Iranian Journal of Blood & Cancer

Iran J Blood Cancer, 2024, 16(3), 70-85
https://ijbc.ir

Review

Microbial Allies: How Gut Microbiota Influence the Effectiveness of Immune Checkpoint Inhibitors

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Citation Azhdari Tehrani H, Yousefi AM, Salari S, Bashash D. Microbial allies: How gut microbiota influences the effectiveness of immune checkpoint inhibitors. Iran J Blood Cancer. 2024 Sep 30;16(3): 70-85.

doi 0

Article info:
Received: 04 July 2024
Accepted: 12 Sep 2024
Published: 30 Sep 2024

Keywords:
Gut microbiota
Immunotherapy
Immune checkpoint inhibitors
ICIs
Cancer

Abstract

The importance of the gut microbiota in human health and disease has been known for a long time. Current investigations involving preclinical and clinical studies have presented numerous lines of evidence indicating that gut microbiota can influence the effectiveness of cancer immunotherapies, particularly immune checkpoint inhibitors (ICIs). The gut microbiota can alter the immune response in the tumor microenvironment (TME) by engaging with innate and adaptive immune cells. Notably, one of the primary methods by which the gut microbiota modulates antitumor immunity is through the production of metabolites, which are small molecules capable of traveling from the gut to other parts of the body and influencing local and systemic antitumor immune responses. This exploration of mechanisms has yielded valuable insights for developing microbiota-based therapeutic strategies such as fecal microbiota transplantation (FMT), probiotics, engineered microbiomes, and specific microbial metabolites. In this review, we explored several possible interventions that could enhance the efficacy of ICIs, thereby potentially restoring or augmenting patient responses to these therapeutic agents.

1. INTRODUCTION

Despite the significant advancements made in the treatment of advanced or metastatic tumors through immune therapy with immune checkpoint inhibitors (ICIs), the impact of these agents on the improvement of progression-free survival (PFS) or overall survival (OS) in cancers remains limited (1). Variability in host and tumoral factors can explain the diversity of responses in immunotherapy in specific tumors (2). For instance, PD-L1 expression and tumor mutational burden (TMB) are two key decision- making markers, so tumors with high TMB and PD-L1 expression usually respond better to immunotherapy. In addition, host-associated factors such as gut microbiota influence antitumor immunity and may justify the disparity in response to ICIs (3).

As we know, each person has exclusive gut microbiota. This commensal microbiota has crucial roles in mucosal integrity, immune response regulation, and synthesis of essential vitamins (B and K) and short-chain fatty acids (SCFA). As one of the main functions of these microorganisms, the gut microbiome plays a pivotal role in the host's immune

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[DOI: 10.61186/ijbc.16.3.70]

response against tumors, and any change to the composition of the original gut microbiota leads to different responses to immunotherapy. On the other hand, the significance of antibiotics on the microbiota, recognized as dysbiosis, and its critical role in the response to immunotherapy has been extensively acknowledged (4). In the review, we aim to explore potential interventions that can restore or increase the response to these agents and may develop newer treatment strategies adjunct to ICIs.

2. OVERVIEW OF IMMUNE CHECKPOINT INHIBITORS

During the replication process of DNA, the enzyme DNA polymerase corrects most errors, but additional mechanisms, such as a mismatch repair (MMR) system, are needed. MMR system consists of proteins such as MLH1, MSH2, PMS2 and MSH6, etc. The essential role of these proteins is to detect the incorrect base pairs. Mutations of the MMR genes are common in various types of cancers. These cancer cells produce neo-antigens that trigger the host immune response. So, MMR-deficient tumors can potentiate immune response due to more expression and production of pro-inflammatory cytokines (5). MMR-deficient tumors are usually resistant to different conventional chemotherapeutic agents such as platinum or fluoropyrimidines. After detecting and processing tumor antigens by antigen-presenting cells (APCs), they present tumor antigens via MHC proteins to T cells. T cells are activated and converted to $CD4^+$ helper and $CD8^+$ killer cells (6). When a naïve T cell activates upon interaction with a new antigen by its T Cell Receptor (TCR), another costimulatory signal via CD28 of T cells and CD80 or CD86 of APCs is transmitted.

