Immune Netw. 2022 Feb;22(1):e3
https://doi.org/10.4110/in.2022.22.e3
pISSN 1598-2629·eISSN 2092-6685

IMMUNE NETWORK

Review Article

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Commensal Microbiota and Cancer Immunotherapy: Harnessing Commensal Bacteria for Cancer Therapy

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OPEN ACCESS

Received: Jan 23, 2022
Revised: Feb 6, 2022
Accepted: Feb 7, 2022
Published online: Feb 14, 2022

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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
A2AR, adenosine 2A receptor; ATP, adenosine 5′-triphosphate; CML, chronic myeloid leukemia; DC, dendritic cell; FDA, Food and Drug Administration; HDAC, histone deacetylase; ICI, immune checkpoint

ABSTRACT

Cancer is one of the leading causes of death worldwide and the number of cancer patients is expected to continuously increase in the future. Traditional cancer therapies focus on inhibiting cancer growth while largely ignoring the contribution of the immune system in eliminating cancer cells. Recently, better understanding of immunological mechanisms pertaining to cancer progress has led to development of several immunotherapies, which revolutionized cancer treatment. Nonetheless, only a small proportion of cancer patients respond to immunotherapy and maintain a durable response. Among multiple factors contributing to the variability of immunotherapy response rates, commensal microbiota inhabiting patients have been identified as one of the most critical factors determining the success of immunotherapy. The functional diversity of microbiota differentially affects the host immune system and controls the efficacy of immunotherapy in individual cancer patients. Moreover, clinical studies have demonstrated that changing the gut microbiota composition by fecal microbiota transplantation in patients who failed a previous immunotherapy converts them to responders of the same therapy. Consequently, both academic and industrial researchers are putting extensive efforts to identify and develop specific bacteria or bacteria mixtures for cancer immunotherapy. In this review, we will summarize the immunological roles of commensal microbiota in cancer treatment and give specific examples of bacteria that show anticancer effect when administered as a monotherapy or as an adjuvant agent for immunotherapy. We will also list ongoing clinical trials testing the anticancer effect of commensal bacteria.

Keywords: Microbiota; Cancer; Immunotherapy; Immune checkpoint inhibitors; Immunity, mucosal; Fecal microbiota transplant

INTRODUCTION

Cancer is primarily caused by unchecked cell proliferation and failure to respond to growth inhibitory signals. It is one of the leading causes of death in most countries and about 20 million new cases and 10 million cancer-related deaths worldwide were reported in 2020 (1). With the global trend of transitioning to an aging society, the number of cancer patients in 2040 is predicted to increase by approximately 50% compared to 2020 (1).

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inhibitor; IDO, indoleamine 2,3-dioxygenase; isoalloLCA, isoallolithocholic acid; JAX, Jackson Laboratory; LCA, lithocholic acid; MAMP, microbe-associated molecular pattern; NSCLC, non-small cell lung cancer; PFS, progression-free survival; PSA, polysaccharide A; RCC, renal cell carcinoma; SAA, serum amyloid A; SCFA, short chain fatty acid; SFB, segmented filamentous bacteria; SPF, specific pathogen-free; TAC, Taconic Farms; Tfh, follicular helper T.

Author Contributions

Conceptualization: Kim YM; Funding acquisition: Kim YM; Supervision: Kim YM; Visualization: Bae J; Writing – original draft: Bae J, Park K; Kim YM; Writing – review & editing: Kim YM

For several millennia, cancer treatment mostly relied on surgical resection which removes primary cancer tissue and local metastasis, but remnant cancer cells and micrometastasis put patients at a high risk of cancer recurrence. Modern medical intervention for cancers started with development of radiotherapy in the 1890s thanks to the discovery of roentgen rays (**Fig. 1A**) (2). Radiotherapy directly damages DNA in cancer cells and induces apoptosis. However, it can be applied only to a limited area of the body and may cause complications such as inflammation and fibrosis. Starting with demonstration of the antitumor effect of nitrogen mustard in non-Hodgkin's lymphoma in the 1940s, systemic administration of cytotoxic agents such as cyclophosphamide, cisplatin, and taxol was introduced to cancer treatment (3). Application of these cytotoxic agents, commonly called 'chemotherapy', inhibits DNA replication and cell division, resulting in cancer growth inhibition. However, chemotherapeutic agents induce numerous adverse effects because they indiscriminately affect all actively dividing cells. Thus, in an effort to find a therapeutic modality with higher efficacy and lower toxicity, targeted therapies exploiting the 'oncogene addiction' of individual cancer types have been developed (4). The targeted therapies often target cancer-specific mutations such as the BCR-ABL translocation in chronic myeloid leukemia (CML), epidermal growth factor receptor mutations in non-small cell lung cancer (NSCLC), and human epidermal growth factor receptor 2 amplification in breast cancer. Continuing advances in molecular biology of cancer have facilitated identification of diverse new molecular targets in cancer cells and novel cancer drugs are continually being developed. However, even targeted therapies rarely cure cancers because most cancers can evolve to establish resistance to the therapeutic interventions directed at themselves.

Cancer immunotherapy takes a very different approach; instead of directly inhibiting cancer cell growth, cancer immunotherapies rely on the immune system's ability to eliminate cancer. To potentiate the host's ability to battle the cancer, various approaches are taken to stimulate endogenous immune cells in cancer patients or to supply specific immune cell types to the patients from outside (5). Among them, immune checkpoint inhibitors (ICIs) such as anti-CTLA-4, anti-PD-1, and anti-PD-L1 monoclonal Abs reinvigorate anticancer T cell responses by interrupting co-inhibitory signaling pathways in exhausted effector T cells and immunosuppressive Tregs (6,7). ICIs demonstrate great efficacy in several cancer types and eradicate cancer in some patients. Currently, there are 7 ICIs approved by US Food and Drug Administration (FDA) for the treatment of 19 different cancer types, including metastatic melanoma, NSCLC, microsatellite instability-high colorectal cancer, head and

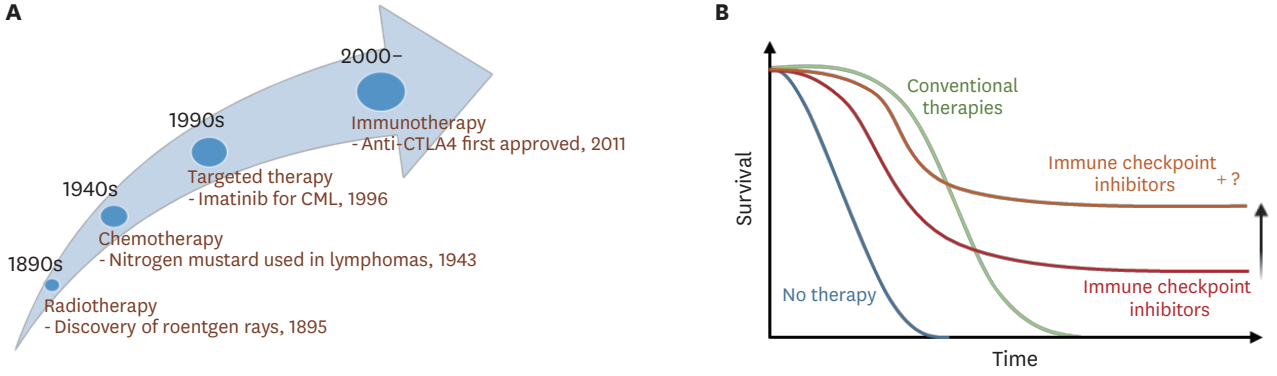

Figure 1. The development of anticancer therapy.
(A) Major breakthroughs in anticancer therapy are depicted. (B) Compared to conventional anticancer therapies, immune checkpoint inhibitors are able to cure cancers in a subset of patients. Still, additional strategies need to be developed to increase the response rate of immune checkpoint inhibitors.

