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Review

Cancer Research

Fine-Tuning Cancer Immunotherapy: Optimizing the Gut Microbiome

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Abstract

The equilibrium linking the intestinal microbiota, the intestinal epithelium, and the host immune system establishes host health and homeostasis, with perturbations of this balance resulting in chronic inflammatory and autoimmune immunopathologies. The mutualistic symbiosis between gut microbiota and host immunity raises the possibility that dysbiosis of the intestinal content also influences the outcome of cancer immunotherapy. Here, we present our recent findings that specific gut-resident bacteria determine the immunotherapeutic responses associated with CTLA-4 checkpoint blockade. This new evidence hints that interindividual differences in the microbiome may account for the significant heterogeneity in therapeutic and immunopathologic responses to immune checkpoint therapies. We discuss how this new understanding could improve the therapeutic coverage of immune checkpoint inhibitors, and potentially limit their immune-mediated toxicity, through the use of adjunctive "oncomicrobiotics" that indirectly promote beneficial immune responses through optimizing the gut microbiome. *Cancer Res; 76(16); 4602–7. ©2016 AACR.*

Introduction

The mammalian immune system is composed of a complex network of innate and adaptive elements that work together to overcome a diverse range of challenges. One of the greatest of these is the requirement for tolerance of the microbiota at mucosal barrier surfaces, allowing maintenance of host homeostasis in the face of potential microbial encounter. Perhaps unsurprisingly therefore, the evolutionary development of the host immune system has been closely associated with the microbial inhabitants of the gut and other surfaces, establishing a symbiotic relationship with the highly diverse microbiome (1). Just as the immune system shapes microbiome composition, the microbiota reciprocally stimulates and regulates multiple aspects of the immune system.

In line with this equilibrium, mounting evidence suggests alterations in the composition of the commensal microbiota are associated with a number of complex diseases, including asthma and allergy, inflammatory bowel diseases, metabolic and cardiovascular disorders, as well as cancer (2, 3). It remains unclear whether such alterations in the microbiome are a cause or consequence of these diseases; however, dysbiosis (and therefore potential exacerbation of disease) can additionally be caused by entry of pathogenic organisms and passenger commensals, aging, and environmental factors, such as diet, smoking, and medication (e.g., antibiotics; ref. 4). Several studies have shown how distinct bacteria, or bacterial products, can promote alterations of immune responses. Several families of bacteria, and metabolites from their bacterial breakdown of indigestible dietary components, have been shown to potentiate the induction of regulatory T cells (Treg), which are essential for the maintenance of gut tolerance (1). IL17-producing $CD4^+$ T helper cells (Th17) primed in the lamina propria to be specific for gut-resident segmented filamentous bacteria (SFB) are also able to exacerbate systemic autoimmune diseases in certain conditions (5). As a result, much effort has been dedicated to the identification of bacteria that may mitigate extraintestinal inflammatory diseases, with the ultimate scope to manipulate the gut microbiota for reducing adverse immune responses.

Dysbiosis and Cancer Therapy

In contrast to autoinflammatory and autoimmune diseases, cancer results from an exacerbated self-tolerance to cancer antigens. Given the recent progress in our understanding of the intertwined nature of the gut microbiome and the immune system, questions have arisen of how gut microbiota constituents could upregulate immune responses instigated by certain cancer therapeutics, and vice versa. We previously demonstrated that

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cyclophosphamide (an immunostimulatory alkylating agent used against multiple hematologic and solid malignancies) alters the microbiota of the mouse small intestine and causes translocation of certain Gram-positive bacteria, notably *Lactobacillus johnsonii* and *Enterococcus hirae*, into secondary lymphoid organs (6). There, these bacteria were observed to stimulate generation of a specific subset of "pathogenic" (p)Th17 cells (which share hallmarks of both therapeutically relevant Th17 and Th1 cells, such as the production of IL17 and IFNγ) and memory Th1 immune responses, underscoring how particular microbial components present in the gut lumen (and occasionally within lymphoid organs) can adjust the polarity of Th responses following cyclophosphamide treatment. Importantly, transfer of *ex vivo*–propagated pTh17 contributed to restoring the lost cyclophosphamide-mediated therapeutic effect in tumor-bearing mice that had been treated with antibiotics to kill Gram-positive bacteria (6). However, the mechanisms at play in this setting appear to be more complicated than they seem, as not all Gram-positive bacteria were able to elicit beneficial Th17/pTh17 immune responses. Moreover, certain bacterial species, such as *Parabacteroides distasonis* (which drive Treg effects) and SFB (which drive Th17 responses), dampened the therapeutic effects of cyclophosphamide administration.