Upon the activation of T cells and their responses to damaged cells, human cells employ various mechanisms to promote immune tolerance towards host tissues. This process involves a range of immune inhibitory proteins known as immune checkpoints (IC). Among the most recognized immune checkpoint proteins are Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and Programmed cell Death proteins 1 and 2 (PD-1 and PD-2). In response to an immune challenge and to prevent damage to normal tissues, the expression of CTLA-4 is upregulated, becoming more prominent on the surface of T cells. This increased expression leads to a greater tendency for CTLA-4 to bind to CD80 or CD86, as it exhibits a higher affinity for these molecules compared to CD28. This interaction results in the inactivation of activated T cells, which subsequently modulates the immune response to prevent autoimmune issues. In addition, the T regulatory cells (T-regs) as CTLA-4-expressing cells can further downregulate immune function. Mice with a deficiency in CTLA-4 expression exhibit exaggerated T cell activation and expansion in most tissues, leading to severe autoinflammation (7).

Moreover, another regulatory mechanism involves the interaction between PD-L1 and PD-L2 present on host tissues and the PD-1 receptors located on T cells, which can inhibit immune responses. The binding of PD-L1 and PD-L2 to PD-1 induces the phosphorylation of its cytoplasmic receptor component (SHP-2), leading to its deactivation and the inhibition of downstream signal transduction of the T cell receptor. Also, SHP-2 blocks the RAS/MEK/ERK pathway that is involved in the proliferation and survival of cells, so in this way, proliferation and survival are restricted (8).

It is worth noting that CTLA-4 expression precedes PD-1 expression during T-cell activation. Deficiency in PD-1 expression in Mice usually does not lead to death in contrast to CTLA-4 deficiency. Also, deficiency in PD-1 expression leads to severe autoimmune disorders such as nephritis, arthritis, and myocarditis (9). Longtime exposure to this ligand also leads to exhaustion and eventually suppression of immune function. Although PD-L2 expression is lower than PD-L1, its affinity for interaction with the PD-1 receptor is higher, but its functional role in cancer immunotherapy is yet unclear (9). There are several activatory and inhibitory receptors on T cells which are summarized in **Figure 1**.

As mentioned above, immune checkpoints are regulators that protect immune responses from damaging normal host tissues due to over-activation, inducing self-tolerance. However, some tumor cells also can protect themselves against immune attack by stimulating immune checkpoint targets. Therefore, Immune Checkpoint Inhibitors (ICIs) that block the interaction between tumor cells and T cells can reverse this process (10). Several ICIs have successfully demonstrated favorable responses in both preclinical and clinical studies. Additionally, some of these agents have received approval from the Food and Drug Administration (FDA) for specific indications. Anti-CTLA-4 antibodies can ignite the flame of immune system responses against tumor cells via several mechanisms like exhausting and decreasing Tregs, especially in the tumor microenvironment (11). In 2011, a new CTLA-4 inhibitor, Ipilimumab, became the first to be approved by the FDA to treat advanced/metastatic melanoma (12).

In the following, two monoclonal antibodies directed against PD-1, pembrolizumab and nivolumab, were approved to treat patients with advanced-stage melanoma (13, 14). In advanced/Metastatic melanoma patients,

[DOI: 10.61186/ijbc.16.3.70]

