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Influence of Immunomodulatory Drugs on the Gut Microbiota

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Abstract

Immunomodulatory medications are a mainstay of treatment for autoimmune diseases and malignancies. In addition to their direct effects on immune cells, these medications also impact the gut microbiota. Drug-induced shifts in commensal microbes can lead to indirect but important changes in the immune response. We performed a comprehensive literature search focusing on immunotherapy/microbe interactions. Immunotherapies were categorized into five subtypes based on their mechanisms of action: cell trafficking inhibitors, immune checkpoint inhibitors, immunomodulators, anti-proliferative drugs, and inflammatory cytokine inhibitors. Although no consistent relationships were observed between types of immunotherapy and microbiota, most immunotherapies were associated with shifts in specific colonizing bacterial taxa. The relationships between colonizing microbes and drug efficacy were not well-studied for autoimmune diseases. In contrast, the efficacy of immune checkpoint inhibitors for cancer was tied to the baseline composition of the gut microbiota. There was a paucity of high-quality data; existing data were generated using heterogeneous sampling and analytic techniques, and most studies involved small numbers of participants. Further work is needed to elucidate the extent and clinical significance of immunotherapy effects on the human microbiome.

Keywords

autoimmune; microbiome; immunomodulatory drug

Introduction

Once a “forgotten organ”, the human microbiome is increasingly recognized as integral to health and disease [1]. Trillions of microbiota colonize all mucosal and barrier surfaces, including the gut, oral cavity, nasopharyngeal, respiratory tract, urogenital tract and skin [2]. Commensal microbiota directly and indirectly shape immune cell development and

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phenotype, with a well-established impact on the pathophysiology of immune-mediated diseases [3, 4]. The microbiome also contributes to medication efficacy, for example by metabolizing drugs [5-7]. Medications, in turn, can impact the microbiota and cause shifts in downstream immune phenotypes which may then impact the microbial composition in a bi-directional manner.

In this review, we explore how immunomodulatory drugs impact the gut microbiota, as other microbial niches, such as the oral cavity, lung, and skin, are poorly studied in this context. We first summarize relevant aspects of the immune system, highlighting components that are relevant to the host-commensal relationship. Next, we discuss the characterization of the healthy gut microbiome and describe important bacterial taxa that modulate host health and disease. Lastly, we examine the complex multidirectional relationships between the host, immunomodulatory drugs, and the gut microbiota (Figure 1).

The Innate Immune System

The innate immune system is characterized by fast, non-specific responses against pathogens. Key players include neutrophils and myeloid-derived cells (e.g. monocyte/macrophages, dendritic cells) which express pattern recognition receptors (PRRs) that bind to microbe-associated molecular patterns (MAMPs) such as Toll-like receptors (TLRs) and Nod-like receptors [8-10]. Natural killer cells are another circulating innate immune cell; these express major histocompatibility complex (MHC) Class I and kill infected cells by releasing cytotoxic granules [11].

Most organ systems include resident innate immune cells, which assist in screening for infections and mounting a rapid immune response [12]. These specialized cells include microglia (CNS) [13], Kupffer cells (liver) [14], alveolar macrophages (lungs) [15], innate lymphoid cells (ILC; mucosal surfaces)[16], and Langerhans cells (skin) [17]. The gut has a particularly robust resident immune system, including innate as well as adaptive cells. The gut-associated lymphoid tissue (GALT), discussed below, contains ILCs, Paneth cells and intestinal epithelial cells which express TLRs and release antimicrobial peptides (α-defensins, β-defensins, C-type lectins) to protect against extracellular pathogens. Commensal gut microbes (e.g. *Lactobacillus)* interact with PRRs on these cells to induce expression of antimicrobial peptides [18, 19].

The Adaptive Immune System

Unlike the general recognition of the innate immune response, the adaptive response is antigen specific. T cells express a unique T cell receptor, and cluster of differentiation (CD) 3. They are divided into two main classes: CD4 helper T (Th) cells and CD8 cytotoxic T cells. Helper T cells are generally categorized into Th1, Th2, Th17, or regulatory (Treg) subsets which express the transcription factors T-bet, GATA-3, RORγT, and Foxp3, respectively [20-25]. Th1 cells provide immunity against intracellular microbes (such as bacteria or viruses) characterized through the release of IFNγ [26, 27]. The cytokine interleukin-4 (IL)-4 skews T-cells towards a Th2 response. These cells protect against parasites/allergens and promote tissue repair through production of IL-4, IL-5, and IL-13

[28-30]. Th17 cells promote immunity against extracellular pathogens, such as fungi, and are pathogenic in a number of autoimmune diseases [31, 32]. Finally, Tregs are important for establishing self-tolerance, suppressing overactive immune responses, and maintaining homeostasis through the production of anti-inflammatory molecules such as IL-10 and TGF-beta [33, 34]. Similar to Th1 cells, CD8 cytotoxic T cells combat intracellular microbes and tumor cells. They do so by secreting IFN-γ/TNF-α, producing cytotoxic granules, and activating the Fas-mediated apoptosis pathway [35]. After their target antigen has been eliminated from the host, effector T cells either die via apoptosis or differentiate into antigen-specific memory T cells [36, 37].

Regardless of induction, T cells communicate with a variety of immune and non-immune cells to drive a robust, protective immune response, which is tightly regulated to limit damage off-target damage. Protective T cell responses can become pathogenic, especially during states of extremely acute or unresolved inflammation, and these phenotypes are influenced by the microbiome [38, 39]. Helper T cells exhibit profound plasticity in response to the cytokine milieu; for example, healthy human Tregs become dysfunctional and express IFNy, a Th1 cytokine, in the presence of IL-12 [40]. Functional and phenotypic imbalances in these T-cell subsets, especially Th17 and Tregs, have been implicated in autoimmune diseases [33, 40, 41]. Conversely, chronically stimulated antigen-specific CD4 and CD8 T cells may become “exhausted”, a state in which the cells are physically present, but no longer functionally active [36, 42]. As a result, antigen clearance and anti-tumor immunity is impaired. These T cell responses can be altered through selective interaction with the microbiome.

B cells comprise the humoral arm of the adaptive immune system. This lineage is best known for antibody production, which can occur in a T cell dependent- or independent manner. B cells also act as professional antigen presenting cells and produce cytokines that further support the adaptive and innate immune responses [43]. The gut microbiome educates the humoral immune response and conversely, mucosal B-cells secrete IgA, which coats potentially pathologic members of the gut microbiome, keeping them in check [44, 45].

The Mucosal Immune System

T cell responses are commonly studied in the context of the blood and secondary lymphoid organs (spleen, lymph nodes), however, the mucosal immune system represents the body’s largest lymphoid organ and directly interfaces with the gut microbiota [46] (Figure 1). Mucosal-associated lymphoid tissue comprises 80% of all immunocytes and is subdivided into gut-associated lymphoid tissue (GALT), bronchus-associated-lymphoid-tissue, and nasal-associated lymphoid tissue [46-48].