A complementary study has shown that gut microbiota influences the antitumor effects of both oxaliplatin chemotherapy and an immunotherapeutic regime of CpG oligodeoxynucleotides with mAb blockade of the receptor for the immunoregulatory cytokine IL10 (7). Optimal oxaliplatin-based chemotherapy was found to require immune system detection of components of the gut microbiota, as mice lacking an intact microbiota or *MyD88* (that encodes critical components for innate immune system sensing of bacteria) had reduced oxaliplatin-mediated tumor infiltration by myeloid cells in comparison with their wild-type counterparts, in turn limiting the antineoplastic effects mediated by these cells. Similarly, CpG/anti-IL10R immunotherapy required the presence of commensal bacteria and TLR4 for optimal TNF production by intratumoral myeloid cells (7).

Alteration of the Gut Microbiota Impacts the Action of Immune Checkpoint Inhibitors

The findings from these earlier studies suggested that other cancer immunotherapies might also fall under the regulation of gut microbiota. Immunotherapy has been among the most exciting developments in cancer care over the past decade, the forefront of this success lying with immune checkpoint blockers (ICB). These inhibitors function as anticancer therapeutics by reactivating T cells driven to an ineffective state by the tumor microenvironment, thus allowing them to respond once again to tumor antigens (8). To date, mAb-mediated blockade of two checkpoints, cytotoxic T lymphocyte–associated protein 4 (CTLA-4) and the programmed cell death protein 1 (PD-1)/programmed cell death ligand 1 (PD-L1) axis, has seen the most clinical success (8), although other ICBs are in the development pipeline.

The preclinical evidence that anti-CTLA-4 antibodies achieve tumor rejection in animal models led to the development of ipilimumab, a fully human mAb directed against human CTLA-4 (8). Ipilimumab induced considerable improvement in the overall survival of patients with metastatic melanoma (9, 10) and has shown encouraging clinical responses in other cancers (8), leading to its approval by the FDA in 2011. CTLA-4, which is present in intracytoplasmic vesicles of resting T cells, is upregulated on T-cell activation and translocates to the plasma membrane to receive signals that ultimately maintain self-tolerance and prevent autoimmunity (11). Tregs express high surface levels of CTLA-4; thus, anti-CTLA-4 mAb therapy acts also to deplete these cells from the tumor microenvironment, resulting in a subsequent release in suppression of antitumoral CTL activity (12).

Anticancer efficacy of CTLA-4 blockade relies on the gut microbiota

Our latest research provides further insight into the therapeutic effects of CTLA-4 blockade, revealing that the immunostimulatory and antitumor effects of this ICB depend on distinct *Bacteroides* species of the gut microbiota (Fig. 1; ref. 13). We found that anti-CTLA-4 mAb loses its therapeutic efficacy against established sarcomas, melanomas, and colon cancers in mice reared under germ-free (GF) conditions or treated with broad-spectrum antibiotics. Coinciding with this, anti-CTLA-4–induced activation of splenic effector $CD4^+$ T cells was significantly suppressed compared with mice having normal [specific pathogen free (SPF)] microflora (or "eubiotic"), with reduced intratumoral accumulation of $CD3^+$ tumor-infiltrating lymphocytes (TIL), Th1 cells, and CTLs. Evidence of similar findings following treatment with select antibiotics implicated Gram-negative bacteria in the anticancer effects of CTLA-4 blockade.