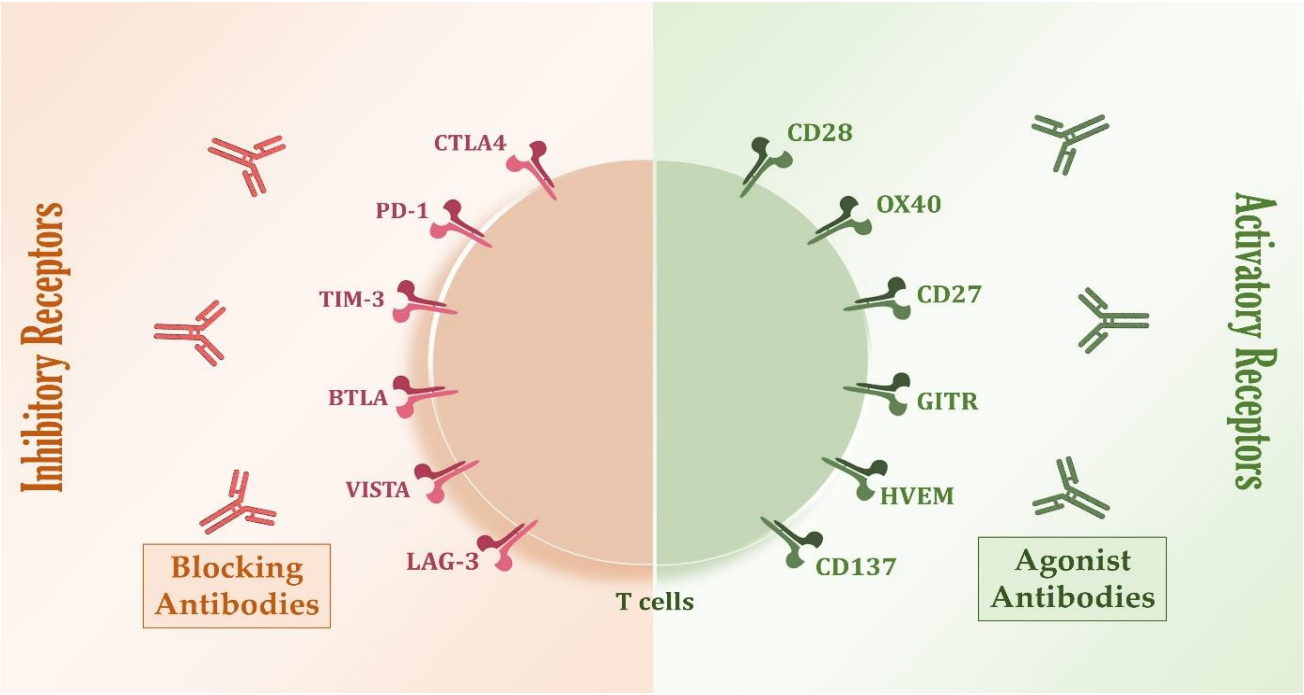

Figure 1. A summary of stimulatory and inhibitory receptors on T cells.

Pembrolizumab versus Ipilimumab monotherapy results in a better objective response rate (30% vs 12%) (15). A recent study also has reported that Nivolumab in combination with Ipilimumab improved PFS than monotherapy with Nivolumab or Ipilimumab (the least PFS was for Ipilimumab) (16). In other malignancies, adding ICIs to the conventional treatment protocol changed the cornerstone of cancer therapy, as shown by the results of the Keynote trials (024 or 042) in non-small cell lung cancer (NSCLC) (17, 18). Furthermore, PD-L1 blocking agents such as Avelumab, Atezolizumab, and Durvalumab are approved to treat metastatic urothelial carcinoma and small cell and non-small cell lung cancer (19, 20). Avelumab can activate natural killer cells (NKs) to induce antibody-dependent cytotoxicity (15).

3. THE EFFECTS OF HUMAN INTESTINAL MICROBIOTA ON IMMUNE SYSTEM

Revised estimates indicate that the number of microbes in the human body significantly exceeds the number of human cells. These microorganisms inhabit nearly all areas of the human body that come into contact with the exterior environment, such as the skin, oral cavity, respiratory system, urogenital tract, and gastrointestinal (GI) tract. The GI tract stands out as the organ with the highest population density when considering the distribution of human flora among organs. The human body relies on the intestinal microbiota to carry out various physiological, immunological, metabolic, and nutritional processes (16). One of the crucial functions of these microorganisms is the extraction of energy from dietary polysaccharides like resistant starch and dietary fibers, which would otherwise be indigestible (16). These metabolic processes produce vital nutrients, encompassing short-chain fatty acids (SCFA), vitamins K, B12, folic acid, and essential amino acids(17). In addition to these functions, the gut microbiota plays a critical role in the development, maturation, and function of both innate and adaptive immune systems (18). Moreover, by manipulating the development and function of APCs, the gut microbiota can control the host's innate immunity locally and systemically. To put it differently, the ability of APCs to defend against pathogens is not only influenced by their interaction with the microbiota, but it also plays a role in regulating immune tolerance towards the normal gut microbiota. Peyer's patches' DCs release significantly higher amounts of IL-10, an anti-inflammatory cytokine, which plays a crucial role in regulating T-cells compared to DCs found in the spleen (19). Additionally, confrontation of intestinal macrophages and PAMPs cannot lead to the production of pro-inflammatory cytokines, which highlights the significance of immune tolerance development over time. Activated DCs by microbe-

[DOI: 10.61186/ijbc.16.3.70]

originated adenosine triphosphate (ATP) induce the differentiation of Th17 cells, which are recognized to be affected by the gut microbiota. Also, the gut microbiome maintains the balance between Th17 cells and Tregs by interacting with various cytokines and metabolites such as SCFAs (20).