The GALT forms a protective immune-barrier surface and is organized within the connective tissue of the lamina propria and dome-shaped Peyer’s patches, which are enriched with antigen-experienced T cells and naïve B cells, respectively [49]. Specialized epithelial cells (M cells) in Peyer’s patches constantly sample gut bacterial epitopes and initiate lymphocyte activation [50]. Plasma cells in Peyer’s patches and mesenteric lymph nodes secrete dimeric secretory immunoglobulin A (IgA), which coats pathogenic/immunogenic bacteria, prevents

their adhesion to the intestinal barrier [51] and promotes host-commensal homeostasis [52]. After activation in Peyer's patches, experienced IgA-secreting B cells travel through the lymphatic system and into the systemic circulation, then return to the lamina propria via the mesenteric lymph nodes [53].

The host immune response has evolved to tolerate commensals. Tolerogenic mechanisms, such as Treg induction and IL-10 secretion, allow beneficial microbes to chronically colonize the host, but the host retains the ability to mount effective immune responses against microbes that breach these barrier surfaces [54, 55].

Commensal gut microbes are necessary for developing normal host mucosal immunity. Germ free mice have severe mucosal, immune, and anatomic abnormalities [56], including shorter ileal villi [57], reduced Peyer's patch size [58], underdeveloped mesenteric lymph nodes [59], fewer IgA-producing plasma cells [60], diminished Th17 and Treg subpopulations [61, 62], and a skewed Th2 to Th1 ratio [63]. These immunological defects impact immune-mediated diseases and highlight the role of commensals in local and systemic immune development and responses [64].

The Human Gut Microbiome

Commensal gut bacteria fall into six main phyla: Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria, Fusobacteria, and Verrucomicrobia [65, 66]. Bacteroidetes and Firmicutes are the dominant organisms, making up the majority of the gut microbiota with Firmicutes predominantly composed of the *Clostridium* genera [65, 66].

The human gut offers a limited number of ecologic niches, which may be filled by different organisms in different individuals. This variation culminates in a high degree of inter-individual heterogeneity in commensal organisms [65, 66]. Moreover, the composition and stability of the gut microbiome is dynamic across a lifetime. In childhood, Firmicutes are enriched, while with increasing age Bacteroidetes become the dominant phylum [67]. Nevertheless, during adulthood the composition of the gut microbiome remains stable unless it is externally perturbed by common medications (e.g. antibiotics, proton-pump inhibitors, metformin [68, 69]) or other factors including drastic dietary changes [70] and geographic relocation [71, 72].

The individual-level heterogeneity in colonizing microbiota necessitates specialized computational techniques for studying the microbiome [73]. *Species richness* quantifies the number of different species represented within an ecologic niche. *Diversity* incorporates both the number of bacterial species and the abundance of each. More specifically, *alpha diversity* quantifies the richness and evenness of bacterial communities within a sample, while *beta diversity* measures diversity between samples (e.g. between different anatomic locations or different individuals).

Alterations in the composition of the gut microbiota, or gut dysbiosis, have been implicated in many diseases including obesity, diabetes, asthma, allergies, inflammatory bowel disease, cancer, autoimmunity, and even neurodegenerative diseases [4]. In this section, we highlight

commensals with immune-modulatory capabilities, some of which have been implicated in disease, and discuss how they are known to modulate the host immune system.

Bacteroidetes

Bacteroidetes are gram-negative anaerobes that comprise a substantial portion of the adult gut microbiota [66]. This phyla contains many immunomodulatory genera. One of the best-studied is Bacteroides. *Bacteroides thetaiotamicron*, a genetically tractable organism, is frequently used to study host-commensal interactions [74, 75]. Another well-studied species is *B. fragilis*, which interacts indirectly with the pattern recognition receptor TLR2 via production of polysaccharide A to induce Treg differentiation and tolerance. Recolonization with *B. fragilis* alone ameliorates the Th1/Th2 imbalance observed in germ free mice [76]. Conversely, some strains of *B. fragilis* produce a pathogenic enterotoxin; these have been implicated in inflammatory bowel disease and colorectal cancer [77]. This phenomenon illustrates the importance of strain-level taxonomic resolution when studying the microbiome.

Prevotella are considered commensals due their contributions to glucose metabolism, although a few strains are opportunistic pathogens. Members of this genus can help prevent inflammation and autoimmune diseases; *P. histicola* strains have been shown to suppress experimental autoimmune encephalitis [78] and collagen-induced arthritis in mice [79] and have recently been discussed as a novel therapeutic option for patients with multiple sclerosis [80]. *Prevotella* have an important role in polysaccharide degradation and energy extraction, and members of this genus are expanded in African children with fiber-rich diets as compared to Italian children [81]. Nevertheless, emerging data have linked mucosal *Prevotella* to low-grade inflammation and a variety of diseases, including periodontitis and rheumatoid arthritis [82, 83]. The strain-specific nature of bacterial immunogenic potential remains to be fully elucidated. *Firmicutes*

Firmicutes represent the other dominant phylum in the human gut. Clostridia are gram-positive endospore-forming bacteria that comprise a large proportion of the Firmicutes. Butyrate-producers in *Clostridium* clusters IV and XIVa, such as *Ruminococcus, Lachnospira*, and *Roseburia*, promote Tregs [84]. They accomplish this directly, by stimulating Treg proliferation, and indirectly, by fermenting dietary fiber to produce butyrate [84]. Butyrate is a histone deacetylase inhibitor that has been shown to boost the generation and function of Tregs and secretion of IL-10 [85-88]. Indeed, gnotobiotic mice reconstituted with *Clostridium* strains have enriched populations of Foxp3+ Treg cells in the colon [62]. However, Clostridial species can also promote disease. For example, *Clostridium difficile* is a gram-positive spore-forming microbe that causes colitis when antibiotics or chemotherapeutic drugs kill other members of the gut flora allowing *Clostridial* overgrowth. *C. difficile* toxins A and B damage colonic epithelial cells and lead to abdominal pain and non-remitting diarrhea. Fecal microbiota transplantation from healthy individuals is an effective treatment for *C.* difficile colitis [89]. Other lactic acid-producing Firmicutes, such as *Lactobacillus* and *Enterococcus*, are also important immune modulators which induce Treg activity and suppress Th1 and Th2 cells [90, 91].

Proteobacteria

Proteobacteria are gram-negative organisms that populate the normal gut in small quantities. Its members are often pathogenic (e.g. gastric ulcer-inducing *Helicobacter pylori* [92]), recognized as immunogenic by the host immune system and are coated by secretory IgA. Proteobacteria dysregulation is associated with pathology, and the Enterobacteriaceae family has been associated with obesity, metabolic diseases and colitis [93]. However, members of this phylum also provide important immune education. For example, *Alicaligenes spp*, are tolerogenic Proteobacteria that inhabit Peyer's patches, where they stimulate secretory IgA and help establish mutualism [94, 95].

