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Authors

Humphries, Audrey

Daud, Adil

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COMMENTARY



The gut microbiota and immune checkpoint inhibitors

Audrey Humphries  and Adil Daud

Department of Medicine, Division of Hematology/Oncology, University of California San Francisco, San Francisco, CA, USA

ABSTRACT

Although immunotherapy has been remarkably effective across multiple cancer types, there continues to be a significant number of non-responding patients. A possible factor proposed to influence the efficacy of immunotherapies is the gut microbiome. We discuss the results and implications of recent research on the relationship between the gut microbiome, our immune systems, and immune checkpoint inhibitor therapies including anti-CTLA-4 Ab and anti-PD-1 Ab. While the investigations all exhibit interesting results and conclusions, we find little congruence in the specific bacteria that were found favorable for antitumor responses. It is unclear whether the inconsistencies are due to differential approaches in study design (pre-clinical or clinical subjects, anti-CTLA-4 Ab or anti-PD-1 Ab), experimental methods and measurements (metagenomics sequencing and clustering variations) or subject population dynamics (differential cancer types and baseline characteristics). Moreover, we note studies regarding particular bacterial commensals and autoimmune diseases, which challenge findings from these investigations. We conclude that with the current research, clinical investigators can appreciate the critical role of gut microbiota in mediating immunostimulant response. However, prospective research exploring the biochemical mechanisms which commensal bacteria communicate with each other and the immune system is imperative to understand how they can be adjusted properly for higher immunotherapy response.

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In recent years there has been increasing recognition that humans harbor an extensive microbial community within the gut and skin, and that alterations of this flora can lead to profound alterations in health. Amongst the contributions of our extensive population of microbial flora, currently estimating to be 30 trillion microbes per human,¹ is an important role in the regulation of the immune system.² Thus, upon the development of immune checkpoint inhibitors for melanoma and other cancers, there was interest amongst translational researchers to explore how the gut microbiota interacts with these agents and whether these interactions influence the safety and/or efficacy of these drugs. In this commentary, we examine the research that links gut flora with response to immune checkpoint inhibitor therapy.

Authored by Zitvogel and colleagues in 2015, one of the earliest publications examined the relationship between the gut microbiota and immunotherapy, specifically between commensal bacteria and anti-Cytotoxic T-lymphocyte Associated Protein 4 (CTLA-4) antibody (Ab) treatment. In mice reared under either specific pathogen free (SPF) conditions, germ-free (GF) conditions, or in antibiotic-treated mice models, the authors found that the efficacy of anti-CTLA-4 Ab was impaired without the presence of gut commensal bacteria and was significantly higher with the colonization of two species from the Bacteroidales order (Bacteroidetes phylum) and one species from the Burkholderiales order (Proteobacteria phylum). Additionally, these studies suggested that two species

from the Bacteroidales and Burkholderiales order significantly reduced the histopathological signs of colitis,³ a common, high-risk, immune-related adverse event associated with anti-CTLA-4 Ab therapy.^{4–7} While there is supportive evidence that species from the Bacteroidales order are associated with decreased incidence of colitis in patients administered anti-CTLA-4 Ab,⁸ there is also data correlating the colonization of certain Bacteroidales species in the gut with colitis^{9,10} and Crohn's disease.^{11,12} Inconsistencies and multiple variables in comparative research force the analysis of factors which might confound the results in this publication, including the possibility of antibiotic resistance and inexplicit microbial baseline values in antibiotic-treated mice models.¹³ Additionally, while mice models are crucial, as they enable experimental procedures that cannot be replicated easily in humans, data extrapolation from mice studies into humans is difficult and fraught to repeat and transfer without accumulating additional variables. Not only are the anatomical structures of a human and mouse gastrointestinal tract and intestinal wall linings significantly different,^{14–16} but it has also been observed that eighty-five percent of gut-colonizing microbes in mice are not found in humans.¹⁷ Furthermore, if prospective research showed that administering live bacterial cultures to patients before and after anti-CTLA-4 Ab treatment yielded significantly favorable results, variables including microbial shifts in species distribution due to individual host diet or lifestyle^{18–21} and interspecies interactions such as competitive exclusion influenced by host gut motility²² between

foreign cultures and baseline microbiota should be considered. Interestingly, this publication demonstrated that after anti-CTLA-4 Ab administration in metastatic melanoma (MM) or non-small cell lung cancer (NSCLC) patients, the population of one microbial group increased in size at the expense of another group, suggestive of interspecies competitive exclusion. Understanding the significance and molecular processes involved in anti-CTLA-4 Ab driving this adjustment in gut bacteria population dynamics might give insight to the overarching mechanisms behind bacteria-mediated boosts in antitumor immune activity.