The linkage of neutrophils, a well-known member of the innate immune system, with intestinal flora has drawn attention. Studies on germ-free (GF) rats have demonstrated that these rats not only showed neutropenia as a quantitative neutrophile disorder but also impaired phagocytosis and malfunction in superoxide anion and nitric oxide generation as qualitative disorders, leading to defective antimicrobial activity. In addition, recognition of gut microbial peptidoglycan increases the killing activity of bone marrow neutrophils (21). However, the precise impact of the gut microbiome on neutrophil function, whether it promotes or suppresses it, remains unclear, requiring further research to clarify this matter. It is worth mentioning that the development of other innate cell types, such as natural killer (NK) and mast cells, are also not independent of the gut microbiota (22).

Moreover, evidence introduces gut microbiota as a pivotal player in the evolution of adaptive immune cells, particularly major subtypes of CD4+ T cells, including Th1, Th2, Th17, and Tregs. Investigations on GF mice claimed an imbalance between Th1 and Th2 responses, with a preference for Th2 responses (23). As a result, this finding is considered noticeable for explaining the association between gut dysbiosis and various atopic conditions like eczema and asthma. In particular, certain bacterial species, such as B. fragilis, stimulate Th1 response through their Polysaccharide A (PSA) molecule and induce Th1 evolution. These findings became the basis of a question of whether the distortion of the gut microbiota leads to an imbalance between Th1 and Th2 responses and its subsequent effects (24). Recently, an investigation on the efficiency of fecal microbiota transplantation (FMT) in individuals suffering from active atopic dermatitis, a disease characterized by a shift towards Th2 responses, has been carried out. The study revealed that most patients experienced considerable amelioration in the severity scores of the disease and reduced dependence on corticosteroids (25). These results are consistent with previous findings from mouse models, which demonstrated that restoring gut microbial diversity and achieving a Th1/Th2 immunologic balance can modulate Tregs, decrease levels of IgE, eosinophils, basophils, and mast cells, and suppress allergic responses induced by atopic dermatitis (26, 27).

Tregs, essential regulators of inflammation, apply their influence by suppressing other cell types, thereby aiding in the prevention of autoimmune disease. Recent exploration indicates that the development of Tregs is affected by the gut microbiota. Specifically, *Clostridia* facilitate their induction, *B. fragilis* can give direction to Tregs for suppressing pro-inflammatory Th17 responses, and colon-related Tregs possess a distinct repertoire of TCRs that distinguish colonic bacterial contents (28, 29). Furthermore, several studies have shown the relationship between the gut flora and intestinal CD8+ T cells and also provide valuable information about the microbiome effect on some intestinal CD8+ T functions, especially modulation of peripheral immune cells such as marginal zone B cells, NK cells, and plasmacytoid DCs (30). In summary, an impaired interplay between the gut microbiota and T cells may result in a more inflammatory environment within the gastrointestinal tract and beyond.

T cells are not the only adaptive cells touched by the gut microbiota. There is evidence about the microbiota's impact on B cell maturation and the production of immunoglobulins. B cells associated with the gut are primarily located in the Peyer's patches and transformed into IgA-secreting plasma cells (31). In GF mice, there is a reduction in the population of plasma cells and, as a result, a decrease in the IgA level. In addition, the splenic germinal center, the place of B cell differentiation and affinity maturation, is smaller in size and number in GF mice. Therefore, GF animals experience a significant reduction in serum natural IgG levels (32). In addition, exposure to microbes induces specific heavy chain repertoires of B cells immunoglobulin, and systemic exposure to microbes leads to varied and expanded IgG production (33). Despite producing a broad spectrum of immunoglobulins in response to the gut microbiome, the mechanism responsible for the preferential tolerance of beneficial commensal bacteria by immunoglobulins is not yet understood. Notably, IgE, the immunoglobulin isotype associated with allergies, is elevated throughout the body, especially in the gastrointestinal tract, which aligns with the predisposition to Th2 response in germ-free animals (34). Although the involvement of the gut microbiota in the evolution and function of B cells is evident, the precise interlinkage of microbes and the diversity of immunoglobulins remains undisclosed and should be the focus of future research. **Figure 2** Summarizes the mechanisms through which gut flora recruits either innate or adaptive immune systems.