Sex Differences in the Human Gut Microbiome

There are sex differences in relative microbial abundance at the phyla level [96]. In humans, Bacteroidetes are decreased in females compared to males [97]. Differences in microbial diversity and composition have also been correlated with serum levels of sex hormones, with high estradiol/testosterone producing individuals hosting more diverse microbial communities [98].

Animal models have provided insight into microbial sex differences in the context of autoimmune disease [96, 99]. Male nonobese diabetic (NOD) mice had expansion of the following bacterial families: *Porphyromonadaceae, Veillonellaceae, Kineosporiaceae, Peptococcaceae, Enterobacteriaceae, Lactobacillaceae, Cytophagaceae, Peptostreptococcaceae, and Bacteroidaceae* [99], Commensal colonization increased serum testosterone and protected males against type I diabetes. Fecal microbial transfer from adult male NOD mice to immature females also protected against the development of type I diabetes by an androgen-dependent mechanism [96].

In humans, androgen deprivation depleted testosterone-metabolizing *Corynebacterium* species and enriched *Akkermansia muciniphila* [100]. These microbial shifts appear to underlie the efficacy of the androgen inhibitor abiraterone acetate in androgen-independent prostate cancer. The full impact of sex hormones on the microbiota and vice versa remains to be well-characterized.

Drug-Microbial Relationships

Gut microbiota directly metabolize many oral drugs via reduction and hydrolysis [101-103]. This may occur either prior to (first-pass metabolism) or directly following absorption (enterohepatic circulation) in the small/large-intestine [101]. Well-studied examples include activation of sulfasalazine by azo-reductase containing bacteria [104], inactivation of digoxin by *Eggerthella lenta* strains [105], and toxification of mycophenolate motefil (MMF) as a result of bacterial β-glucorinodiase (GUS) activity [106]. Leveraging microbial metabolism has major clinical implications for improving therapeutic responses in patients with cancer and autoimmune diseases. For example, inhibition of GUS-containing bacteria via antibiotics ameliorates GI-related side-effects and improves antitumor effect in mice treated with MMF [106], or irinotecan [107], respectively.

In addition to metabolizing medications, commensal gut bacteria can be directly impacted by oral and parenteral medications. Antibiotics substantially reshape the gut microbiome [108], but many other drugs also have downstream effects on the microbiota. Well documented examples include metformin, which leads to expansion of *Akkermansia muciniphila* and other short chain fatty acid producing microbes [109], and proton pump inhibitors, which lead to expansion of *Lactobacillus* species [110]. Immunomodulatory medications are beginning to be recognized as another class of medications that impacts the gut microbiota with downstream effects on the underlying disease. We will review the existing literature on interactions between immunomodulatory medications and commensal microbiota.

Anti-Proliferative Immunotherapies

Cyclophosphamide

Cyclophosphamide is a cytotoxic alkylating agent that impairs transcription and translation in rapidly proliferating cells [111]. It is used as a chemotherapeutic agent for a variety of malignancies and as an immunosuppressant for numerous autoimmune conditions. Cyclophosphamide profoundly depletes circulating B and T cells, with a predilection for CD4+ T cells. Rodent research demonstrated that cyclophosphamide caused translocation of gram-positive commensals (mainly *Lactobacillus* and *Enterococcus* species) to secondary lymphoid organs, where they promoted Th1 and Th17 differentiation. These immune cells were necessary for the antitumor effect of cyclophosphamide [112]. To date, there have been no human studies investigating its impact on composition or function of the microbiome.

Mycophenolate Mofetil

Mycophenolate mofetil (MMF) inhibits *de novo* guanine nucleotide synthesis, eliciting a cytostatic effect on B- and T-lymphocytes [113]. It is an important immunosuppressant used for transplant recipients and a variety of autoimmune conditions [114]. MMF-treated mice had decreased gut alpha diversity. This was driven by increased pathogenic *Escherichia/Shigella* and a decrease in three gut-protective genera: *Clostridium, Akkermansia*, and *Parabacteroides*. Concurrently, mice had rapid weight loss and increased colonic inflammation that was dependent on the colonizing microbiota [115]. MMF has been associated with erosive enterocolitis in human transplant recipients, but no specific drug effects have yet been shown on the human microbiome [116]. Of note, MMF is directly metabolized by B-glucuronidase (GUS)-expressing gut microbiota during enterohepatic circulation [117]. Inhibiting GUS-expressing bacteria can improve MMF-associated GI toxicity [106].

Methotrexate

Methotrexate (MTX) is a first-line drug in treating RA and other autoimmune diseases and an anti-neoplastic agent. It is a folate antimetabolite that competitively inhibits dihydrofolate reductase, interfering with purine and pyrimidine nucleotide synthesis and suppressing rapidly dividing cells. At lower doses, MTX inhibits 5-aminoimidazole-4-carboxamide ribonucleotide, which leads to adenosine accumulation [118]. Adenosine can suppress neutrophil and macrophage recruitment and reduce proinflammatory cytokines such as

TNFα and IFNγ [119, 120]. Microbiota containing carboxypeptidase glutamate 2 enzymes (CPG2) catabolize MTX and help decrease nephrotoxicity [121, 122].

MTX may modulate the composition of the gut microbiota in both mice and humans, but the patterns of change have been inconsistent [123-126]. In mice, *Bacteroides fragilis* decreased after MTX treatment in a time-dependent manner [123]. In another study, MTX-associated changes to the microbiome were dose-dependent. At low doses, the relative abundance of Firmicutes compared to Bacteroidetes increased, but the opposite trend was observed at high doses [121]. In humans, a small cohort of MTX-treated RA patients had significantly decreased Enterobacteriales [125] compared to treatment-naive patients (Table 1); these findings need to be validated in a larger cohort. In a metagenomic sequencing study, researchers compared the effects of MTX on the oral, salivary, and gut microbiomes [126]. In the dental plaque microbiome, MTX responders had increases in healthy control-enriched microbial linkage groups (MLGs) such as *Prevotella maculosa*. In the salivary microbiome, MTX responders had reductions in *Veillonella* MLGs, which were elevated in RA patients prior to treatment. In the fecal microbiome, *Holdemania filiformis* MLGs were increased after treatment with MTX compared to baseline.

Some MTX-associated bacterial shifts decrease the gut microbiota's capacity for drug detoxification, leading to gastrointestinal toxicity [121]. The complex interplay between drug and bacteria may explain the inter-individual heterogeneity of patient responses and outcomes, but the multi-directional relationships make this difficult to study. Overall, MTX treatment appeared to partially restore the microbial composition of RA patients to resemble that of healthy controls. Immunomodulatory therapies thus have widespread effects on the microbiota, impacting many environmental niches.