We next focused on potential anti-CTLA-4–mediated effects at the gut–microbiota interface, this being where dysbiosis is initially sensed by the immune system and equally where ipilimumab can cause immune-related adverse events (irAE; ref. 14). Alteration of the mucosal barrier occurred following administration of anti-CTLA-4 mAb in mice, consistent with a "subclinical colitis." This pathology was more prominent in SPF than in GF animals, suggesting a role for gut-resident commensals. Anti-CTLA-4 mAb treatment additionally increased the proliferation and the T cell–mediated apoptosis of intestinal epithelial cells (IEC). We used intestinal crypt–derived enteroid cultures to show that this apoptosis is inducible by intraepithelial lymphocytes (IEL) from anti-CTLA-4 mAb–treated (but not isotype mAb-treated) mice and that it also requires the presence of microbial products. Coinciding with the perturbed mucosal barrier, FISH analysis revealed an accumulation of distinct *Bacteroides spp*. in the ileum (corroborated by qPCR of the mucosal-associated bacteria), potentially within reach of mucosal dendritic cells (DC). Taken together, these findings signified that CTLA-4 mAb induces a dysregulation of the equilibrium among IECs, IELs, and the microbiota at the intestinal barrier.

To identify particular bacterial species affected by this dysregulation, we performed high-throughput pyrosequencing of 16*S* ribosomal RNA gene amplicons from murine feces. This analysis indicated that a single injection of anti-CTLA-4 mAb could significantly affect the microbiome at the genus level. CTLA-4 blockade induced a rapid underrepresentation of both *Bacteroidales* and *Burkholderiales* family members in feces (corroborated by qRT-PCR analyses), with a relative increase in *Clostridiales*. Using this information, we investigated how recolonization of antibiotics-treated or GF mice with a selection of *Bacteroides* and *Burkholderia spp.* might affect the antitumoral efficacy of anti-CTLA-4 mAb. *Bacteroides fragilis*, *Bacteroides thetaiotaomicron*, *Burkholderia cepacia*, and a combination of

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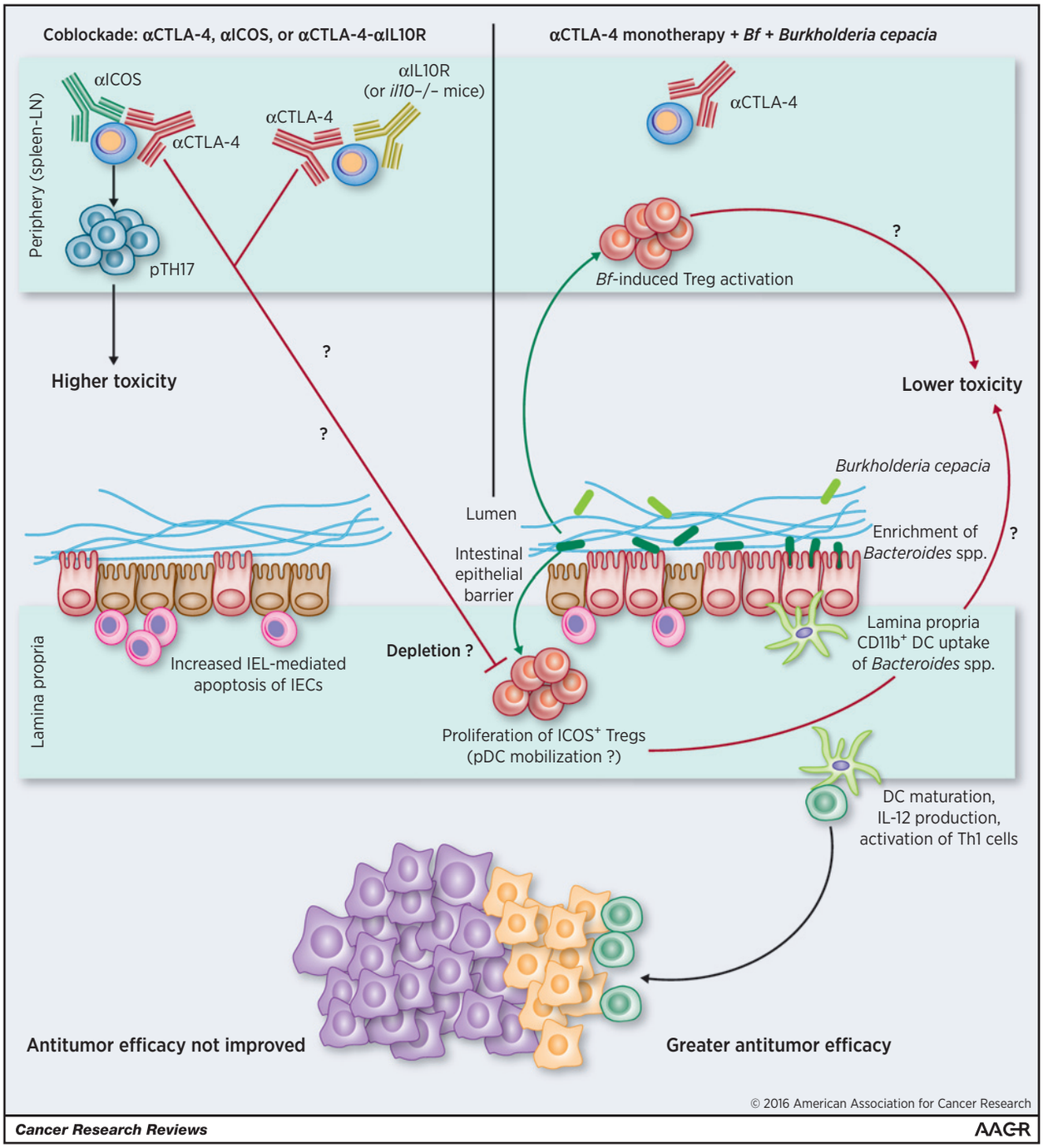