neck cancer, bladder cancer and renal cell carcinoma (RCC) (8). However, the therapeutic efficacy of ICIs wildly varies among cancer types and even among patients with the same type of cancer (9-13). Therefore, development of innovative new strategies that are based on comprehensive understanding of various factors regulating the responsiveness to ICIs in individual cancer patients are required to improve the efficacy of ICIs and immunotherapy in general (**Fig. 1B**). Multiple factors including host genetics, cancer mutation burden, and tumor microenvironment composition are shown to contribute to the variability of immunotherapy response rates (9,14,15). Among such factors, commensal microbiota inhabiting patients have been identified as one of the most critical factors determining the success of immunotherapy (16). In this review, we will summarize how intestinal bacteria generally affect the host immune system and provide specific examples of commensal bacteria demonstrated to improve the efficacy of ICIs. We will only focus on modulation of intestinal bacteria and will not cover bacteria therapies that directly inject bacteria or bacterial toxins into tumor tissues or methods that use bacteria as vectors to deliver therapeutic agents (17,18).

INTESTINAL BACTERIA AND THE IMMUNE SYSTEM

Humans have evolved together with commensal microorganisms, such as bacteria, fungi, and virus, that establish a unique ecosystem in the human body. Microorganisms are found in every surface area of the body and the intestinal tract is especially the richest habitat for the commensal microorganisms. It is estimated that trillions of commensal bacteria and more than 1,000 microbial species are resident in the healthy human gut and many of them steadily interact with host cells (19). According to the Human Genome Project and the Human Microbiome Project, the total number of the human genome was identified to be 20,000 to 25,000, whereas the number of microbial genes is estimated to be about 2 million, at least 100 times that of human genes (20,21). Moreover, when comparing two individuals, the overall sequence differences in the human genome are about 0.1%, whereas the collection of microbial genes, termed microbiome, shows the variation of up to 80%–90% (22). Therefore, it is plausible that the large differences in microbiota composition greatly influence the health maintenance and disease progression of human hosts through complex interactions between host cells and microorganisms.

Since the postulations of Elie Metchnikoff on the role of intestinal bacteria in the 1900s, it has been demonstrated that commensal bacteria conduct many important physiological roles such as digestion of foods, biosynthesis of micronutrients, and defense against pathogens (23,24). More recently, it also became certain that intestinal bacteria control the function of local immune cells and further influence the immune responses in distal organs for host defense and organismal homeostasis (25). The importance of commensal bacteria in the maturation of the host immune system was clearly evidenced by the small size of Peyer's patches and the reduction in $CD4^+$ T cells and IgA-producing plasma cells in the intestine of germ-free mice (26). Initial development of the 'immune tone' in newborn babies is mostly attributed to the exposure to commensal bacteria during vaginal delivery or cesarean section and intake of breast milk (27). Throughout life human microbiota composition can be highly influenced by aging, diet, lifestyle, infection, and antibiotics treatment, and variabilities in gut microbiota differentially shape the individuals' immune system and calibrate the 'immune tone'.

Detailed mechanisms on how particular commensal bacteria modify the host immune system is being unveiled but the research on this topic is still in its infancy. In general, the

various pattern-recognition receptors expressed in intestinal epithelial cells and immune cells are thought to recognize microbe-associated molecular patterns (MAMPs) of commensal bacteria and mediate the interaction between host and commensal bacteria (28). Intestinal dendritic cells often take a center stage in these processes and orchestrate the polarization of $CD4^+$ T cell differentiation by expressing appropriate cytokines and co-stimulating molecules. In addition to MAMPs, metabolites secreted by bacteria can directly or indirectly affect differentiation, activation, and recruitment of immune cells (29). MAMPs or bacterial metabolites can also stimulate enteric neurons and consequently neurotransmitters that are secreted from the affected neurons can regulate the intestinal immune cell function (30). The effect of intestinal bacteria is not limited to local immune cells since the intestinal immune cells can exit from the intestine and travel to distal organs via blood circulation (25,31). In addition, MAMPs such as bacterial LPS and peptidoglycan as well as several bacterial metabolites were shown to enter the systemic circulation. They directly affect hematopoiesis in bone marrow and differentiation, activation, and trafficking of mature immune cells in peripheral organs (32,33).

Regulation of immune cells by particular commensal bacteria species is best recognized in differentiation of intestinal $CD4^+$ and $CD8^+$ T cells (**Fig. 2**). *Bacteroides fragilis* was shown to direct the development of inducible $Foxp3^+$ Treg cells without affecting natural Tregs (34). Polysaccharide A (PSA) produced by *B. fragilis* directly stimulates TLR2 in mouse Tregs and promotes $CD103^+$ dendritic cell-mediated human Treg differentiation (35,36). *Clostridium* species also increase colonic Treg differentiation by increasing epithelial secretion of TGF-β and indoleamine 2,3-dioxygenase (IDO) (37,38). In contrast to the Treg-promoting bacteria, adhesion of segmented filamentous bacteria (SFB) to the terminal ileum induces epithelial production of serum amyloid A proteins (SAAs) and secretion of IL-22 by innate lymphoid cells 3, which in turn promote SFB-specific Th17 cell differentiation (39-43). Introduction of *Akkermansia muciniphila* into gnotobiotic mice colonized with altered Schaedler flora also resulted in generation of *A. muciniphila*-reactive $CD4^+$ T cells in Peyer's patches, but in this case majority of cells adapted follicular helper T (Tfh) cell fate (44). On the other hand, colonization of specific pathogen-free (SPF) mice with *A. muciniphila* led to differentiation of *A. muciniphila*-specific $CD4^+$ T cells into Th1, Th17, and Treg subsets in addition to Tfh cells in the small intestinal and colonic lamina propria, suggesting that microbial contextual signals critically influence T cell responses to specific commensal bacteria (44). Context-dependent differentiation of bacteria-specific $CD4^+$ T cells was also demonstrated with *Helicobacter* species. During homeostasis, *Helicobacter* species induce differentiation of $ROR\gamma t^+Foxp3^+$ Tregs producing IL-10, but in the inflammatory condition they induce Th17 cell differentiation and contribute to exacerbation of colitis (45,46). *Klebsiella* species is normally resident in the oral cavity. However, when the intestinal microbiota is dysbiotic, *Klebsiella* species can ectopically colonize the intestine and potently induce IFN-γ^+ Th1 cell differentiation, again illustrating the context-dependent regulation of intestinal immune cells by commensal bacteria (47).