In 2015, Gajewski and colleagues published research suggesting that commensal microbial compositions might promote the response to immunotherapy.²³ Through noted differences in immune checkpoint response in mice reared in different microbial flora colonies and fecal microbial transplantation (FMT) experiments, they found the bifidobacterium species (Actinobacteria phylum) to be significantly more abundant in mice that had higher antitumor responses to anti-Programmed Cell Death-1 (PD-1) Antibody (Ab).²³ Gajewski and colleagues added to their previously published research in their recent publication where they analyzed baseline fecal samples from metastatic melanoma patients treated with anti-PD-1 Ab. Through both 16s ribosomal RNA (rRNA) sequencing and shotgun sequencing, they concluded that a patients' gut microbiome composition, with the bifidobacterium species being highly overrepresented in responding patients, often predicted their outcome to immunotherapy.²⁴ Given the significant differences between the mechanism of action of anti-CTLA-4 Ab versus anti-PD-1 Ab, (the former work at the priming stage of the immune response while the latter are predominantly involved peripherally activating "exhausted" or chronically activated T-cells)²⁵ the differences between the microbial flora associated with response to immunotherapies are interesting. Other known distinctions between the CTLA-4 and PD-1 receptor proteins include their membrane location and endocytosis (for CTLA-4), immune cell location, associated antigen presenting cells, and the phase in which each regulates T-cell proliferation.²⁶ Moreover, whether the impact of commensal bacteria on systemic immunity in humans is dependent solely on bacterial characteristics or rather on the host-bacterial ecosystem remains a critical question for prospective research.

To better appreciate the functional biomolecular mechanism by which the bifidobacterium species instigate an immune anti-tumor response either alone and in combination with anti-PD-1 Ab, Spranger et al. contextualized the research done by Gajewski and colleagues with other findings. Predominantly based off the function of genes expressed on tumor infiltrating dendritic cells (DCs) of bifidobacterium-hosting mice, critical immunologic processes for tumor detection and destruction were present. While the proposed model is provisional, the authors emphasize that certain dendritic cells are clearly dependent on bifidobacterium species for proper priming and proliferation of CD8+ effector T cells.²⁷ Supporting the authors' claims, some bifidobacterium species have been recognized as immunomodulators.^{28,29} However, at what concentration or in what situation do gut microbiota stimulate the immune system so that healthy tissue is at stake? As other investigations reveal that certain bifidobacterium species influence the development

of autoimmune thyroid diseases³⁰ and allergic disorders in infants and children,^{31,32} further research, especially research focusing on the factors that establish the immunomodulating function of the microbiome, is clearly required to resolve the inconsistencies amongst present data.

Interestingly in contrast, research by Rutkowski et al. in 2015 revealed evidence of TLR-5 dependent commensal bacteria, which are species originating from the Bacteroidetes and Tenericutes phylum, stimulating the growth of tumor cells. Through increasing IL-6, these microbes send a cascade of signals that increase the concentration of suppressor Treg-cells in the tumor microenvironment.³³ Although apparently contradictory, the diversity of the gut microbiome could include some species of bacteria as suppressive while others could function to stimulate an immune inflammatory response.³⁴ Rutkowski and colleagues did not find a significant difference in the quantity of PD-1 between Tlr5-/- and wild-type mice models, however, it could be of further interest to measure responses to anti-CTLA-4 Ab. Even more intriguingly, the functional role of TLR-5 immunosuppressive bacteria might be useful for research regarding the efficacy of immunosuppressive drugs such as anti-CD3 mAb, or teplizumab, supported by preliminary investigations focusing on the synergistic functionality between gut commensal bacteria and immunosuppressive drugs.^{35,36} A study by Herold and colleagues in December 2017 found systemic immune activation in humanized mice models given anti-CD3 mAb in combination with antibiotics, shown through elevated numbers of effector T-cells and IFN- γ , decreased production of IL-10, and the presence of anti-nuclear antibodies.³⁵ While there are other investigations demonstrating that gut dysbiosis, or a microbial imbalance or modification in the host gut, may counteract the effects of both immunosuppressive³⁵ and immunostimulatory drugs,^{3,23,27,37,38} it may be that microbiota have a more potent or direct interaction with the immune checkpoint drugs rather than the immunomodulatory cells involved per se. All in all, these apparent contradictions reinforce that the function and mechanisms by which commensal bacteria communicate with the immune system are poorly understood and remain a fascinating challenge for prospective researchers.