Figure 1.

Certain gut microbiota determine the level of efficacy and toxicity during CTLA-4 checkpoint blockade. Cancer immunotherapy with anti-CTLA-4 antibodies modulates the microbiota–intestinal barrier equilibrium by inducing intestinal epithelial cell (IEL)–mediated apoptosis of IECs, resulting in barrier perturbation. Barrier perturbation is increased further during coblockade of IL10 signaling or ICOS signaling in experimental settings [possibly due to pathogenic Th17 cells (pTh17)], resulting in higher gut toxicity resembling early signs of colitis (left). (Re)colonization of antibiotics-treated mice with *B. fragilis* (*Bf*) and *Burkholderia cepacia* reduces anti-CTLA-4 mAb–induced toxicity [possibly through the capacity of *B. fragilis* to promote the proliferation of ICOS$^+$ Treg in the lamina propria, via plasmacytoid DC (pDC) mobilization], while maintaining strong antitumor efficacy (right). Increased uptake of bacterial species, such as *B. fragilis*, by lamina propria DCs, or a potential uptake by DC of soluble bacterial products, results in DC maturation and IL12 production, in turn allowing for priming/activation of T cells, such as Th1 cells (facilitated by the ongoing immune checkpoint blockade). These T cells, which are presumably cognate for tumor antigens or cross-reactive bacterial antigens, participate in antitumoral immune responses.

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B. fragilis and *B. cepacia* were each identified as species that could restore anti-CTLA-4 mAb–mediated anticancer responses, contrasting with several other isolates that failed to do so. Notably, the restoration of efficacy seen on oral feeding with *B. fragilis* correlated with induced Th1 immune responses in tumor-draining lymph nodes and greater DC maturation in tumor beds. In addition, we identified that the microbiota-dependent immunostimulatory effects produced by CTLA-4 blockade required IL12, with this cytokine likely produced by mobilized *B. fragilis*–stimulated $CD11b^+$ DC from the lamina propria.

Next, we searched for memory T-cell responses specific for the most immunomodulatory species among *Bacteroidales* during CTLA-4 blockade in mice and humans (15–20). $CD4^+$ T cells harvested from spleens of anti-CTLA-4 mAb–treated mice or from blood taken from ipilimumab-treated cancer patients were restimulated with various strains of *Bacteroides* loaded onto DCs. Both human and mouse memory Th1 responses consisting in the production of IFNγ were observed against *B. fragilis* and *B. thetaiotaomicron*, with little concomitant IL10 production. Of note, no memory response could be detected in recall responses to *B. distasonis* or *B. uniformis* (two main commensal bacteria from the gut metagenomic core; ref. 21) in anti-CTLA-4 mAb–treated mice. Together, these findings suggested that anti-CTLA-4 mAb elicited immune responses against some *Bacteroides* species that may, in turn, have skewed the intestinal microbiome repertoire.