Many commensal bacteria in the intestine digest dietary fibers and produce high concentrations of short chain fatty acids (SCFAs) such as acetate, propionate, and butyrate. SCFAs potently inhibit the activity of histone deacetylases (HDACs) and activate G protein-coupled receptors, GPR41 and 43. Both HDAC inhibition and GPR43 activation by SCFAs were shown to enhance differentiation of $Foxp3^+$ Tregs in the intestine (48,49). Secondary bile acids are other bacterial metabolites abundantly present in the intestine. It was shown that two derivatives of lithocholic acid (LCA), 3-oxoLCA and isoallolithocholic acid (isoalloLCA),

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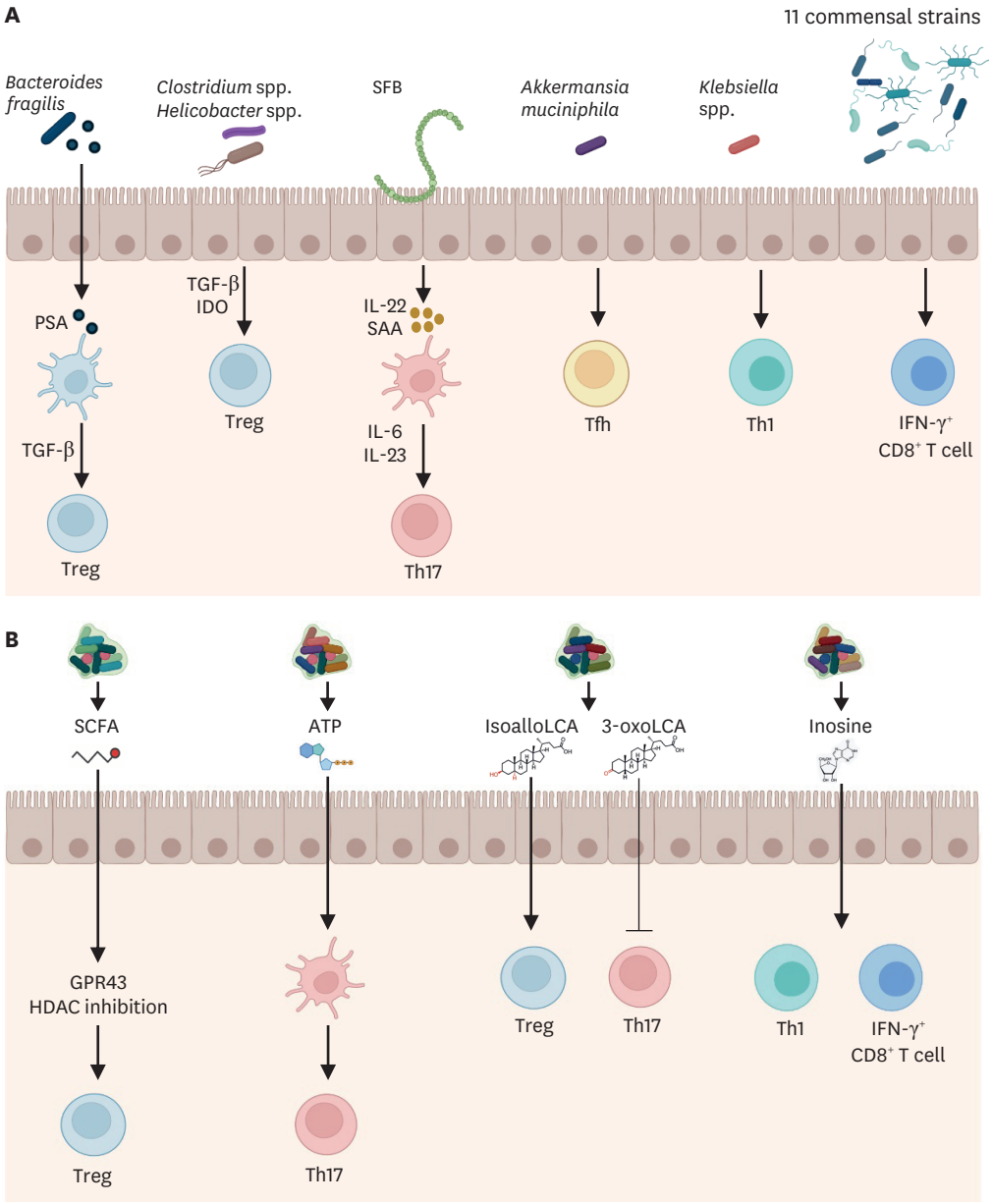

Figure 2. Regulation of intestinal immune cells by commensal bacteria.
(A) PSA released by *B. fragilis* makes dendritic cells to secrete TGF-β and induces Treg cell differentiation. *Clostirdium* spp. and *Helicobacter* spp. increase Treg cells by upregulating TGF-β and IDO. SFB increase production of IL-22 and SAA and promote dendritic cell-mediated Th17 cell differentiation. *A. muciniphila* induces differentiation of Tfh cells whereas *Klebsiella* spp. enhance Th1 cell differentiation. The mixture of 11 specific bacteria increases IFN-γ$^+$CD8$^+$ T cells in the intestine. (B) SCFA produced by bacteria-mediated fermentation of fibers promote Treg differentiation by activation of GPR43 and inhibition of HDAC. ATP generated by commensal bacteria induces Th17 cell differentiation. Intestinal bacteria metabolize bile acids to produce secondary bile acids. isoalloLCA increases Treg cells while 3-oxoLCA inhibits Th17 cell differentiation. Some commensal bacteria produce inosine which promotes differentiation of Th1 cells and increases IFN-γ$^+$CD8$^+$ T cells. Parts of figures were created with BioRender.com.

control the balance of Treg and Th17 cells by distinct mechanisms (50). The 3-oxoLCA directly binds to the transcription factor RORγt and inhibits Th17 cell differentiation whereas isoalloLCA facilitates Treg differentiation by promotion of mitochondrial ROS generation and FoxP3 expression (50). In addition, commensal bacteria-derived adenosine 5′-triphosphate (ATP) was shown to activate CD70^{high}CD11c^{low} cells in the intestinal lamina propria and enhance Th17 cell differentiation without affecting Th1 cells (51).

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In the case of $CD8^+$ T cells, Honda and colleagues (52) isolated a consortium of 11 bacterial strains from feces of a healthy human donor and showed that it strongly induces IFN-γ^+ $CD8^+$ T cells in the mouse intestine without causing inflammation. Induction of intestinal $CD8^+$ T cells by those 11 bacterial strains was dependent on $CD103^+$ dendritic cells and MHC class I molecules. Colonization of mice with the 11 bacterial mixture enhanced host defense against *Listeria* infection as well as the therapeutic efficacy of ICIs (52). Gut microbiota-derived butyrate was also shown to increase expression of IFN-γ, granzyme B, and other effector molecules of $CD8^+$ T cells in a manner that is dependent on HDAC inhibition (53). Additionally, butyrate promotes memory cell differentiation of $CD8^+$ T cells via uncoupling of the TCA cycle from glycolysis and promotion of fatty acid oxidation (54). The positive effect of butyrate on $CD8^+$ T cell memory response was dependent on GPR41 and GPR43. Another SCFA, pentanoate produced by human commensal bacteria *Megasphaera massiliensis* induced production of IFN-γ and TNF-α in $CD8^+$ T cells *in vitro* and enhanced anti-tumor activity of Ag-specific T cells in mice (55).

