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Checkpoint Checkmate: Microbiota Modulation of Cancer Immunotherapy

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In recent years, immune checkpoint inhibitors (ICIs)[5], which block the ability of cancer cells to evade killing by $CD8^+$ T cells, have begun to revolutionize cancer treatment. Since 2011, 6 different ICIs targeting T-cell (PD-1 or CTLA-4) or tumor (PD-L1) surface proteins have been approved for the treatment of several cancers, including non-small cell lung cancer and metastatic melanoma, with dozens of additional clinical trials ongoing. However, despite impressive clinical outcomes in some patients, the efficacy of ICI therapy remains highly variable, and the key drivers of this heterogeneity are not fully understood. Three recent articles *(1–3)* provide intriguing and compelling evidence that patients' gut microbiotas, or the communities of microbes that inhabit their gastrointestinal tracts, affect their responsiveness to ICIs. Here, we highlight key elements of these studies and discuss outstanding questions and future directions.

The mammalian gut is home to a complex microbial ecosystem that continuously interacts with and regulates its host's immune system. Early in life, the developing gut microbiota helps train and shape the immature immune system, and improper immune responses later in

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life (e.g., Crohn disease and allergies) are often associated with an altered gut microbiota. Similarly, germ-free and antibiotic-treated animals are functionally deficient in both innate and adaptive immunity. Although the human gut microbiota is consistently dominated by 2 phyla, Firmicutes and Bacteroidetes, no microbial strains are universally conserved among all individuals. Thus, interpersonal variation within the gut microbiota is 1 plausible explanation for the range of clinical outcomes observed during ICI therapy.

This putative connection between the microbiota and immunotherapy was greatly strengthened in 2015 with the observation that specific gut bacteria can influence ICI efficacy in preclinical mouse models of cancer. These 2015 studies did not encompass data from patient cohorts, but in 2018, 3 articles by Routy et al. *(1)*, Gopalakrishnan et al. *(2)*, and Matson et al. *(3)* independently extended this paradigm to human patients undergoing anti-PD-1 immunotherapy, 1 of the most widely used ICIs. Despite some differences in methodologies and conclusions (Table 1), all 3 studies followed a similar framework. In each study, patients' stool samples were collected prospectively before ICI therapy and sequenced to identify specific microbial signatures associated with ICI responders and nonresponders. To establish causation vs correlation, these stools—and, in some cases, individual discriminatory taxa—were used to produce microbiota-humanized mice by fecal microbiota transfer (FMT) into germ-free or antibiotic-treated animals. These mice were then challenged with cancer xenografts and monitored for tumor progression and responsiveness to ICIs. Finally, the authors surveyed the immunophenotypes of these animals to establish putative mechanisms by which the microbiota might mediate the effectiveness of checkpoint blockade.

Routy et al. observed that cotreatment with antibiotics significantly accelerated tumor progression in cancer xenograft mice receiving ICI therapy. This led the authors to study a cohort of human patients undergoing anti-PD-1 or anti-PD-L1 therapy for non–small cell lung cancer, renal cell carcinoma, or urothelial carcinoma, approximately one-third of whom had been given antibiotics for various conditions. Patients who received antibiotics immediately before or during ICI therapy had greatly diminished survival relative to matched controls (median overall survival of 11.5 months vs 20.6 months, respectively). In these cohorts, 2 specific bacterial species, *Akkermansia muciniphila* and *Enterococcus hirae*, were particularly predictive of ICI responsiveness, and germfree mice colonized with these bacteria or with responder FMTs experienced significantly diminished tumor growth in multiple xenograft models. Remarkably, the addition of these 2 species to mice treated with antibiotics or colonized with microbiotas from nonresponding patients was sufficient to restore efficacy of anti-PD-1 therapy. These phenotypes were attributed to the enhanced recruitment and infiltration of specific $CD4^+$ helper T-cell subsets into tumors, although the mechanisms by which *A. muciniphila* and other commensals drive this phenomenon remain unclear.

Gopalakrishnan et al. further established the importance of the gut microbiota to successful immunotherapy. In this study, patients with metastatic melanoma responsive to anti-PD-1 therapy harbored significantly more diverse microbiotas than did nonresponders, and patients with a high abundance of a single bacterial genus, *Faecalibacterium*, were more than twice as likely to remain progression-free after 600 days than were patients with low

Faecalibacterium abundance. Germ-free mice colonized with responder FMTs, injected with melanoma cells, and treated with anti-PD-L1 checkpoint blockade had significantly smaller tumors—and significantly greater $CD8^+$ T-cell infiltration into those tumors—than did mice that received nonresponder FMTs. Importantly, *Faecalibacterium* abundance was directly correlated with $CD8^+$ T-cell intrusion in these animal models.