[DOI: 10.61186/ijbc.16.3.70]

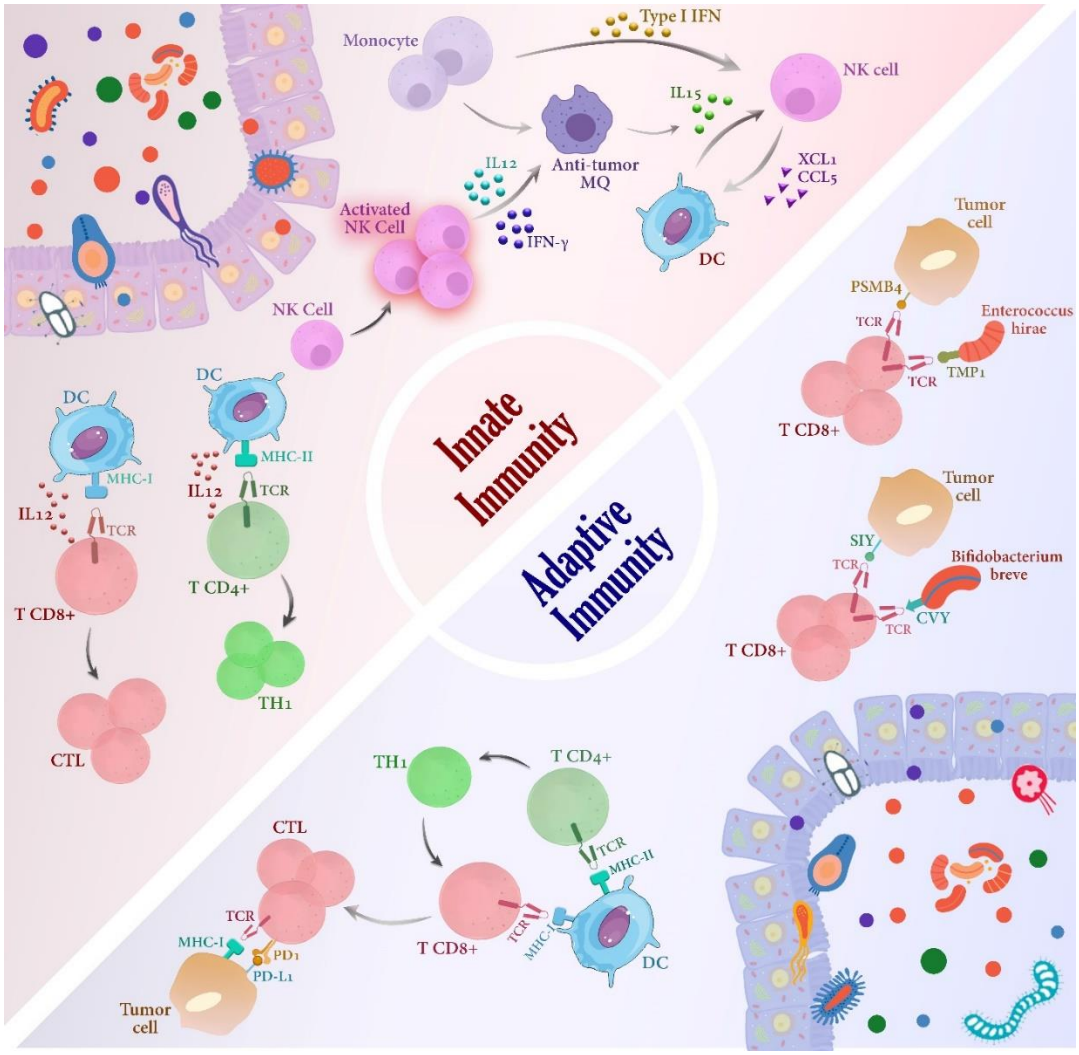

Figure 2. Fundamental roles of gut microbiota in the immune system.