COMMENSAL BACTERIA AND CANCER THERAPY

The use of bacteria for cancer therapy dates back to the late 19th century (56,57). In 1868, Wilhelm Busch first described the spontaneous regression of tumors in patients who suffered from erysipelas, a skin infection by *Streptococcus pyogenes*. This phenomenon was later confirmed by Friedrich Fehleisen and others (56,57). Inspired by these observations, American surgeon William Bradley Coley purposefully injected the mixture of live or heat-inactivated *S. pyogenes* and *Serratia marcescens*, called "Coley's toxin", into patients' tumors and achieved tumor regression or complete cure in many cases. However, this practice did not receive a wide acceptance in the medical community due to the lack of mechanistic understanding and the risk of infecting patients with highly pathogenic bacteria (56,57). Although "Coley's toxin" was no longer used in clinical practices after the 1950s, other bacteria-derived agents are currently in use for cancer treatment. Intravesical injection of Bacillus Calmette-Guerin, an attenuated form of *Mycobacterium bovis*, is a standard therapy of high-risk superficial bladder cancer (58). Monophosphoryl lipid A derived from LPS of *Salmonella enterica* serovar Minnesota R595 is being used as an adjuvant in a vaccine for human papilloma virus causing cervical carcinoma (59).

In the last decade, advancement in metagenomic sequencing technologies and growing appreciation of the functional importance of commensal bacteria in regulating the immune system have led to fundamental discoveries linking commensal bacteria with cancer therapies. In a landmark paper, Zitvogel and colleagues (60) reported that chemotherapy with cyclophosphamide depends on the presence of gut microbiota. Tumors in germ-free mice or in mice depleted of gram-positive bacteria by vancomycin treatment were resistant to antitumor effects of cyclophosphamide (**Fig. 3A**). They further showed that cyclophosphamide disrupts the gut mucosal barrier and permits translocation of intestinal bacteria into secondary lymphoid organs, which presumably leads to stimulation of anticancer T cell responses (60). Similarly, Goldszmid and colleagues (61) reported that another chemotherapeutic agent, oxaliplatin, loses its antitumor efficacy in germ-free or antibiotics-treated mice. In addition, immunotherapy using the combination of CpG-oligodeoxynucleotide and anti-IL-10 receptor Ab was shown to require presence of commensal microbiota (61). Subsequently, efficacy of ICIs such as anti-CTLA-4, anti-PD-1, and anti-PD-L1 Abs was also demonstrated to be dependent on the commensal microbiota

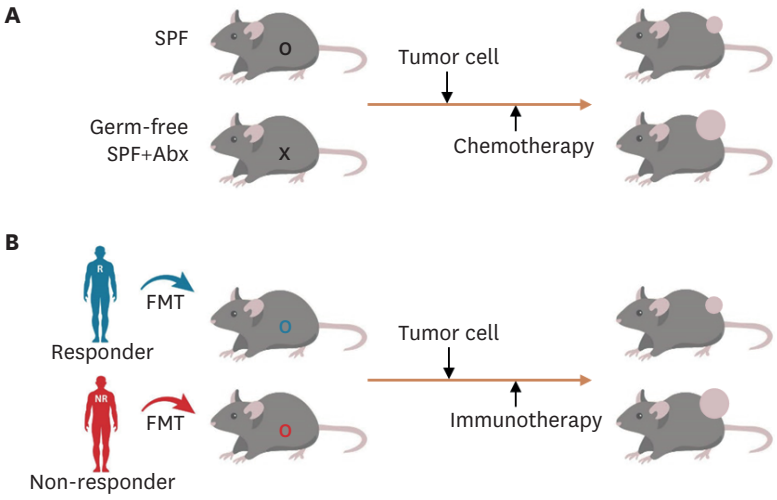

Figure 3. Experimental strategies for establishing causal relationship between commensal microbiota and efficacy of cancer therapeutics.
(A) Chemotherapy is effective in controlling tumor growth in mice raised in SPF conditions but not in germ-free or antibiotics (Abx)-treated mice which lack commensal bacteria. (B) Mice carrying human microbiota can be generated by transplanting feces from immunotherapy responders (N) or non-responders (NR) to germ-free mice. The immunotherapy is effective in mice having microbiota of the responders but not in mice having the non-responder's microbiota.

in both mice and humans (62-66). Especially, variable responsiveness of individual cancer patients to anti-PD-1 therapy was nicely mirrored in mice which received fecal bacteria transplant from each patient, strongly suggesting that the composition of microbiota determines the therapeutic efficacy of immunotherapy (**Fig. 3B**) (64-66). In these and following studies, metagenome analyses of intestinal bacteria of therapy responders and non-responders have identified several bacteria species, abundance of which correlated with the efficacy of the particular immunotherapeutic agents. By administering respective bacteria into tumor bearing mice and comparing the efficacy of the therapy, the cause and effect of a single or a combination of commensal bacteria on the enhancement of anticancer immune responses can be assessed in preclinical settings. Below we describe representative commensal bacteria that are found to be beneficial for cancer therapy. Regulation of various immune cell types by specific intestinal bacteria species in the context of immunotherapy or chemotherapy is also depicted in **Fig. 4**.

Bifidobacterium species

Bifidobacterium is a genus of gram-positive, anaerobic bacteria belonging to the Actinobacteria phylum (67). It is one of the most dominant bacteria genera in the intestine and also inhabits the oral cavity and vagina (68). Some *Bifidobacterium* species have been used as probiotics that provide various health benefits (69).

Sivan et al. (63) noticed that subcutaneous B16.SIY melanoma growth was significantly faster in mice that originated from Taconic Farms (TAC) than in mice of Jackson Laboratory (JAX). By cohousing and fecal transplant experiments, they found that the disparate tumor growth rates in mice from the two vendors were due to the differences in the intestinal microbiota composition. Subsequently, they identified that *Bifidobacterium* spp. were significantly more abundant in the fecal contents of JAX mice and were positively associated with anticancer T cell responses. Oral administration of a cocktail of commercially available *Bifidobacterium*