To confirm that these findings were of clinical relevance, we analyzed the gut microbiome composition in metastatic melanoma patients before and after ipilimumab treatment. Three distinct microbiome clusters were revealed, for which segregation was determined by the *Bacteroides* and *Prevotella* genera (*Alloprevotella*/*Prevotella* driving cluster A and distinct *Bacteroides* spp. driving clusters B and C). We next performed fecal microbial transplantation of feces representing each cluster into GF mice that were subsequently treated with anti-CTLA-4 mAb. In this model, the microbial composition of cluster C, rich in immunogenic *Bacteroides* species (e.g., *B. fragilis*), was seen to restore anti-CTLA-4 mAb efficacy, while cluster B (enriched in tolerogenic *Bacteroides* species, including *Parabacteroides distasonis* and *Barnesiella intestinihominis*) resulted in a complete resistance to treatment. This highlighted the possibility that ipilimumab may facilitate its antitumor efficacy through adjusting levels of immunostimulatory *Bacteroides* spp. in the gut.

Distinct gut microbiota may uncouple CTLA-4 blockade toxicity from its efficacy

Given the exceptional clinical outcomes and lengthened overall survival that can be induced by ipilimumab, it is unfortunate that many patients receiving this immunotherapy develop irAEs, which often dictate the cessation of treatment (14). Remarkably, we identified that recolonization of antibiotics-treated animals with *B. fragilis* and *B. cepacia*, mandatory to restore anti-CTLA-4 efficacy, failed to induce signs of "subclinical" colitis on CTLA-4 blockade but instead afforded protection against anti-CTLA-4–induced intestinal lesions. This protection may be associated with the capacity of *B. fragilis* to promote the proliferation of $ICOS^+$ Treg in the lamina propria, possibly via mobilizing plasmacytoid DC seen to accumulate and mature in mesenteric lymph nodes after *B. fragilis* monocolonization of GF mice treated with anti-CTLA-4 mAb (13, 15). In support of this, blockade of ICOS or IL10 plus anti-CTLA-4 mAb treatment resulted in an overt and deadly colitis in tumor bearers reared in SPF conditions. Therefore, efficacy and toxicity following CTLA-4 blockade could be uncoupled in this model of *B. fragilis and B. cepacia* bicolonization. Supporting these experimental findings, a recent report described the protective role of the *Bacteroidetes* phylum (namely *Barnesiellaceae unclassified, Rikenellaceae unclassified*, detected by 16*S* ribosomal RNA sequencing of feces) against the development of colitis during ipilimumab therapy in melanoma, associated with modules of genes encoding the spermidine/putrescine polyamine transport system and biosynthesis of B vitamins (detected by shotgun metagenomic sequencing analyses; ref. 22).

A role for other gut microbiota in the efficacy of immune-mediated tumor control and other ICBs

Like ipilimumab, antibodies that inhibit human components of the PD-1/PD-L1 signaling pathway have also been subject to successful clinical testing, with two anti-PD-1 antibodies, pembrolizumab (23) and nivolumab (24), having already received FDA approval for several cancers. These therapies inhibit the interaction between PD-L1, present on the surface of tumor or antigen-presenting cells, and PD-1, present on the surface of activated T cells, and in doing so overcome the suppression of antitumor T-cell activity mediated by this pathway (8). In a parallel study to ours, a role for other distinct gut microbiota has been identified for host tumor control and response to anti-PD-L1 mAb immunotherapy (25). In this study, Sivan and colleagues compared the antitumor CTL responses in genetically similar C57BL/6 tumor bearers derived from two different mouse facilities with differing microbiota. Comparison of mice from the Jackson Laboratory and from Taconic Farms revealed significant differences in the growth kinetics of subcutaneously implanted melanomas, with more aggressive tumors in Taconic Farms mice attributable to lower tumor-specific T-cell responses, and poor intratumoral accumulation of CTLs. The aggressive tumor growth in Taconic Farms mice was reduced following either fecal microbiota transplantation from The Jackson Laboratory mice or following cohousing of Taconic Farms mice with The Jackson Laboratory mice. 16*S* ribosomal RNA sequencing identified *Bifidobacterium* to be associated with the enhanced tumor control, confirmed by findings that oral feeding of Taconic Farms mice with *Bifidobacterium* restored CTL responses and tumor control. An enhanced activation of the processing and presentation machinery of intratumoral DCs mediated these effects. Notably, *Bifidobacterium*-induced TIL enrichment of tumors also facilitated an augmentation in antitumor responses mediated by anti-PD-L1 mAb immunotherapy (25).