4. INTERRELATION OF THE GUT MICROBIOME AND RESPONSE TO ICIS

Several studies have suggested that the gut microbiota alters the response to ICIs by recruiting the innate and adaptive immune systems, directing immune responses against tumor cells in the tumor microenvironment (TME) (18). The gut microbiome influences the types of innate immune cells, such as NK cells, DCs, monocytes, and macrophages, regulating a patient's response to ICIs. To reverse the immune tolerance caused by immature DCs, researchers used antigens or metabolites from the gut microbiota along with immunomodulators (39). For instance, *Bacteroides fragilis* can improve the effectiveness of CTLA-4 blockade by promoting DC maturation and provoking interleukin 12 (IL-12) -dependent TH1 immune responses (35). Another study revealed that oral consumption of *Bifidobacterium* stimulates tumor-specific CD8+ T cells following DC activation (36). Contrarily, a tumor-friendly environment driven by imbalances in the levels of interferon-I (IFN-I) and mononuclear phagocytes is one of the mechanisms that minimize the efficacy of immunotherapies (40). The favorable gut microbiota reprograms monocytes in the TME toward anti-tumor macrophages, while TME skews toward pro-tumor macrophages in unfavorable microbiota. Intratumoral monocytes produce type I interferon (IFN-I) in response to a microbiota-derived stimulator of interferon genes (STING) agonists to regulate macrophage polarization and NK cell-DC crosstalk, leading to improved efficacy of ICIs (41).

Furthermore, *Bacteroides fragilis* can promote innate immunity by stimulating the differentiation of macrophages into the M1 phenotype and inducing CD80 and CD86 expression (42). It is worth noting that regulation of DC and

[DOI: 10.61186/ijbc.16.3.70]

CD8+ T cell population within the TME is the most well-known mechanism through which NK cells can impact the outcomes of ICIs, and recently, a growing body of evidence has discovered the interrelation of the gut microbiota and NK cells (43, 44). Another study by Jin et al. found that patients suffering from NSCLC with a broad spectrum of microbiota have more unique memory CD8+ T cells and NK cell subsets in response to receiving PD-1 suppressors (44). Also, *Lactobacillus plantarum* and intratumorally *Bifidobacterium* effectively promote NK cell activation, thereby triggering innate anti-tumor immunity (45, 46). Multiple studies have confirmed that specific gut microbes stimulate the production of CD8+ T cells in systemic circulation and the TME (43, 47, 48). In the case of refractory metastatic melanoma, FMT enriched with *Enterococcus* is related to CD8+ T cell infiltration to the TME and causes tumor cell necrosis (49). Likewise, *Bifidobacterium* and 11 other strains increase the number of CD8+ T cells facilitated by DCs, which makes ICIs medications more efficient (36, 50). In parallel, studies enumerate amplified antigen presentation and amended function of effector CD4+ T cells and CD8+ T cells as main mechanisms recruited by *Clostridiales*, *Ruminococcaceae*, or *Faecalibacterium* to improve the anti-tumor effects of ICIs in melanoma patients (37). Additionally, *Faecalibacterium* is associated with long-term clinical benefits of ipilimumab via increasing quantity of CD4+ T cells and serum CD25 while reducing Treg cells abundance in peripheral blood (51). Interestingly, synergic effects of a combination of ICIs therapies and *B. pseudopodium* metabolite, inosine, induce Th1 differentiation and magnified anti-tumor immune responses (52).