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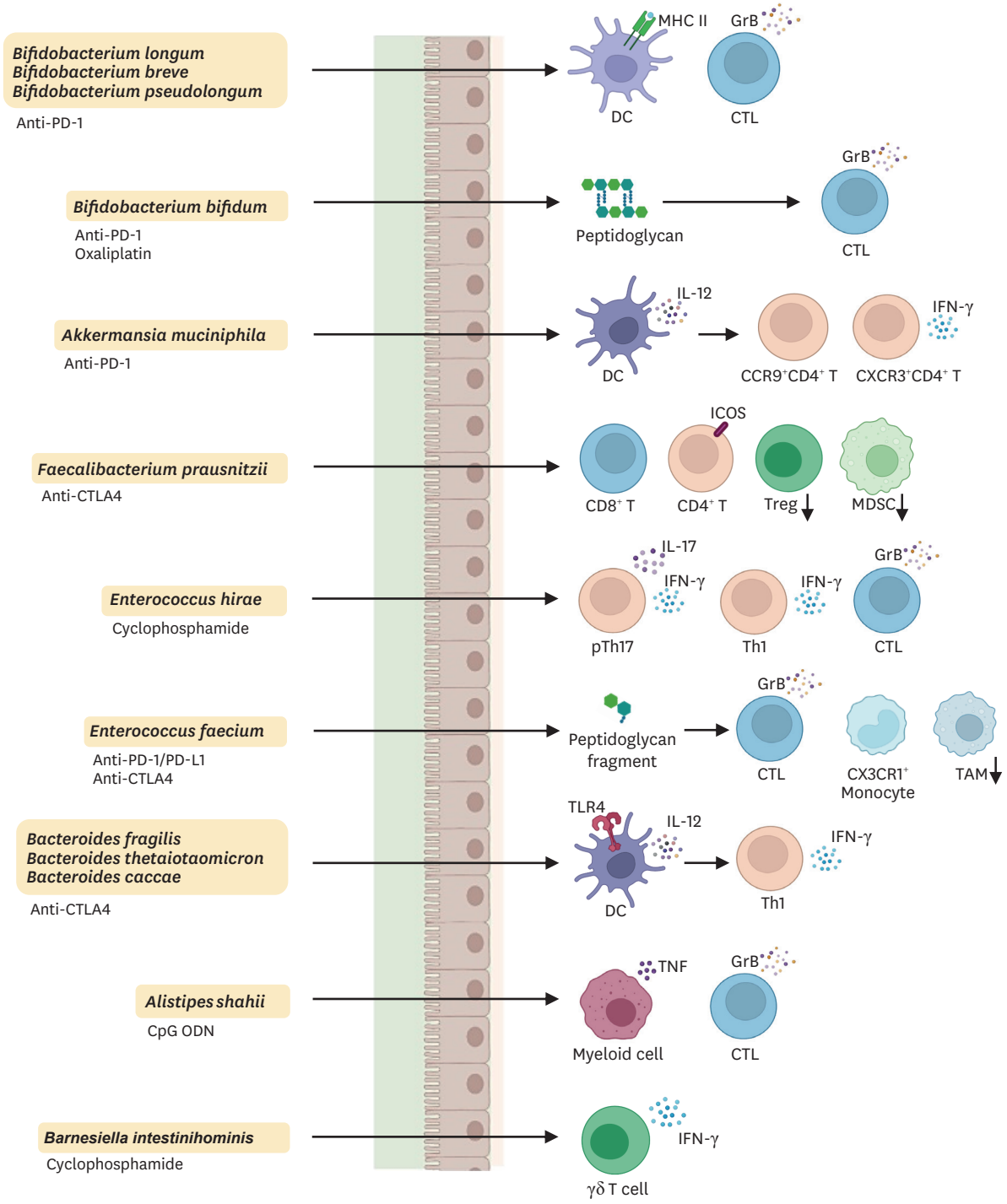

Figure 4. The immune modulation by commensal bacteria in the context of anticancer therapy.
Regulation of various immune cell types by specific bacteria species in the context of immunotherapy or chemotherapy is depicted. Parts of figures were created with BioRender.com. DC: dendritic cell, GrB, granzyme B; MDSC, myeloid-derived suppressor cell; pTh17, pathogenic Th17; TAM, tumor-associated macrophage.

species (*Bifidobacterium breve* and *Bifidobacterium longum*) to TAC mice resulted in tumor growth retardation to the same degree as anti-PD-L1 Ab therapy. The combined treatment of *Bifidobacteria* and anti-PD-L1 Ab was significantly more effective than the single treatment of either agent. Tumor infiltrating dendritic cells (DCs) from *Bifidobacterium*-treated TAC mice

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showed upregulation of genes associated with DC maturation, Ag cross presentation, type I interferon signaling, and $CD8^+$ T cell activation, compared to DCs from untreated TAC mice. Therefore, *Bifidobacterium* spp. seem to exert antitumor effect by enhancing DC functions and stimulating the effector function of tumor-specific $CD8^+$ T cells (63).

In humans, *B. longum* and *Bifidobacterium adolescentis*, along with *Enterococcus faecium* and a few other bacteria species, were found to be more abundant in stool samples from metastatic melanoma patients who positively responded to anti-PD-1-based immunotherapy compared to the samples from non-responders (66). Importantly, the stool samples were taken before the initiation of the immunotherapy, suggesting that the baseline microbiota composition of individual patients is highly associated with the clinical response to the immunotherapy. Germ-free mice successfully colonized with the fecal microbiota from the responders showed a better control of tumor growth and enhanced response to the anti-PD-1 Ab therapy than mice colonized with the microbiota of non-responders (66). Similarly, *Bifidobacterium bifidum* was enriched in stool samples from NSCLC patients who responded to cancer therapeutics such as platinum-based chemotherapy, ICIs, and epidermal growth factor receptor kinase inhibitors (70). Many *B. bifidum* strains were shown to suppress tumor growth in several mouse syngeneic tumor models when orally administered before the tumor inoculation. However, not every *B. bifidum* strain exhibited synergistic antitumor effect in a combination therapy with oxaliplatin or anti-PD-1 Ab. Moreover, the synergistic effect was not seen in TLR2-deficient mice, suggesting that peptidoglycan-mediated signaling is a key factor determining the strain-specific synergistic effect of *B. bifidum* on cancer therapeutics.

The beneficial effect of *Bifidobacteria* was also shown in the anti-CD47 immunotherapy (71). Oral administration as well as intravenous injection of a *Bifidobacterium* cocktail (*B. bifidum, B. longum, Bifidobacterium lactis, and B. breve*) restored the antitumor efficacy of CD47-based therapy in germ-free or TAC mice which originally showed resistance to the therapy. Interestingly, in both oral and systemic administration, live *Bifidobacterium* were detected in tumor tissues, but not in the lung. Furthermore, local injection of low dose antibiotics into the tumor abolished the antitumor effect of oral or systemic *Bifidobacterium* administration, suggesting that *Bifidobacterium* exerts its immunostimulatory effects directly in the tumor tissue. It was hypothesized that the hypoxic condition of the tumor core mimics the anaerobic condition of the colon and permits the survival of *Bifidobacterium.* In addition, *Bifidobacterium*-mediated facilitation of anti-CD47 immunotherapy was dependent on the activation of the STING-type I interferon pathway (71).

Despite many studies demonstrating the beneficial role of *Bifidobacteria*, exact bacterial components responsible for their immunostimulatory function are not well known. Recently, McKoy and colleagues showed that *Bifidobacterium pseudolongum* is frequently found in tumor tissues of mouse orthotopic colorectal cancer models after immunotherapy with anti-CTLA-4 or anti-PD-L1 Ab (72). Monocolonization of *B. pseudolongum* in germ-free mice enhanced efficacy of both anti-CTLA-4 and ant-PD-L1 therapy whereas *B. pseudolongum* alone without the immunotherapy did not show significant tumor growth inhibition in spite of its ability to increase Th1 and $CD8^+$ T cells in the intestine. Notably, the immunotherapy-promoting activity of *B. pseudolongum* could be transferred to germ-free mice by simply transferring the serum of the anti-CTLA-4-treated, *B. pseudolongum*-monocolonized mice. It turned out that inosine made by *B. pseudolongum* can directly enhance Th1 cell differentiation via adenosine 2A receptor ($A_{2A}R$) signaling in T cells. In the same study, it was shown that *A. muciniphila*, another bacterium known to increase the immunotherapy efficacy (see below), also produces

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a high amount of inosine and utilizes the inosine-$A_{2A}R$ signaling for its immunotherapy-promoting activity (72).

A. muciniphila

A. muciniphila is a gram-negative bacterium belonging to the Verrucomicrobia phylum (73). It is a strict anaerobe and is well-known for its mucin-degrading activity in the intestine as its name implies (73). *A. muciniphila* has received a lot of attention due to its potentially beneficial effect in preventing obesity and type 2 diabetes (74,75).