Targeted Immunoablative Therapies

Rituximab/Ocrelizumab

Rituximab and ocrelizumab are monoclonal antibodies that target CD20, a surface antigen expressed on most of the B cell lineage [127]. They are effective against hematologic malignancies and a variety of autoimmune conditions including multiple sclerosis, vasculitis, myasthenia gravis and some types of autoimmune encephalitis. B cells are a major contributor to the GALT and are necessary for ongoing IgA production/secretion; the tight relationship between the mucosal immune system and the gut microbiota might suggest that these medications would shift the composition of the microbiome. However, parenteral anti-CD20 monoclonals may incompletely deplete tissue-resident lymphoid cells [128, 129].

No studies have yet examined whether anti-CD20 medications directly impact the gut microbiota in humans. Germ-free mice mono-colonized with *B. fragilis* and treated with anti-CD20 had decreased IgA coating of intestinal bacteria and could be readily invaded by wild-type bacteria, losing their single-strain stability [130]. This study reveals that IgA coating is not only important for pathogen clearance, but also is required to maintain stable colonization by commensal bacteria such as *B. fragilis*. Additional research is needed to identify how these medications impact other members of the gut microbiome.

Alemtuzumab

Alemtuzumab (ALZ) targets the surface molecule CD52, which is expressed on B cells, T cells, and a variety of innate immune cells. It is used for hematologic malignancies, organ transplantation and multiple sclerosis [131]. Antibody binding to CD52 induces lysis of circulating cells; the affected cellular populations then recover gradually over time. Monocytes recover first, after about one month. B cells begin to repopulate around three months, while CD8/CD4 T cells subsets can take a year or longer to repopulate [132].

A mouse study has suggested that ALZ increases intestinal permeability by decreasing intestinal intraepithelial lymphocytes [133]. Similarly, a study using Cynomolgus monkeys observed transient changes in gut microbial composition. These shifts appeared at 1 day post ALZ-treatment, normalized by 9 days and were maintained until day 56 [134]. In the ileal mucosa, there was an enrichment in Enterobacteriales (*E. coli, S. flexneri*) and *Prevotella* (*P. copri, P. dentalis*) directly following treatment and on day 6, respectively. The colonic mucosa showed similar increases in Enterobacteriales. In the fecal microbiota, members of the Clostridiales order increased between days 1-9, except for *Faecalibacterium prausnitzii* which tended to decrease after lymphocyte depletion. ALZ may affect intestinal permeability via depleting intraepithelial lymphocytes, precipitating concurrent effects on microbial composition.

Immunoenhancing Therapies

Immune Checkpoint Inhibitors: Anti-PD1 and Anti-CTLA-4

Immune checkpoint proteins are cell-surface markers that prevent immune overactivation. Checkpoints such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) are activated by interaction with specific ligands. Following activation, these checkpoints inhibit T cell proliferation and effector function [135]. Tumors that express the corresponding ligands (e.g. PD-L1, PD-L2) are able to evade immune surveillance by activating these checkpoints and avoiding anti-tumor immunity.

Immune checkpoint inhibitors (ICIs) such as anti-PD1 and anti-CTLA-4 agents are monoclonal antibodies that block these checkpoints and restore anti-tumor immunity. Unlike the immunoablative therapies, ICIs upregulate the adaptive immune system [135, 136]. By unleashing the full power of the adaptive immune response against tumor cells, ICIs have revolutionized cancer therapy, dramatically improving outcomes in melanoma and other malignancies [135, 136].

While recent studies have been investigating the impact of the baseline gut microbiome on immunotherapy efficacy [137-140], there is a paucity of research exploring how the microbial composition is impacted by treatment. Anti-CTLA4 antibodies led to an increase in Clostridiales, and a rapid decline in Bacteroidales and Burkholderiales. The therapeutic effect of the anti-CTLA4 antibody was dependent upon bacterial colonization of the gut mucosa [141]. When multiple melanoma patients were clustered into distinct enterotypes (A, B, C), anti-CTLA4 therapy tended to increase the proportion of patients in cluster C and decrease those in cluster B (clusters were characterized by distinct *Bacteroides* species). In a

human study, ICI treatment for renal cell carcinoma resulted in an increase in stool richness after 2 months [142].

Perhaps more importantly, gut microbial composition appears to play an important role in ICI efficacy [142-144]. In one retrospective study in patients with advanced non-small lung cancer, ICI responders (those with longer times to treatment failure) were significantly enriched with *Lactobacillus, Clostridium*, and *Syntrophococcus*, although the time at which stool samples were obtained post-ICI treatment was not specified [143]. In another study, ICI responders were characterized by increased baseline abundance of Firmicutes and Bacteroidetes (*Alistipes*) [142]. In both cancer patient cohorts, responders had overrepresentation *of Akkermansia muciniphila*. In order to better examine a cause-effect relationship between anti-tumor effects of ICIs and specific microbiota, researchers transplanted fecal microbiota from human anti-PD-1 responders and nonresponders to germ-free/antibiotic-treated mice. Only mice colonized from anti-PD-1 responders were subsequently sensitive to PD-1 blockade. Oral gavage with *A. muciniphila* was also able to rescue the efficacy of the cancer immunotherapy in mice [142].

Gut microbial composition also influences ICI-related side effects. Despite being associated with therapeutic efficacy, baseline abundance of Firmicutes has been associated with ICI-induced colitis and other GI-related side effects [140, 144]. In contrast, higher levels of Bacteroidetes have been observed in individuals who experience no side effects [140, 144]. The ratio of Bacteroidetes/Firmicutes may predict not only the clinical response to immunotherapy, but also whether a patient experiences side effects.

Lymphocyte Trafficking Inhibitors

Natalizumab

Natalizumab (NTZ), a monoclonal antibody against alpha4beta1 integrin, blocks lymphocyte adhesion and extravasation [145, 146]. It is a highly effective therapy for MS and Crohn’s disease. The single published study of NTZ-associated microbial effects demonstrated that NTZ reduced lipopolysaccharide binding protein and lipopolysaccharide (LPS) levels in the blood, brain and spinal cord of rats with experimental autoimmune encephalitis, an animal model of MS [147]. High levels of LPS lead to mucosal barrier dysfunction and a proinflammatory state [148]. Since LPS is a major enterotoxin secreted by gram negative bacteria, especially those belonging to Proteobacteria phyla, this suggests that NTZ may alter Proteobacteria composition. Further work specifically examining bacterial taxonomy and metabolic function in response to NTZ, is needed.

Fingolimod

Fingolimod is a modulator of sphingosine-1-phosphate receptors 1, 3, 4 and 5; it sequesters lymphocytes in secondary lymphoid tissue, thus preventing CNS infiltration [149]. It is used for the treatment of multiple sclerosis. Very little is known about fingolimod’s effect on gut microbiota. A small Russian study observed that fingolimod-treated patients tended to have an increased Bacteroidetes/Firmicutes ratio [150], which is consistent with findings seen for

other MS immunomodulators [151, 152]. They also suggested shifts in *E. coli* strains in response to fingolimod (Table 1); however these results remain to be replicated.