In another study, Sivan et al. reported that tumors in mice with certain *Bifidobacterium* species in their gut had slower progression and showed a positive response to PD-1/PD-L1 blockade. As a result, using FMT or co-housing can convey these beneficial effects to other mice. Furthermore, they reported that giving oral probiotics containing *Bifidobacterium* to mice harboring impaired gut microbiota improved the anti-tumor efficiency of PD-L1 inhibition by enhanced DCs maturation and tumor-specific CD8+ T cell activity (36). Consistently, Vetizou et al. stated that the efficacy of anti-CTLA-4 therapy was boosted by administering *Bacteroides fragilis* orally along with either *Bacteroides thetaiotaomicron* or *Burkholderia cepacian*. This combination stimulated a Th1 immune response and facilitated the maturation of DCs. Contrarily, administering anti-CTLA-4 antibodies to GF mice or specific pathogen-free (SPF) mice treated with broad-spectrum antibiotics significantly reduces its efficacy. However, this decreased effect can be corrected by performing FMT on patients harboring the predominant *Bacteroides* species (35). In research conducted by Gopalakrishnan et al. on 112 melanoma patients who received anti-PD-1 therapy, there were significant differences in the population and composition of the gut microbiome between responders and non-responder individuals. Authors suggested that patients with a beneficial gut microbiome, characterized by high diversity and the presence of *Ruminococcaceae* and *Faecalibacterium,* have enhanced antigen presentation and improved function of effector T cells in both the periphery and tumor microenvironment, resulting in boosted systemic immune responses, particularly anti-tumor functions. Contrarily, patients with an "unfavorable" gut microbiome, characterized by low diversity and a high relative number of *Bacteroidales*, experienced weakened systemic and anti-tumor immune responses due to limited infiltration of lymphoid and myeloid cells within tumors and reduced antigen presentation capacity (37). In another study in the field of epithelial tumors, administering anti-PD-1/PD-L1 treatment led to a remarkably higher survival rate in patients who were not subject to routine antibiotics compared to those who did. These results suggest that antibiotics may compromise the efficiency of patients' anti-tumor immunity and ICIs by depleting the gut microbiota. Analysis of stool samples revealed that patients with higher levels of *Akkermansia* and *Alistipes* in their gut bacterial compositions responded much better to PD-1 blockade (38). **Tables 1** and **2** present a compilation of preclinical and clinical research that has examined the impact of miscellaneous elements found in the gut flora on the efficacy of ICIs therapy.

4.1. Preclinical studies

As previously stated, mounting evidence suggests that gut microbiota can either enhance or diminish the effectiveness of immunotherapy by influencing the innate and adaptive immune system. Nevertheless, the precise mechanisms by which the gut flora enhances the efficacy of immunotherapy remain incompletely comprehended. For example, observations from various methods of gut microbiome modifications, encompassing probiotics, fecal transplantation, consortia, and dietary interventions, have demonstrated that favorable microbiota modulation is associated with more CD8+ effector T cell infiltration within the TME. The infiltration of CD8+ T cells augments the function of TH1 cells and DCs settled in TME while reducing the number of immunosuppressive cells (59). Now, we delve into the potential mechanisms in which the gut microbiome might impact the function of immune cells through at least three interconnected mechanisms:

[DOI: 10.61186/ijbc.16.3.70]

Table. 1 Preclinical investigations of microbiota-associated interventions in conjunction with immune checkpoint inhibitor therapy.