Intestinal colonization of *A. muciniphila* starts in early life and develops within a year to a level found in adults (76). It is one of the major intestinal bacterial species in healthy adults and its proportion decreases in the elderly. In a study comparing the gut microbiota of NSCLC and RCC patients who received anti-PD-1 therapy, a relatively higher level of *A. muciniphila*, together with *Enterococcus hirae*, was observed in therapy responders compared to non-responders (64). The abundance of *A. muciniphila* was further associated with the increase in peripheral blood Th1 and Tc1 responses and with better prognosis. Fecal microbiota transplantation (FMT) using stools from responders or oral administration of *A. muciniphila* alone into germ-free or ABX-treated mice resulted in improved response to anti-PD-1 therapy in subcutaneous tumor models. It was further shown that *A. muciniphila* stimulated IL-12 secretion from DCs and increased $CCR9^+CD4^+$ T cells and $CXCR3^+$ Th1 cells in tumor tissues (64). *A. muciniphila* was also found to be enriched in anti-PD-1 therapy responders among hepatocellular carcinoma patients (77). However, another study found that *A. muciniphila* was rather enriched in stool samples from Korean NSCLC patients who did not respond to various cancer therapeutics including anti-PD-1 therapy and chemotherapy, which might be due to functional variations in different *A. muciniphila* strains (70). Therefore, regulation of the anticancer immune responses by *A. muciniphila* might depend on the ethnicity of patient groups or other factors.

E. hirae and *E. faecium*

Enterococcus species are gram-positive, facultative anaerobes belonging to the Firmicutes phylum (78). *E. faecium* and *E. faecalis* are the two predominant gram-positive cocci in human stools and some *Enterococci* are also found in the urogenital tracts and the oral cavity. *Enterococci* can cause a variety of infections, including urinary tract infections, endocarditis, meningitis, and bacteremia. Moreover, vancomycin-resistant enterococcus is a leading cause of health care-associated infection (78). However, commensal strains of *E. faecium* and *E. faecalis* have been safely used as probiotics in humans for a long time (79).

E. hirae, together with other gram-positive bacteria, were found to translocate from the gut lumen to secondary lymphoid organs after treatment of mice with cyclophosphamide which disrupts the intestinal barrier (60). Near depletion of intestinal microbiota with broad spectrum antibiotics abolished the anticancer efficacy of cyclophosphamide, demonstrating that the microbiota mediates the cancer inhibitory effect of cyclophosphamide. In a following study, it was shown that colonization of *E. hirae* alone was sufficient to restore the efficacy of cyclophosphamide in antibiotics-treated mice and *E. hirae* was designated as "oncomicrobiotics" which are immunogenic commensals influencing the host-cancer equilibrium (80). The anticancer effect of *E. hirae* was attributed to induction of IFN-γ^+CD4^+ T cells, pathogenic Th17 cells, and cytotoxic T cells (80). As previously mentioned, *E. hirae* was also found to be enriched in responders of anti-PD-1 therapy compared to non-responders among NSCLC and RCC patients (64).

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E. faecium was relatively more abundant in immunotherapy-responders of metastatic melanoma patients (66). A recent study found that several strains of *E. faecium* can synergize with anti-PD-1 therapy in mouse syngeneic tumor models while administration of *E. faecium* alone did not exhibit a significant tumor inhibitory effect (81). In addition to *E. faecium*, other *Enterococci*, such as *E. hirae*, *Enterococcus durans*, and *Enterococcus mundtii* but not *Enterococcus faecalis*, also showed an immunotherapy-enhancing activity. These active *Enterococci* commonly expressed a conserved NlpC/p60 peptidoglycan hydrolase SagA that generates immune-stimulating muropeptides and required the innate immune sensor NOD2 for their anticancer effects. Expression of SagA in non-protective *E. faecalis* was sufficient to enhance efficacy of ICIs including anti-CTLA-4, anti-PD-1, and anti-PD-L1 Abs. Moreover, heterologous expression of SagA in probiotic bacteria *Lactococcus lactis* endowed the bacteria with anticancer activity, suggesting that SagA or its enzymatic products such as muramyl dipeptides can be utilized for improving response to immunotherapy.

Bacteroides thetaiotaomicron and *B. fragilis*

Bacteroides species belong to the *Bacteroidetes* phylum and they are obligate anaerobic, gram-negative bacteria (82). *Bacteroides* spp. were estimated to account for up to 25% of intestinal anaerobic bacteria and they maintain a complex and generally beneficial relationship with the host (82).

In a SPF condition, *B. thetaiotaomicron* and *Bacteroides uniformis* were found to be enriched in the small intestine mucosa of tumor-bearing mice 24 to 48 h after anti-CLTA-4 Ab injection and oral administration of *B. thetaiotaomicron* and *B. fragilis* into antibiotics-treated mice recovered the anticancer efficacy of anti-CLTA-4 therapy (62). Additionally, colonization with *B. fragilis* restored therapeutic response of germ-free mice to anti-CLTA-4 Ab by inducing maturation of DCs in the tumor tissue and increasing Th1 immune responses in the tumor-draining lymph nodes. Furthermore, adaptive transfer of *B. fragilis*-specific memory Th1 cells into germ-free mice partially restored the efficacy of the immune checkpoint blocker. Similarly, anti-CTLA-4 therapy in metastatic melanoma patients caused a significant increase in IFN-γ^+CD4$^+$ T cells that are reactive to *B. thetaiotaomicron* and *B. fragilis* (62). Melanoma patients who responded to anti-CTLA-4 plus anti-PD-1 therapy also possessed more *B. thetaiotaomicron* in their fecal samples than non-responders and *B. caccae* was enriched in the responders to all types of ICI therapy (83). Collectively, these data imply a potential role of *Bacteroides*-specific T cells in antitumor immune responses.

Faecalibacterium species

Faecalibacterium is an obligate anaerobic, gram-positive bacterium belonging to the Firmicutes phylum (84). Along with *Bacteroides, Bifidobacterium,* and *Clostridium, Faecalibacterium* is a predominant bacterial genus in the human gut. Especially, *Faecalibacterium prausnitzii* was estimated to represent up to 5% of total fecal bacteria in healthy adults but its proportion seems to decrease in patients suffering from metabolic disease or intestinal disorders (84). Similarly, *F. prausnitzii* was also found to be reduced in cancer patients with NSCLC (70).

Among metastatic melanoma patients, the higher abundance of *Faecalibacterium* spp. were observed in responders to anti-CTLA-4, anti-PD-1, or the combination of anti-CTLA-4 and anti-PD-1 therapy and the enrichment of *Faecalibacterium* was correlated with prolonged progression-free survival (PFS) (65,83,85,86). One study found that *F. parausnitzii* level was negatively associated with serum concentration of butyrate which was shown to inhibit the antitumor effect of anti-CTLA-4 by preventing dendritic cell maturation and downregulating

https://doi.org/10.4110/in.2022.22.e3

the accumulation of $ICOS^+CD4^+$ T cells in mice (85). In metastatic melanoma patients, the abundance of *Faecalibacterium* in the baseline microbiota that are present before the anti-CTLA-4 treatment was correlated with upregulation of $ICOS^+CD4^+$ T cells and downregulation of Treg cells in the peripheral blood. It was also positively correlated with clinical benefit (86). However, most studies on *Faecalibacterium* to date showed only correlation with immunotherapy efficacy and did not prove their causal relationship.

Other bacteria

Many other commensal bacteria have been reported to be associated with efficacy of cancer therapeutics in mice and humans and some of them were directly shown to promote anticancer immune responses in preclinical models.