Immunomodulatory Therapies

Dimethyl Fumarate

Dimethyl fumarate (DMF) and its active metabolite monomethyl fumarate are small molecules used for the treatment of relapsing MS. DMF activates the Nrf2 antioxidant stress pathways, reducing axonal degradation and tissue damage [153]. It also shifts the immune system from a Th1/Th17 state to a Th2 state [153, 154]. This is accompanied by selective depletion of lymphocytes, predominantly CD8+ and CD4+ T cells [153, 155].

DMF affects both gut microbial composition and intestinal barrier integrity [147, 151, 156-158]. The specific bacterial species affected by DMF vary across studies (Table 1). One study found that *Bifidobacterium* decreased and *Faecalibacterium* increased after two and twelve weeks of DMF treatment, respectively [156]. In contrast, Sand and colleagues reported that DMF reduced Firmicutes (e.g. Lachnospiraceae, Veillonellaceae, Clostridiales) and Fusobacteria with a concurrent increase in Bacteroidetes [151]. Metabolic changes, including changes in retinols, amino acids, methane metabolism and ethylbenzene degradation, were also associated with DMF. In mice, DMF shifted the relative abundance of both Bacteroidetes and Firmicutes phyla depending on the region of the intestines studied [158]. DMF treatment also increased villi height in the jejunum and the ileum, suggesting improved absorption efficiency. These studies suggest that there are multidirectional interactions between DMF and the microbiota, but the influence of microbial composition on drug efficacy is unknown. While there are global microbial shifts occurring between treated and untreated individuals, there may be more subtle shifts that could be tied to responders or non-responders within a treatment group. Existing studies were small and lacked the power to address these patient outcomes. Future longitudinal studies may help dissect the relationship between microbial composition and treatment response.

Glatiramer Acetate

Glatiramer acetate (GA) is a synthetic myelin analogue used for the treatment of MS. Like DMF, GA promotes differentiation of anti-inflammatory Th2 cells and reduces the activity of autoreactive T cells [159, 160]. It also induces differentiation and proliferation of Tregs [161, 162] and CD8 T cells [163, 164]. Several human studies have investigated the influence of GA on the gut microbiota [150-152, 165] (Table 1). Katz-Sand and colleagues observed that GA treatment was associated with enrichment in seven Firmicutes/Proteobacteria genera and concurrent reductions in seven other Bacteriodetes/Proteobacteria/Firmicutes genera [151]. Others corroborated shifts in Firmicutes (Clostridia, *Faecalibacterium, Clostridium, Ruminococcus*; Lactobacillaceae) and Bacteriodetes (Bacteriodaceae) among GA-treated patients [165]. A small Russian study noted that patients treated with GA were more likely to report constipation, which they attributed to higher levels of atypical forms of Proteobacteria (e.g. *Proteus* species) [150]. However, all these studies were small and lacked the power to identify significant effects of GA on the microbiome.

Interferon Beta

Interferon beta (IFNβ), a type 1 interferon, treats MS through multiple mechanisms of action including reducing T cell activation, promoting Treg differentiation, inducing cytokine shifts, altering matrix metalloprotease expression, strengthening the blood-brain-barrier, and regulating B cell activity [166, 167]. Treatment was associated with increased Bacteroidetes among RRMS patients [168, 169] (Table 1). A small Spanish study found a trend for reduced *Prevotella* (*P. copri*) in MS patients that reversed with IFNβ treatment [169]. In order to further discern the effects of IFN, Reynders and colleagues sub-classified multiple sclerosis patients based on their disease phenotype. Microbial richness appeared to be lower in IFNβ-treated relapsing-remitting MS patients compared to benign and primary progressive MS patients [168]. Specific microbial composition changes also differed based on the phenotype subclassification with the *Bacteriodetes 2* enterotype more prevalent in IFN β-treated relapsing-remitting MS patients compared to other clinical subgroups. *Butyricicoccus*, a genus in the Clostridia order, was observed to be inversely correlated with patient-reported symptoms. This study highlights the importance of studying microbial changes on a disease-subtype level in order to discern effects that IFN may be having on the gut microbiome and its impact in patient outcomes.

Anti-Cytokine Immunotherapies

TNF inhibitors

TNF inhibitors (TNFi) revolutionized the treatment of RA and other systemic autoimmune disorders including ankylosing spondylitis, Crohn's disease, ulcerative colitis, and psoriasis [170]. This class of molecules antagonizes tumor necrosis factor alpha (TNFα), a proinflammatory cytokine secreted by activated macrophages and T cells that contributes to the pathophysiology of multiple systemic autoimmune diseases. TNFi include neutralizing monoclonal antibodies, fusion proteins, and pegylated fragments. In general, TNFi bind and inhibit both soluble and transmembrane TNFα [171]. These have divergent functions. Soluble TNFα is implicated in inflammatory diseases and expressed by activated cytotoxic T cells. In contrast, transmembrane TNFα is expressed on many adaptive immune cells. The lack of receptor specificity may contribute to the lack of TNFi efficacy in certain diseases. Indeed, TNF inhibition worsens central demyelinating diseases like MS [172]. One study reported that Cyanobacteria increased, while Deltaproteobacteria and Clostridiaceae decreased after TNFi treatment in RA patients [125]. Decreased Proteobacteria and increased Clostridiales were associated with successful TNFi treatment in inflammatory bowel disease [173, 174] (Table 1).

Relatively little, however, is known about TNFi and microbiota interactions with respect to drug efficacy. Blocking TNF alpha systemically has been linked to increase in fungal infections [175] and may possibly increase susceptibility to other types of pathobionts. A study found that TNFi may shift the diversity of the fecal microbiome in patients with inflammatory bowel disease (IBD) to resemble that of healthy controls [176]. The researchers also showed that the levels of butyrate and substrates involved in butyrate synthesis were diminished among IBD patients who were non-responders to TNFis but were enriched in IBD patients in clinical remission. This shows the advantage of using functional

assays like metabolomics in addition to taxonomic characterization of the microbiome to parse out differences between responders and non-responders.

Discussion

Host-Drug-Microbiota Interactions

Immunotherapies directly affect the host immune system, but indirect drug effects on the gut microbiota may also contribute to their therapeutic efficacy. Moreover, the composition of the gut microbiome may impact whether individuals respond to immunotherapy. Most immunomodulators were not associated with measurable changes in alpha or beta diversity. However, shifts in the relative abundance of bacterial taxa were observed in response to most drugs studied.

