and oral microbiome samples along with a battery of questionnaires, are listed as secondary outcomes in order to better gauge how the microbiome changes in relation to behavior patterns in cancer patients. Blood samples are also being collected at the same time points to gain insight into mechanistic changes associated with lifestyle modifications and microbial shifts over the course of the intervention and therapy. Although both studies are in their infancy, they will provide valuable information on how lifestyle factors modulate the gut microbiome, disease markers, and patient outcomes.

Administration of Bacterial Consortia or "Designer Probiotics"

Although dietary interventions may seem relatively simple to design and implement, the effects on the microbiota can be modest, and patient compliance is difficult to enforce and monitor. Administration of bacterial consortia or "designer probiotics" could provide a more feasible method of microbial manipulation in the clinical setting. Several trials using probiotics in cancer patients have been initiated with some completed, and most studies have focused on safety and biomarker-related endpoints, with a minority including cancer-related outcomes (such as disease-free survival) as a primary endpoint (NCT02079662).

Some of the first studies initiated involved treatment with probiotics in patients with colorectal cancer (CRC) (Table 1). This includes a trial where patients with CRC were treated with probiotics containing strains of *Lactobacillus acidophilus* and *Bifidobacterium lactis*, and were shown to have an increased abundance of butyrate-producing bacteria (particularly *Faecalibacterium* and other *Clostridiales*) within the tumor, and its associated non-tumor colonic mucosa and stool (NCT03072641) (Hibberd et al., 2017). Another study assessed preoperative probiotic therapy on mucosal immunity in CRC patients, demonstrating altered cytokine profiles within the colonic mucosa at the time of colon resection, with lower IL-1β, IL-10, and IL-23A mRNA levels in the patients treated with probiotics compared with controls who received no probiotics (NCT01895530) (Consoli et al., 2016). These studies demonstrate mixed changes in colonic mucosa—with a decreased production of both pro-inflammatory and anti-inflammatory cytokines within healthy colonic mucosa; thus, it is difficult to interpret what these findings might imply in regard to CRC development and progression or response to therapy. Nonetheless it is proof of principle that probiotic therapy can alter immunity locally.

In addition to these studies in CRC, there are ongoing trials assessing the impact of administration of probiotics on other cancer types, including a trial investigating the effects of probiotics on intratumoral $CD8^+$ T cell infiltrate in patients with stage I–III breast cancer (NCT03358511). This is a single-arm study and all patients will receive the same probiotic (Primal Defense Ultra multi-strain probiotic formula). Additional studies are currently in development, and it is important to highlight that assessment of changes to the microbiome as well as anti-tumor immunity in these studies is paramount. Certainly, there is wide variability in probiotic formulations available with regard to their composition, stability, and authenticity (Huys et al., 2013); thus significant caution should be taken in advocating their use to cancer patients until these can be carefully tested. Efforts to identify "ideal" bacterial consortia to be administered to cancer patients to enhance responses to cancer therapy are underway but have yet to be defined.

FMT

FMT represents the most direct means to manipulate the microbiota, and FMT preparations can be administered to patients via oral administration of lyophilized or frozen pills or via direct delivery by colonoscopy or gastroscopy. FMT has already been employed in other patient populations, showing significant success in curing *Clostridium difficile* infection resistant to conventional therapies (Borody et al., 2004).

Clinical trials utilizing FMT in cancer patients are in their infancy, but, based on results from pre-clinical studies discussed above, they have generated much excitement. Autologous FMT in acute myeloid leukemia is being trialed in patients undergoing intensive treatment in an attempt to prevent dysbiosis and to increase diversity of the gut microbiota during the course of treatment (NCT02928523). Furthermore, FMT is being considered in patients undergoing immunotherapy for solid tumor malignancies, specifically those treated with immune checkpoint inhibitors (Table 1). A phase 1 single-center trial for metastatic melanoma patients who failed prior immunotherapy opened recently (NCT03353402) wherein FMT from patients with a good response to immunotherapy is administered to refractory patients. Primary outcomes include safety and time to microbiota engraftment, while secondary outcomes include immune cell shifts, alterations in immune cell activity, and objective response. Design of additional trials is currently underway to test the hypothesis that modulation of the gut microbiota will improve response to treatment with immune checkpoint blockade (Gopalakrishnan et al., 2018).

Conclusions and Future Directions

The age of the microbiome is upon us, and seminal reports incorporating pre-clinical and clinical studies on the role of microbiota in cancer have brought this topic to light as a potentially dominant mediator in response to cancer therapy. We have gained insights into the influence of the microbiome on immunity and cancer—however, there is still a great deal to learn with regard to the inherent mechanisms, as well as optimal strategies to modulate the gut microbiome to enhance responses to cancer immunotherapy.

Provocative clinical questions are also raised from these studies and call into question the potential need and utility of microbiome profiling in patients on cancer therapy. However, complexities exist with regard to optimal methods for profiling (16S rRNA sequencing versus metagenomic shotgun sequencing and choice of reference databases). In addition to this, significant additional questions remain regarding how other factors affect the gut microbiome–such as diet, medications (including

probiotics, antibiotics, and other medications), mental health or other environmental factors–and how they affect cancer therapy and also call to question the potential need to monitor these factors during cancer therapy.

Furthermore, additional complexities exist as we move forward with efforts to modulate the gut microbiome to enhance therapeutic responses. It is not yet clear what composition of the gut microbiome is optimal to facilitate anti-tumor immune responses and a diverse range of therapeutic options exist to change the microbiome that need to be tested carefully in the context of clinical trials. The use of preparative regimens prior to modulation of the gut microbiome (e.g., with antibiotics) and methods to sustain changes (via dietary and prebiotic supplementation) is also of important consideration. It is only through a comprehensive understanding of these interactions (in preclinical models and in the context of these clinical trials) that we will learn to optimally modulate the gut microbiota to enhance anti-tumor immunity and immunity as a whole, with the potential to enhance immune surveillance and cancer treatment.

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DECLARATION OF INTERESTS

J.A.W. and V.G. are inventors on a US patent application (PCT/US17/53,717) submitted by The University of Texas MD Anderson Cancer Center that covers methods to enhance checkpoint blockade therapy by the microbiome. J.A.W. is a clinical and scientific advisor at Microbiome DX and a consultant at Biothera Pharma, Merck Sharp and Dohme. V.G. is a consultant at Microbiome DX. C.N.S., A.R., and B.A.H. report no relevant conflicts of interest or financial disclosures.

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