Monocolonization of *Alistipes shahii* in antibiotics-treated mice restored TNF production from tumor-infiltrating myeloid cells and controlled cancer growth upon immunotherapy using combination of CpG-oligodeoxynucleotide and anti-IL-10 receptor Ab (61). *Barnesiellar intestinihominis* facilitated cyclophosphamide-mediated cancer inhibition by promoting the infiltration of IFN-γ^+ γδT cells into tumor tissues of mice (80). In addition, memory Th1 immune responses to *B. intestinihominis* was shown to predict the prolonged PFS in advanced lung and ovarian cancer patients after platinum-based chemotherapy (80). Anticancer immune responses were studied not only for individual commensal bacterium species but also for a consortium of several bacteria. Administration of a mixture of 11 bacteria isolated from a healthy human donor strongly induced IFN-γ^+CD8^+ T cells in the colon of germ-free mice and prevented tumor growth with or without combined immunotherapeutics such as anti-PD-1 and ant-CTLA-4 Abs (80). Several species of *Bacteroides* and *Parabacteroides* were included in the 11 bacterial mixture along with *Alistipes senegalensis*, *Paraprevotella xylaniphila*, *Eubacterium limosum*, *Ruminococcaceae bacterium* cv2, *Phascolarctobacterium faecium*, and *Fusobacterium ulcerans*. The mixtures of 7 selected bacteria out of 11 or the other 4 bacteria were less efficient in inducing IFN-γ^+CD8^+ T cells, suggesting that the IFN-γ^+CD8^+ T cell-enhancing activity of the 11 bacteria mixture does not solely depend on one particular bacteria species and rather requires combined action of two or more bacteria included in the mixture (80).

CLINICAL TRIALS OF FECAL MICROBIOTA TRANSPLANT FOR CANCER THERAPY

A number of clinical trials are currently underway to test the effect of commensal bacteria for boosting the efficacy of pre-existing cancer drugs, especially of ICIs (**Table 1**). Among them, the preliminary results of two clinical trials that examined the effect of FMT from immunotherapy-responders to non-responders were published last year (87,88).

In a single-arm clinical study (NCT03341143) carried out by Davar et al. (87), 16 melanoma patients who did not respond to previous anti-PD-1 therapy were recruited as recipients of FMT. FMT donors were selected from patients who showed complete or partial response to the anti-PD-1 therapy. A single donor-derived FMT was administered to individual recipients by endoscopy together with one cycle of anti-PD-1 Ab (pembrolizumab), followed by anti-PD-1 therapy every three weeks. Treatment-related adverse events were minimal and, out of 15 patients who were evaluated for response, object responses were noted in 3 patients (1 complete response and 2 partial responses) and 3 other patients had durable stable disease for more than 12 months.

https://doi.org/10.4110/in.2022.22.e3

Table 1. List of the clinical trials testing the efficacy of commensal bacteria for cancer therapy

Intervention	Combination	Phase	Status	Cancer types	NCT identifier	Ref.
Single strain						
EDP1503 (*Bifidobacterium animalis* subsp. *lactis*)	Anti-PD-1 Ab	Phase 1, 2	Completed	Colorectal cancer, gastroesophageal cancer, renal cell carcinoma, bladder cancer, breast cancer, lung cancer	NCT03775850	
GEN-001 (*Bifidobacterium bifidum*)	Anti-PD-L1 Ab	Phase 1	Recruiting	NSCLC, head and neck cancer, urothelial carcinoma	NCT04601402	(70)
MRx0518 (*Enterococcus gallinarum*)	Anti-PD-1 Ab	Phase 1, 2	Recruiting	NSCLC, renal cell carcinoma, melanoma, bladder cancer	NCT03637803	
MRx0518 (*Enterococcus gallinarum*)	Anti-PD-L1 Ab	Phase 2	Not yet recruiting	Urothelial carcinoma	NCT05107427	
MRx0518 (*Enterococcus gallinarum*)	Radiation therapy	Phase 1	Recruiting	Pancreatic cancer	NCT04193904	
CBM588 (*Clostridium butyricum*)	Anti-PD-1 Ab, anti-CTLA-4 Ab	Phase 1	Recruiting	Renal cell carcinoma	NCT03829111	
CBM588 (*Clostridium butyricum*)	Anti-PD-1 Ab, tyrosine kinase inhibitor	Phase 1	Recruiting	Solid cancers	NCT05122546	
Kex02 (*Lactobacillus rhamnosus*)	Anti-PD-1, platinum-based chemotherapy	Not applicable	Recruiting	NSCLC	NCT05094167	
M9 (*Lactobacillus rhamnosus*)	Anti-PD-1 Ab	Not applicable	Recruiting	Liver cancer	NCT05032014	
Consortia and others						
MET-4 (more than 30 commensal strains)	Anti-PD-1/PD-L1 Ab	Early Phase 1	Recruiting	All solid tumors	NCT03686202	
VE800 (11 commensal bacterial strains)	Anti-PD-1 Ab, vancomycin	Phase 1, 2	Active, not recruiting	Melanoma, gastric cancer, colorectal cancer	NCT04208958	(52)
SER-401 (multifunctional bacterial consortia)	Anti-PD-1 Ab	Phase 1	Active, not recruiting	Melanoma	NCT03817125	
BIFICO (mixture of *Enterococcus faecalis*, *Bifidobacterium longum*, *Lactobacillus acidophilus*)	Chemotherapy, targeted therapy	Not applicable	Not yet recruiting	Colorectal cancer	NCT04131803	
Fecal microbiota transplantation						
Fecal contents from responders	Anti-PD-1 Ab	Phase 2	Active, not recruiting	Melanoma	NCT03341143	(87)
Fecal contents from responders	Anti-PD-1 Ab	Phase 1	Unknown	Melanoma	NCT03353402	(88)
Fecal contents from responders	Immune checkpoint inhibitors	Not applicable	Recruiting	Melanoma	NCT04577729	
Fecal contents from pooled-donor	Anti-PD-1 Ab, anti-CTLA-4 Ab	Phase 2	Not yet recruiting	Melanoma	NCT04988841	
Fecal contents from a healthy donor	Immunotherapy	Phase 1	Active, not recruiting	Melanoma	NCT03772899	
Fecal contents from responders	Anti-PD-1 Ab	Phase 1, 2	Recruiting	Melanoma, lung cancer	NCT04521075	
Oral restorative microbiota therapy	Anti-PD-L1 Ab, chemotherapy	Phase 2	Not yet recruiting	Lung cancer	NCT04105270	
Fecal contents form healthy individuals or responders	Anti-PD-1/PD-L1 Ab	Not applicable	Recruiting	Lung cancer	NCT04924374	
Fecal contents from responders	Immune checkpoint inhibitor	Phase 2	Recruiting	Lung cancer	NCT04951583	
Fecal contents from responders	Anti-PD-1/PD-L1 Ab, chemotherapy	Phase 1	Not yet recruiting	Lung cancer	NCT05008861	
Fecal contents from responders	Anti-PD-1 Ab, anti-CTLA-4 Ab	Phase 1	Recruiting	Renal cell carcinoma	NCT04163289	
Fecal contents from responders	Immune checkpoint inhibitors	Phase 1, 2	Recruiting	Renal cell carcinoma	NCT04758507	
Fecal contents from responders	Anti-PD-1 Ab, androgen receptor antagonist	Phase 2	Recruiting	Prostate cancer	NCT04116775	
Fecal contents from responders	Anti-PD-1 Ab	Phase 1	Recruiting	Gastrointestinal system cancer	NCT04130763	
Fecal contents from responders	Anti-PD-1 Ab	Early Phase 1	Recruiting	Gastrointestinal system cancer	NCT04729322	
Fecal contents from responders	Surgical resection	Early Phase 1	Not yet recruiting	Pancreatic ductal adenocarcinoma	NCT04975217	
Oral microbiome restoration therapy	Surgical resection	Early Phase 1	Recruiting	Breast cancer	NCT04139993	
Fecal contents from responders	Immunotherapy	Not applicable	Recruiting	Solid carcinoma	NCT04264975	