The overall shifts in commensal microbiota differed depending on the immunomodulator. Medications used to treat autoimmune diseases have been associated with overall reductions of Firmicutes (e.g. *Clostridium*) [125, 151, 152] and increases in Bacteroidetes (e.g. *Bacteroides*) [151, 152, 169], leading some to hypothesize that the Firmicutes:Bacteroides ratio was fundamentally altered in autoimmune diseases. However, with time, it has become clear that both Firmicutes and Bacteroides play divergent roles and generalizing at the phylum level will likely not be scientifically accurate. Multiple studies reported that immunotherapy led to “normalization” of the gut microbiome; in other words, treated patients’ microbiota assumed a taxonomic distribution more similar to that of healthy controls than to untreated, diseased individuals [124-126, 151, 152, 156, 165, 169, 173, 176]. In several studies, *Prevotella* species increased among patients taking interferon beta [152, 169]. Counterintuitively, these organisms have sometimes been associated with inflammation and autoimmune pathology [82]. Changes in *Prevotella* were not observed with the other immunomodulatory medications studied to date (Table 1).

Perhaps unsurprisingly, ICIs and immune suppressing medications had distinct effects on gut microbiota. Although existing data have not demonstrated consistent relationships between ICI administration and downstream changes in gut microbial ecology, the baseline composition of the microbiome substantially impacted the therapeutic efficacy and side effect profile of ICIs. High baseline levels of Firmicutes were associated with greater therapeutic efficacy and increased progression-free survival [142, 143], while the relationship between Bacteroidetes and anti-PD-1 treatment response was more heterogeneous [142-144]. For instance, high levels of baseline *Alistipes* and *Akkermansia* were observed in responders to PD-1 targeted therapies, while *Parabacteroides* were abundant in non-responders [142]. Further elucidation of the mechanisms underlying the relationship between baseline gut ecology and treatment efficacy could ultimately lead to treatments designed to optimize the gut microbiota in advance of immunotherapy. This may further improve treatment efficacy.

Prophylactic antibiotics are common among cancer patients, which further complicates interpretation of microbiota-ICI interactions. Antibiotic administration before, during, or after the initiation of immunotherapy has generally been associated with negative outcomes in cancer patients [177-180]. A recent meta-analysis concluded that ICI-treated non-small

cell lung cancer patients on antibiotics had shorter progression-free survival and overall survival compared to those with no antibiotics [178]. A negative association was also observed between antibiotics and survival in urothelial carcinoma patients treated with atezolizumab, an anti PDL-1 immunotherapy, but not for patients taking traditional chemotherapy [179]. This suggests that gut microbes are necessary for maximal ICI efficacy. However, these observations could be confounded by patient characteristics. Those needing antibiotics are often weaker, more immunodeficient and have previous infections/comorbidities. The heterogeneity of the cancer patient cohorts, the variable time-window when antibiotics were taken, and the retrospective nature of these studies also confound interpretation of these results.

Comprehending the functional significance of immunomodulatory drug-induced changes in microbial populations will require better understanding of host-commensal interactions, specifically the immune interactions. There are a plethora of immunologic mechanisms that may explain the positive or detrimental functional relevance of drug-induced microbiome shifts. For instance, specific bacteria and metabolites have been demonstrated to alter barrier permeability both in the gut (alternatively referred to as ‘leaky gut) [181] and non-gut barriers, such as the blood-brain barrier [182]. In mice, the gut and the blood-brain barrier have been altered in the presence of microbiome-derived metabolites, notably, tryptophan-derived aryl hydrocarbon receptor reactive metabolites [183]. Short chain fatty acids are bacterial metabolites that promote intestinal epithelial barrier integrity [184-186], B cell IgA production [187], regulatory T cell differentiation [188], and anti-inflammatory IL-10 production [189]. Many of the bacteria apparently impacted by immunotherapies are short-chain fatty acid producers, including multiple Clostridial species [190] *Lactobacillus* and *Bifidobacterium*. In addition to generating immunoactive metabolites, commensals themselves can translocate across mucosal barrier surfaces and directly promote inflammation and T cell polarization [191-194]. Another mechanism by which commensals could mediate differential anti-tumor and autoimmune responses is cross-reactivity, or molecular mimicry. Commensal antigens, by chance or homology, have linear and conformational epitopes with enough similarity to host antigens that commensal-specific T and B cell responses are able to recognize and react to host tissue, thereby promoting autoimmunity and anti-tumor immunity [195-202]. Taken together it is plausible that microbial alterations due to immunomodulators may have unintended side-effects on host-immune physiology.

The rapid evolution of sequencing technologies and analysis methods used for microbiome research has led to methodological challenges in data interpretation (Figure 2). Most researchers utilize 16S rRNA sequencing, but the hypervariable regions used (e.g. V1-3, V3-V4, V3-V5, V4) and the selected primers vary between studies with different amplicons preferentially identifying different bacterial genera [203, 204]. Moreover, alternate sequencing methodologies, such as shotgun metagenomics and long-amplicon 16S sequencing, are emerging. This methodological variance makes it extremely challenging to compare results between studies. Another pitfall of human microbiome research is that subjects are often incompletely characterized; variables like diet, medical comorbidities and medication usage substantially impact the microbiome yet are rarely captured by the study design [70, 73, 205]. Cross-sectional studies therefore become problematic, as it is difficult

to assure comparable controls. Statistical tools to calculate power for microbiome experiments and analyze the data are also still evolving. The human studies reviewed were all relatively small. Given the heterogeneity of the substrate, it is likely that substantially more patients will be needed to detect differences between groups. Indeed, a similar phenomenon was observed during the advent of genetic research for immune diseases. For years, studies found only minimal genetic substrate for diseases such as MS and RA [206, 207]. It was only when multicenter, multinational consortia standardized data collection techniques and pooled thousands of cases that a clear picture emerged [208, 209], and now well over 230 genes have been linked to MS risk [210]. As with all biomarkers, best practice would mandate that microbiota of interest should be first identified in a discovery cohort and subsequently verified using a separate validation cohort. However, to date human microbiome research has not achieved this level of rigor. Consensus regarding best practices for microbiome experimental design and collaboration across centers will be needed to fully elaborate the role of the microbiome in human disease. Such large-scale studies will additionally allow meta-analyses to define reproducible microbiota-host interactions [211].

We hypothesized that immunotherapies with shared mechanisms of action might elicit similar changes in the gut microbiota. This was not substantiated by the existing data. However, the literature in this field is striking for its heterogeneity. Many immunomodulators have never been studied in the context of the gut microbiome, or they have been examined in only one or two small studies. Given the expected level of microbial heterogeneity between individuals, the variability imposed by nonstandard methodology, and disease-specific microbial shifts , we expect that as the field matures, more clarity will emerge about the bidirectional relationships between immunotherapies and the gut microbiota. We anticipate that pharmacomicrobiomics is an important component of immunomodulator efficacy. Although the field is in its infancy, further elucidating drug/microbial interactions and how these impact the host immune response will afford opportunities to personalize treatment and achieve better treatment efficacy for autoimmune diseases and malignancies.