Expanding current knowledge on what explicit profile of commensal bacteria governs a favorable response to anti-PD-1 Ab, Zitvogel and colleagues published another interesting report in November 2017 suggesting that the efficacy of anti-PD-1 Ab depends on the host gut microbiome. Utilizing quantitative metagenomics by shotgun sequencing, fecal matter from 67 renal cell carcinoma (RCC) and 140 NSCLC patients was collected before and after anti-PD-1 Ab administration. In responders, there was significantly more bacteria from the species *Akkermansia Muciniphila* (Verrucomicrobia phylum), the genus *Alistipes* (Bacteroidetes phylum), and more generally in the phylum Firmicutes.³⁷ The microbiome composition result of anti-PD-1 Ab responders found in mice by Gajewski and colleagues is different from that of human patients in this publication. As previously discussed, animal studies are inherently different and thus yield results which cannot be easily applied to human studies. Additionally, differences between how bacteria are tested and measured from fecal samples among researcher teams complicates formal comparisons, as there is

currently no single, standardized test for analyzing bacterial metagenomics and an unclear comparative system declaring which phylogenetic level should be measured. For instance, while Gajewski and colleagues approach genus level analyses with 16s rRNA sequencing, Zitvogel and colleagues utilize shotgun sequencing of larger DNA strands, incorporating assessments at the family, genus, and species level. Advantages in gene prediction, accurate bacterial species detection, and thorough diversity measurements in shotgun sequencing DNA have been recognized over 16s rRNA sequencing.³⁹ Furthermore, there are variations in the techniques of 16s ribosomal RNA sequencing and many proposed algorithms for clustering of genetic sequences into operational taxonomic units (OTUs) to measure the diverse profile of the microbiome, some of which are found to have a negative impact on downstream analyses.⁴⁰⁻⁴⁸ Zitvogel and colleagues proceeded to demonstrate the functional relationship between microbiota and antitumor T-cell immunity by performing a series of FMT tests on mice using the patient stool samples. One involved administering the stool from 8 NSCLC patients, including both responders and non-responders, in the form of oral gavages to mice inoculated with MCA-205 sarcoma cells. Even though the test yielded positive results, it is unclear why the authors used sarcoma rather than NSCLC mice models, given that the hosts had NSCLC. Comparable to what was discovered in their 2015 publication, the authors measured an increase in stool richness, at the metagenomics species level, of the NSCLC and RCC patients after two months since their first injection of anti-PD-1 Ab. Moreover, a response to anti-PD-1 Ab has been shown to be associated with significantly higher alpha diversity of gut microbiota³⁸ and interestingly, increased levels of CTLA-4^{hi} PD-1^{hi} CD8+ T-cells.²⁵ Corresponding proportions are thus suggestive of a situational positive feedback mechanism where TILs or other systemic lymphocytes signal the proliferation of certain bacteria or possibly enrich the ecosystem in the gut to establish a more tolerable living environment for the gut microbiome.

Finally, we described the 2017 publication by Wargo and colleagues, which argues that the gut microbiome is so closely associated with the immunomodulatory system that certain microbiota profiles can essentially indicate what kind of response patients have to immunotherapy. Through comparing fecal samples from 112 MM patients taking anti-PD-1 Ab therapies, Wargo and colleagues found that responders had a significantly higher abundance of commensals of the Clostridiales order and the Bacteroidales order, belonging to the Firmicutes phylum and Bacteroidetes phylum respectively.³⁸ Even though there was a clear difference in the cancer types studied between this publication and the 2017 publication by Zitvogel and colleagues, there were key similarities between the microbial profiles associated with response. Therefore, the species of commensal bacteria found essential for response may have little to do with the type of cancer which anti-PD-1 Ab targets. If this is the case, then what characterizes the profile of gut microbiota that assists in eliciting response to immune checkpoint inhibitors? After this preliminary question is answered, investigators might have a better understanding of how the gut microbiota can be manipulated for therapeutic benefit. As discussed previously, Wargo and colleagues also measured a high incidence of

alpha diversity in the gut microbiome of anti-PD-1 Ab responders. As much of the current research regarding specific microbiome species or profiles with response from immunostimulants is contradictory, the level of bacterial diversity could play a more critical role than a specific species or enterotype. However, Frankel et al. in 2017 detected the opposite to Wargo and colleagues, where there were no significant differences in gut microbial diversity between responders and non-responders of immunostimulants.⁴⁹ This raises the question of how the baseline diversity of both research groups of non-responders and responders from each publication compared, especially while incorporating patient demographics. Moreover, it would be reasonable to measure the average baseline differences of an individual's profile of gut microbiota relatively amongst different sexes, ages, health conditions, lifestyle, and environment to develop a more standard measurement for future FMT investigations.

Clearly, the identification of molecular mechanisms that facilitate microbial communication with immunomodulatory cells is critical to further our understanding of how to functionally characterize and measure the role of commensal bacterial communities in mediating immunotherapy success. Our review of literature that has been published, while encouraging, reveal several inconsistencies in the results of recent research publications. Thus, it is established that the relationship between commensal gut bacteria and the immune system has only begun to be appreciated and remains poorly understood. Prospective research studies directed at understanding both the functional properties of different gut microbiome species and the mechanisms by which certain commensal communities interact with the immune system will allow us to better characterize, measure, and ultimately manipulate the human gut microbiome to improve patient response to immune checkpoint inhibitors.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

ORCID

Audrey Humphries  <http://orcid.org/0000-0002-0605-8460>

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