Shotgun metagenomic sequencing of fecal samples from donors and recipients collected before and after FMT showed that the gut microbiota composition shifted significantly toward donor microbiota in responders but not in non-responders (87). Bacteria belonging

to phyla Firmicutes (*Lachnospiraceae* and *Ruminococcaceae* families) and Actinobacteria (*Bifidobacteriaceae* and *Coriobacteriaceae* families) were significantly increased in responders and species associated with the clinical response included *B. longum* and *F. prausnitzii*. In contrast, most of the bacteria decreased in responders after FMT belonged to phylum Bacteroidetes. Analysis of peripheral blood mononuclear cells by single-cell RNA sequencing showed that $CD8^+$ T cells and mucosal-associated invariant T cells were more activated in responders compared to non-responders. In addition, FMT altered serum metabolites and cytokines in responders (87).

Another clinical trial (NCT03353402) involved metastatic melanoma patients who had previously failed in anti-PD-1 therapy (88). Native microbiota of the recipients was first depleted by antibiotics and then fecal microbiota from two donors (donor 1 and 2) who had achieved a complete remission for more than a year was transplanted into recipients by a colonoscopy on day 0 and the oral administration of stool capsules at day 1. At day 12, the patients further received the combination of stool capsules and anti-PD-1 Ab which were repeated every 2 weeks for a total of 6 cycles and were followed by regular anti-PD-1 monotherapy. FMT recipients developed only mild adverse effects and 3 out of 10 recipients demonstrated clinical responses (1 complete response and 2 partial responses). All three responders turned out to have received FMT from the donor 1.

Stool metagenome analysis by 16S rRNA gene sequencing demonstrated that gut microbiota composition of all recipients significantly changed after FMT. Although there was no statistically significant difference in the pre-treatment microbiota composition between recipients in the donor 1 group and those in the donor 2 group, post-treatment microbiota compositions between the donor 1 group and the donor 2 group recipients differed from each other and the donor 1 group was characterized by a higher relative abundance of taxa like *B. adolescentis* (88). Although responders among the donor 1 group had a higher relative abundance of *Enterococcaceae*, *Enterococcus*, and *Streptococcus australis*, no clear association between those taxa and clinical response to therapy was established. Bulk RNA sequencing analysis of gut samples showed that the donor 1 group recipients upregulated gene sets related to Ag-presentation, innate immunity, and IL-12, whereas the donor 2 group recipients did not upregulate any immune-related genes. Similarly, upregulation of immune-related gene sets including IFN-γ signaling pathway, T cell activation, dendritic cell maturation was only observed in tumor samples of the donor 1 group recipients (88).

Combined, these two clinical studies have demonstrated that restructuring the gut microbiota by FMT was effective in activating anticancer immune responses and overcoming the resistance to immunotherapy in a subset of anti-PD-1-refractory melanoma patients. Currently, many other clinical trials are underway to evaluate the anticancer effect of FMT and commensal bacteria (single bacteria or mixture) in various types of cancer patients (**Table 1**).

FUTURE DIRECTIONS

Now it is indisputable that intestinal bacteria are key factors regulating anticancer immune responses and determining efficacy of cancer therapeutics. Consequently, interests in improving the commensal bacteria composition in cancer patients have been rapidly growing in all sectors involved in novel cancer therapy, including academia, hospitals, and drug industries. Experimental results obtained from animal models are being translated into

humans and some early clinical trials have yielded encouraging outcomes (87,88). However, there are still many issues to be resolved for successful development of therapeutic strategies utilizing commensal bacteria for cancer therapy.

Despite the rapidly accumulating metagenomic data from studies comparing the differences in intestinal bacteria compositions between cancer patients and healthy controls or between therapy responders and non-responders, it is far from clear what exactly constitutes the 'beneficial' and 'harmful' microbiota for cancer treatment. Moreover, it is plausible that 'beneficial' microbiota for cancer therapy might turn out to be 'harmful' microbiota for other diseases such as autoimmune diseases. In addition to obvious consideration of cancer types and tumor microenvironments, incorporation of various factors including patients' genetics, ethnicity, age, and lifestyle is needed for fully integrated interpretation of metagenomic data from diverse cohorts and it may be aided by adaptation of artificial intelligence.

Another critical factor to consider is functional variations of different strains belonging to same bacteria species. Short replication cycles of bacteria lead to inevitable accumulation of genetic changes and emergence of divergent strains, which may cause functional diversification of a single bacteria species and its dissimilar influences on the host immune system. Some of the conflicting reports describing positive or negative association of a particular bacteria species with immunotherapy responsiveness in cancer patients might be due to a failure in distinguishing different strains in metagenome analysis. The popular method of bacterial 16S RNA sequencing for metagenome analysis cannot detect strain differences. Instead, shotgun metagenomic sequencing combined with metatranscriptomic analysis will be a useful tool for overcoming such a problem and help us gain more accurate insights on the role of individual bacteria strains in cancer immunotherapy.

The current approach of FMT using fecal bacteria from healthy donors or responders to a particular therapy carries a small but unavoidable risk of pathogen infection. In fact, drug-resistant *Escherichia coli* infection-related sepsis and even mortality were reported in patients who received FMT for *Clostridium difficile*-associated colitis despite the donor stool screening following the protocols approved by FDA and the local institutional review board (89). Therefore, more comprehensive and stringent donor stool screening protocols need to be established to increase the safety of FMT. Eventually, alternative methods of using a single or a mixture of selected bacteria instead of the whole fecal microbiota will be preferred although clinical evidence for efficacy of a single or combination of bacteria in cancer treatment is currently lacking. Additionally, efforts to identify particular bacterial components or metabolites that mediate the beneficial effects of selected bacteria need to be continued for potential development of new cancer drugs. Identification of such factors will also facilitate understanding of molecular mechanisms that underly the positive actions of those bacteria in cancer treatment.

Another important issue for live bacteria-based pharmaceuticals is the relative difficulty of CMC (chemistry, manufacturing, and controls). As living organisms, bacteria can change their genetic makeup in a relatively short time period and adjust gene expression patterns according to variable environments. Therefore, establishment of adequate quality control protocols is essential to continuously maintain the intended efficacy of therapeutic bacteria from different batches of production. Improvement in culture conditions and production yield is another factor to consider, especially for strictly anaerobic bacteria.

https://doi.org/10.4110/in.2022.22.e3

Regardless of many hurdles for actual clinical application, commensal microbiota is certainly a very promising target of intervention for cancer treatment and prevention. Commensal microbiota can also provide valuable information for cancer diagnosis and prognosis. Successful harnessing of the power of commensal bacteria will be a game changer for cancer therapy in the future.

ACKNOWLEDGEMENTS

We thank Tirhas Niguse Gebre for proofreading this manuscript. This work was supported by grants from the National Research Foundation of Korea (2017M3A9F3047085, 2020R1A2C2011307) and the 2020 Joint Research Project Grant of Institutes of Science and Technology.

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