Finally, in a separate cohort of metastatic melanoma patients, Matson et al. identified 8 and 2 taxa preferentially enriched in anti-PD-1 responders and nonresponders, respectively. As in the studies described above, FMT into germ-free mice recapitulated donors' phenotypes, and several of the species associated with anti-PD-1 responsiveness (e.g., *Enterococcus faecium* and *Collinsella aerofaciens*) have been previously associated with T-helper 1 (T_H1) polarization, Forkhead box P3 ($FoxP3^+$) regulatory T-cell reduction, and other aspects of robust antitumor immunity. Indeed, the authors documented increased $CD8^+$ T-cell infiltration into tumors of mice colonized with responder FMTs, which was similarly observed in their human cohort.

Collectively, Routy et al., Gopalakrishnan et al., and Matson et al. strongly suggest a role for the commensal microbiota in modulating the outcome of ICI therapies in human cancer patients. However, although some general trends (e.g., overrepresentation of phylum Firmicutes in responders) were independently observed, the 3 studies did not converge on the same specific causal taxa and, in some cases, yielded seemingly contradictory results. For example, although both studies examined anti-PD-1 responsiveness in metastatic melanoma patients, Gopalakrishnan et al. identified the family Ruminococcaceae as enriched in responders, whereas Matson et al. found the species *Ruminococcus obeum* (of the Ruminococcaceae family) to be predictive of nonresponders. Similarly, Gopalakrishnan et al. consistently associated *Bacteroides* spp. with nonresponsiveness, whereas Routy et al. found some *Bacteroides* spp. to be enriched in responders and others in nonresponders. It is unclear whether these discrepancies stem primarily from differences in cohort composition or in methodology (e.g., patients' diets and treatment schedules, sequencing platforms, and analysis pipelines), but they highlight the importance of species- and strain-level identification. More broadly, the correlation between ICI responsiveness and microbial richness (Routy et al.) and diversity (Gopalakrishnan et al.) suggests that the overall architecture of the microbiota, rather than the relative representation of specific taxa, may best differentiate responders from nonresponders.

Although all 3 studies observed microbiota-dependent infiltration of T cells into tumors, a prerequisite for successful ICI therapy, the relevant interactions underlying this phenomenon remain to be elucidated. In particular, it is unclear which microbial products are immunomodulatory in the specific context of checkpoint blockade. Although genome sequencing provides useful information on phylogenetic structure (16S rRNA gene sequencing) and functional potential (whole-genome shotgun sequencing), it does not explicitly illuminate microbial metabolism and production. As such, incorporating high-throughput metabolomics and proteomics into similar future studies could identify additional biomarkers that correlate with ICI responsiveness or nonresponsiveness. A fully integrated "multiomics" approach involving robust characterization of hosts and microbes

alike will likely be critical for establishing the mechanisms by which microbial communities stimulate antitumor immunity in situ.

Additional functional approaches could further link key components of the microbiota and the immune system. For instance, the use of BugFACS (IgA-Seq), a flow cytometry-based technique that selects for IgA-bound bacteria, identifies taxa that are under observation by the host immune system and potentially proinflammatory *(4)*. Given the assumption that the microbiota ultimately affects ICI efficacy via immune modulation, this approach has particular relevance for immunotherapy. Finally, bacterial triangulation *(5)* could further establish the relative importance of key taxa identified in these studies. This technique exploits the microbial mixing that inevitably occurs when mice with different microbiotas (e.g., responder and nonresponder) are cohoused. Triangulation experiments result in mice colonized with hybrid consortia distinct from their donors, and by analyzing microbiota overlap in hosts displaying a given phenotype (e.g., ICI responsiveness), effector taxa can be discerned with greater power.

Along with their implications for the basic biology of the gut microbiota, these studies offer the possibility of improving cancer immunotherapies themselves. Most obviously, the relative ease and apparent predictive power of microbiota analysis could, along with tumor genetics and other factors, help to inform the selection of customized patient treatment strategies. Although a much broader set of studies encompassing various immunotherapies, cancers, and patient demographics would be required, an ability to identify likely nonresponders a priori would maximize their likelihood of effective treatment and prognosis. Moreover, direct manipulation of the microbiota before or during ICI therapy could enhance clinical outcomes. FMTs, for example, have been successfully used to manage recalcitrant *Clostridium difficile* infections and could be similarly deployed to colonize cancer patients with appropriately immunomodulatory taxa. Personalized regimens of probiotics, perhaps including the 8 taxa identified by Matson et al. as being enriched in responders, could be delivered before initiating therapy, as could prebiotics that encourage the in vivo expansion of favorable taxa already established in a patient's microbiota. Many probiotics persist only transiently in vivo, but if responsiveness to ICIs is ultimately linked to specific microbial metabolites, hyperpersistent probiotics engineered to stably produce those molecules could be leveraged for long-term antitumor immunity.