Implanting a Gut Microbiota That Advances Therapy Outcome

Although anticancer therapy with ICBs has seen unprecedented clinical efficacy, a major drawback is that they fail to control neoplasia in the majority of patients and are often associated with irAEs that require clinical management (26). As described, this variability in clinical responses suggests that additional host factors must influence ICB activity, with interindividual microbiome differences potentially having a significant impact here.

It is interesting to note that the recent studies into the microbiota's impact on immunotherapy each differ with respect to the

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specific bacteria identified that cause these effects. In addition, whereas an optimized gut microbiota augmented the antitumor response to anti-PD-L1 mAb in one case (25), this was absolutely required for anti-CTLA-4 mAb efficacy in our study (13). Although this may reflect background microbiome differences between the C57BL/6 strains from distinct commercial suppliers, there is a complexity here that must be addressed in future studies. Nonetheless, a similar observation between the above-mentioned checkpoint blockade studies was that translocation of bacteria did not occur after immunotherapy (13, 25) and that microbial-dependent DC activation (i.e., increased MHC-I and MHC-II expression, IL12 production) appears to be indispensable for the anticancer efficacy of ICBs. An additional possibility, beyond providing these secondary costimulatory signals for T-cell priming (i.e., via activation of TLR/NLR/inflammasome innate signaling pathways), is that immunogenic bacteria might provide a primary cognate MHC–peptide complex interaction with the TCR to elicit adaptive immune responses that can crossreact with tumor antigens (27–29). This theory warrants investigation into shared epitopes between commensals and tumor antigens that could engage cross-reactive TCRs of high avidity.

Putting these studies together suggests that a dedicated selection of a particular bacterium or a select cocktail of bacteria and/or bacterial products may be required for clinical use beside individual or combined ICBs (and other immunotherapies), as adjunctive "oncomicrobiotics." The future implementation of oncobiotics in microbiota-conditioning strategies will depend largely upon an expansion in our knowledge regarding cancer-associated intestinal dysbiosis and how interindividual differences may influence immunotherapy efficacy and toxicity, this making another step toward truly personalized cancer therapy. Toward achieving this, integrated catalogs of reference genes of the human gut microbiome have recently been reported from metagenomics and metatranscriptomics analyses from the Meta-HIT and the HMP projects (30, 31), alongside "culturomics" coupled with MALDI-TOF mass spectrometry (32). When an optimized microbiota has been identified for a given immunotherapeutic, the corresponding oncomicrobiotic will presumably be formulated as one or more of the following clinical products: (i) formulations of live immunogenic commensals (i.e., probiotics), given (re)colonization with live (and not dead) immunogenic bacteria is required to reinstate anticancer adaptive T-cell responses (6, 13, 25); (ii) derivatives from such commensals or other bacterial products; and (iii) antibiotics that selectively eliminate immunosuppressive microbes and those identified to be detrimental to antitumoral immune activity or that contribute to immunotherapy-associated irAEs.

Conclusions

The recent evidence presented by our group and others, revealing that constituents of the gut microbiota facilitate the efficacy of CTLA-4 blockade and other immunotherapies, presents an important new concept through which the heterogeneity of antitumor immunity observed in the clinic may be explained. These findings may also point to direct implications in the clinical management of cancer patients receiving immunotherapy (e.g., the choice and usage of antibiotics regimens) and have potentially opened up a new field of therapeutic research, a field of "adjunctive oncomicrobiotic," aimed at strengthening and maintaining therapeutically mediated immune responses against cancer. Hence, there now exists the possibility to (re)establish a favorable enteric microflora in patients with an ineffective microbiome (e.g., dysbiosis), which may otherwise be associated with poor prognosis to immunotherapy or with therapy-mediated toxicity. The search is now on for microbes that maximize the clinical therapeutic coverage and benefit of cancer immunotherapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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