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Abbreviations:

CD	cluster of differentiation
Th	helper T cells
Treg	regulatory T-cell subsets
GALT	gut-associated lymphoid tissue
TLR2	Toll-like receptor-2
MMF	Mycophenolate mofetil
MTX	Methotrexate

RA	Rheumatoid Arthritis
MS	Multiple Sclerosis
MLGs	microbial linkage groups
ALZ	Alemtuzumab
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
PD-1	programmed cell death protein 1
PD-L1	programmed cell death protein 1 ligand 1
PD-L2	programmed cell death protein 1 ligand 2
ICIs	Immune checkpoint inhibitors
NTZ	Natalizumab
LPS	lipopolysaccharide
DMF	Dimethyl fumarate
GA	Glatiramer acetate
IFNβ	Interferon beta
TNFi's	TNF inhibitors
TNFα	tumor necrosis factor alpha
IgA	immunoglobulin A
IL	interleukin

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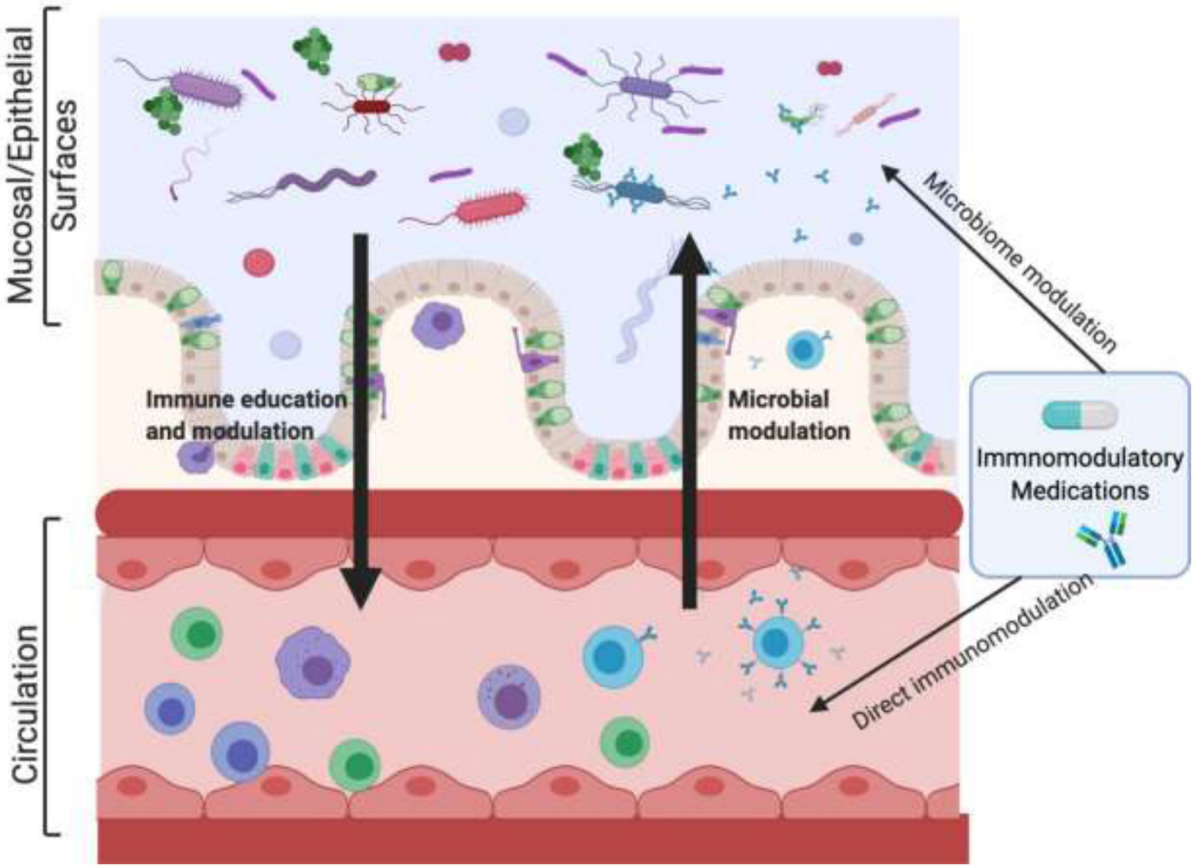

Figure 1. Host-Drug-Microbiota Interactions.

The interface between microorganisms and the immune is complex and multidirectional, and it is further influenced by immunomodulatory medications. Commensal organisms at mucosal/epithelial surfaces influence immune education and modulation both locally and systemically. These microbes also influence drug metabolism and efficacy. Inversely, the immune system shapes the composition of the microbiome. Immunomodulatory medications impact circulating immune cells directly, via a variety of mechanisms. Emerging data suggest that these medications also function indirectly, by shifting the composition of the microbiome. These multi-directional relationships are complex and remain poorly understood. Figure created with BioRender.com