In summary, Routy et al., Gopalakrishnan et al., and Matson et al. provide exciting new evidence of a potential role for the microbiota in predicting—and, through targeted modulation, even enhancing—the clinical success of ICIs and other cancer immunotherapies. These studies will not be the last to address the biological basis and biomedical implications of interactions between the microbiota, the immune system, and cancer.

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5 Nonstandard abbreviations:

ICI	immune checkpoint inhibitor
PD-1	programmed cell death protein 1
CTLA-4	cytotoxic T lymphocyte-associated protein 4
PD-L1	programmed death ligand 1
FMT	fecal microbiota transplant
FoxP3	Forkhead box P3

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Table 1.

Key parameters of presented studies.[a]

	Routy et al. *(1)*	Gopalakrishnan et al. *(2)*	Matson et al. *(3)*
Human data			
Cancer type(s)	Non–small cell lung cancer (60), renal cell carcinoma (40)	Metastatic melanoma (43)	Metastatic melanoma (42)
ICI type(s)	Anti-PD-1 (100), anti-PD-L1 (not included in microbiota analysis)	Anti-PD-1 (43)	Anti-PD-1 (38), anti-CTLA-4 (4)
Response criteria	RECIST 1.1	RECIST 1.1	RECIST 1.1
General gut microbiota measures	↑ species and gene richness in R	↑ α-diversity (Shannon, Simpson, and Chao1) in R; ↑ anabolic pathways in R	Not reported
Sequencing method	WGS (all)	16S (all), WGS (subset)	16S (all), WGS (subset)
Key R-associated bacterial species	*Akkermansia muciniphila, Enterococcus hirae, Eubacterium* spp., *Alistipes* spp.	*Faecalibacterium* spp., *Eubacterium* spp., *Clostridium* spp., *Oscillibacter* spp., *Ruminococcus* spp.	*Enterococcus faecium, Collinsella aerofaciens, Bifidobacterium adolescentis, Klebsiella pneumoniae, Veillonella parvula, Lactobacillus* spp., *Parabacteroides merdae, Bifidobacterium longum*
Key NR-associated bacterial species	*Parabacteroides distasonis, Bacteroides nordii, Bacteroides clarus, Clostridium botteae*	*Bacteroides thetaiotamicron, Escherichia coli, Anaerotruncus colihominis, Gardnerella vaginalis*	*Ruminococcus obeum, Roseburia intestinalis*
Key R-associated immunophenotypes	↑ $CD8^+$ T_C1 T-cell reactivity against *A. muciniphila* and *E. hirae*, ↑ $CD4^+$ T_H1 T-cell reactivity against *A. muciniphila*	↑ $CD8^+$ T cells in tumors, ↑ systemic $CD8^+$and $CD4^+$T cells, ↓ systemic $FoxP3^+$ T_{regs}	↑ $CD8^+$ T cells in R trended but did not reach significance
Effect of antibiotics on ICI efficacy	↓ ICI efficacy (↓ progression-free survival and ↓ overall survival)	Not performed	Not performed
Mouse colonization data			
Cancer model(s)	MCA-205 sarcoma, RET melanoma, RENCA carcinoma	BP melanoma	B16.SIY melanoma
ICI type(s)	Anti-PD-1, anti-CTLA-4	Anti-PD-L1	Anti-PD-L1
ICI efficacy after FMT	↓ ICI efficacy (↑ tumor size and ↓ survival) in antibiotic-treated mice, ↑ ICI efficacy (↓ tumor size and ↑ survival) on R FMT, ↑ ICI efficacy (↓ tumor size) in NR FMT supplemented with *A. mucinophila* and/or *E. hirae*	↑ ICI efficacy (↓ tumor size) on R FMT; $CD8^+$ T-cell tumor infiltration proportional to *Faecalibacterium* colonization	↑ ICI efficacy (↓ tumor size) in R FMT *(C. aerofaciens, K. pneumoniae, Lactobacillus* spp., *P. merdae,* and *B. longum* successfully transferred from R FMT)
Proposed mechanism(s) from animal models	↑ $CCR9^+CXCR3^+CD4^+$ T-cell infiltration into tumors	↑ $CD8^+$ T-cell infiltration into tumors	↑ SIY^+ $CD8^+$ T-cell infiltration into tumors; no significant difference in $FoxP3^+$ $CD4^+$ T-cell infiltration

[a] R, responder; NR, nonresponder; WGS, whole-genome shotgun sequencing; FMT, fecal microbiota transplant; ICI, immune checkpoint inhibitor.