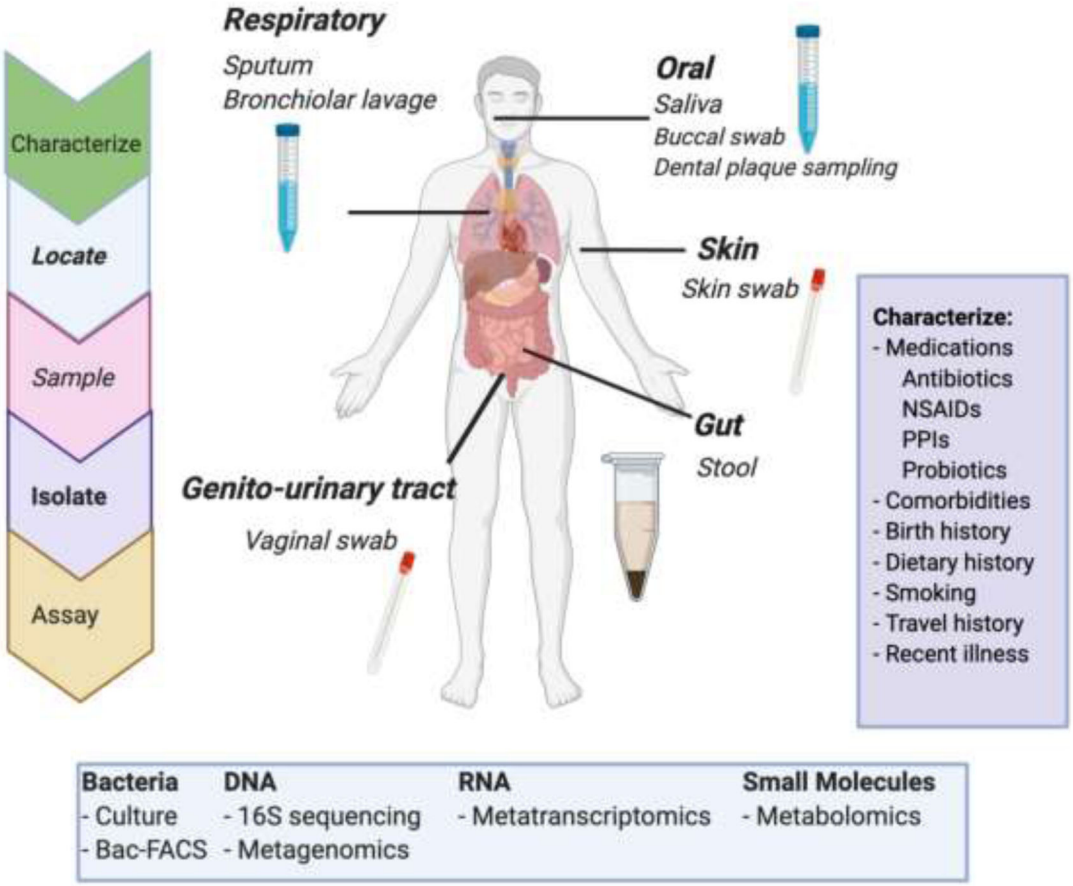

Figure 2. Microbiome Study Design.

Human microbiome studies require identifying the anatomic region of interest, selecting a sampling methodology, and isolating the specimen. Analytic assays will be targeted to the research question and may include microbial characterization at the cellular, DNA, RNA, or metabolic level. Human microbiome studies require subject-level characterization to adequately control for environmental variables known to impact the microbiome. NSAIDs: non-steroidal anti-inflammatory drugs. PPIs: proton pump inhibitors. Figure created with BioRender.com

Table 1.

Bacterial effects in the first comparison group are reported. The changes in bacterial composition in the human gut microbiome are reported as changes in terms of phyla and their specific class and family names are in parentheses. Numbers of patients in each comparison group is written in parentheses. R = Responder; NR= Nonresponder; Tx=Treated; BL = Baseline; UTx = Untreated; HC = Healthy Control; SE = side effects; NSE = no side effects; NS = not significant; NR = not reported; Δ = Changed; ↑ = Increased; ↓ = Decreased.

Drug Name	Disease	Immunomodulatory Type	Comparison (n)	Bacterial Phyla (Family, *Genus*)	Diversity
Methotrexate	RA	Anti-Proliferative	Tx (11) vs. UTx (11) [85]	↓ Proteobacteria (*Enterobacteriales*)	NS
			Tx (9) vs. BL (9) [86]	↑ Firmicutes (Erysipelotrichaceae, *Holdemania*) microbial linkage groups	NS
			Tx (24) vs. HC (32)[84] †	NR	↑ Alpha
Anti-PD1	Cancer	Immunoenhancing	Tx (42) vs. BL (36) [102]	↑ Firmicutes (*Clostridium; Streptococcus; Eubacterium*) ↑ Bacteroidetes (*Alistipes*) ↓ Firmicutes (*Roseburia; Oscillibacter; Lachnoclostridium*) ↓ Bacteroidetes (*Alistipes; Coprobacter*)	↑ Richness
			R (6) vs. NR (11) [103]	↑ Firmicutes (Lactobacillaceae, *Lactobacillus*; Clostridiaceae, *Clostridium*; Lachnospiraceae, *Syntrophococcus*) ↓ Bacteroidetes (*Parabacteroides*) ↓ Proteobacteria (*Bilophila; Sutterella*)	NS
Anti-CTLA-4	Cancer	Immunoenhancing	Tx (26) vs BL (26)[104]	Shifts in bacterial proportions were not linked to treatment	NS
			Colitis (7) vs. BL (7) [104]	↓ Firmicutes (*Ruminococcus*; Lachnospiraceae, *Blautia; Clostridium IV; Eubacterium; Pseudoflavonifractor*)	↓Alpha
			Tx (25) vs. BL (25)[101]	Bidirectional shifts in *Bacteroides*	NR
Glatiramer Acetate	MS	Cellular	Tx (60) vs. UTx (75) [111]	↓ Firmicutes (Lachnospiraceae, *Roseburia*; Veillonellaceae) ↓ Proteobacteria (*Sutterella, Aggregatibacter, Haemophilus*) ↑ Firmicutes (*Enterococcus, Acidaminococcus*) ↑ Proteobacteria (*Enterobacter, Pseudomonas, Sphingoblum, Burkholderiales*)	NS
Dimethyl Fumarate	MS	Cellular	Tx (33) vs. UTx (75) [111]	↓ Firmicutes (*Anaerococcus, Finegoldia, Peptoniphilius; Lachnospiraceae, Blautia*; Veillonellaceae, *Megasphaera*) ↑ Bacteroidetes ↓ Fusobacterilia (*Fusobacterium*) ↓ Proteobacteria (*Campylobacter*) ↓ Actinobacteria (*Varibaculum, Corynebacterium, Rothia*)	NS
			Tx (23) vs. BL (25) [116]	↑ Firmicutes (*Faecalibacterium*) ↓ Bacteroidetes ↓ Actinobacteria (*Bifidobacterium*) *	NS
Interferon-β	MS	Cellular	Tx (15) vs. UTx (15) [129]	↑ Bacteroidetes (Prevotellaceae, *Prevotella*)	NR
			Tx (24) vs. UTx (20-24) [128]	Δ Bacteroidetes enterotype distribution	↓ Richness

Drug Name	Disease	Immunomodulatory Type	Comparison (n)	Bacterial Phyla (Family, *Genus*)	Diversity
Combined INF-β/GA			Tx (32) vs. UTx (28) [112]	↑ Bacteroidetes (Prevotellaceae, *Prevotella*) ↑ Proteobacteria (Sutterellaceae, *Sutterella*) Firmicutes (Clostridiaceae, *Sarcina*)	NS
TNF inhibitors	IBD	Anti-Cytokine	Tx (20) vs. BL (20) R (13) vs. NR (7) [133] †	↓ Proteobacteria	NR
			R (9) vs. NR (7) [134]	↑ Firmicutes (Lachnospiraceae, *Anaerostipes*; Veillonellaceae, *Veillonella*; Acidaminococcaceae, *Acidominococcus*)	↑ Alpha
			Tx (12) vs BL (12) R (9) vs. NR (3) [136] †	↑ Firmicutes (Lachnospiraceae, *Coprococcus, Roseburia*) NS	↑ Alpha, beta NS
	RA		Tx (17) vs BL (17) R (11) vs. NR (6) [136] †	↑ Firmicutes (Erysiopelotriahaceae; Lachnospiraceae, *Dorea*) NS	↓ Beta NS
			Tx (10) vs. UTx (11) [85]	↓ Proteobacteria (Class: Deltaproteobacteria) ↓ Firmicutes (Clostridiaceae) ↑ Cyanobacteria (Class: Nostocophycideae, Order: Nostocales)	NS

† A subset of patients was on other immunomodulators including azathioprine, leflunomide.

* Changes were transient and reversed with longer times on drug