Study	Year	Cancer type	Bacterial strain	Intervention	ICIs	Key findings	Refs
Sivan et al.	2015	MM (B16.SIY)	*Bacteroidales* spp.	Oral gavage	Anti-PD-L1	Bifidobacterium augments dendritic cell action resulting in enhanced CD8+ T cells and facilitates anti–PD-L1 efficiency	(36)
Vétizou et al.	2015	Sarcoma (MCA205); MM (Ret); CRC (MC38)	*B.thetaiotaomicron* *B. fragilis* *Burkholderiales*	Oral gavage FMT	Anti-CTLA4	Oral administration of *Bacteroides* spp. and *Burkholderia* spp. can promote the anti-CTLA4 anti-tumor activity. Response to human-to-mice fecal transplantation is associated with increased levels of Bacteroides supplement.	(35)
Routy er al.	2018	Sarcoma (MCA205) MM (Ret); NSCLC; RCC	*Akkermansia muciniphila*	Antibiotic cocktail: Ampicillin, Streptomycin & Colistin Oral gavage FMT	Anti-PD1± Anti-CTLA4	Oral administration of *Akkermansia muciniphila* brings back the effectiveness of anti-PD1 in antibiotic-treated mice. Restoration of sensitivity to anti-PD-1 inhibitors in mice pretreated with antibiotics preceded by FMT from ICI-sensitive mice.	(38)
Matson et al.	2018	MM (B16.SIY)	*Enterococcus faecium longum* *Collinsella aerofaciens* *Bifidobacterium*	FMT	Anti-PD-L1	A strong correlation was detected between medication sensitivity and the composition of commensal microbes. Recompositing germ-free mice with fecal material obtained from responsive patients, hindered tumor growth, incited T-cell functions, and enhanced sensitivity to anti–PD-L1 drugs.	(53)
Tanoue et al.	2019	CRC (MC38)	*Bacteroidales* spp.	Oral gavage	Anti-PD1± Anti-CTLA4	Elevated levels of IFN-γ, boosted CD8+ T cell production, and amplified ICI effects can be induced following colonization of 11 bacterial strains.	(50)
Xu et al.	2020	MSS-type CRC (CT26)	*Akkermansia muciniphila* *Bacteroides*	Antibiotic cocktail: Colistin & Streptomycin & Ampicillin Colistin, Vancomycin (alone).	Anti-PD1	Colistin-treated mice showed poor anti-tumor responses compared to vancomycin-treated ones. In comparison to enriching non-responding mice with Bacteroides, *Akkermansia muciniphila* uplifted levels were accompanied by strengthened anti-tumor functions in mice treated with vancomycin. Weakened anti-PD-1 effects are observed following antibiotic treatment because of glycerophospholipid-modified metabolism.	(54)
Cutzac et al.	2020	CRC (MC38, CT26); MM	*Faecalibacterium* *Gemminger*	Oral gavage	Anti-CTLA4	Increased blood levels of butyrate through bacterial fermentation in the colon are associated with resistance to CTLA4 inhibitors.	(55)
Mager et al.	2020	CRC (MC38); RCC (MB49); MM (B16-F10)	*Bifidobacterium pseudolongum, Lactobacillus johnsonii, and Olsenella*	Oral gavage	Anti-CTLA4	Inosine derived from microbes provokes T cells in two ways: binding to adenosine A_{2A} receptor, and costimulation of T cells by MAMPs.	(52)
Dees et al.	2021	GM (HuM1-HuM5)	*Bacteroides cellulosilyticus* *Bacteroides caccae*	FMT	Anti-PD1	Impeded tumor growth and higher survival rate after subjecting to anti-PD1 agents are seen in mice with an appropriate abundance of *Bacteroides cellulosilyticus* and *Bacteroides caccae*.	(56)
Gao et al.	2021	CRC (CT26)	*Lactobacillus rhamnosus* (Probio-M9)	Oral gavage (Probiotic administration)	Anti-PD1	A synergic effect on hampering tumor growth is seen following the combination of Probio-M9 and immune checkpoint	(57)

[DOI: 10.61186/ijbc.16.3.70]

						inhibitors. Probiotic supplementation also populates more beneficial bacteria (such as *Bifidobacterium pseudolongum*, *Parabacteroides distasonis*, and some *Bacteroides*).	
Messaoudene et al.	2022	Sarcoma (MCA205) BC (E0771)	*A. muciniphila and Bifidobacterium* (Myrciaria dubia)	Oral gavage (Probiotic administration) FMT	Anti-PD1	Oral consumption of Castalagin consisting of certain bacteria (*Ruminococcaceae* and *Alistipes*) that reinforce immune responses and improve the balance CD8+/FOXP3+CD4+ ratio. Castalagin oral supplementation after FMT restores the anti-PD-1 effect in ICI-refractory models.	(58)
Fong et al.	2023	CRC (CT26 & MC38)	*Lactobacillus gallinarum* (Indole-3-carboxylic)	Oral gavage	Anti-PD1	*L. gallinarum*-derived Indole-3-carboxylic acid made anti-PD1 therapy efficient in CRC patients by retardation of Treg differentiation and fortifying CD8+T cell function.	(59)

FMT: fecal microbiome transplant; **CRC**: colorectal cancer; **RCC**: renal cell carcinoma; **MM**: metastatic melanoma; **BC**: Breast cancer; **GM**: glioma; **NSCLC**: non-small cell lung cancer.

[DOI: 10.61186/ijbc.16.3.70]

Table 2. Clinical Studies exploring the effects of microbiota-related interventions alongside immune checkpoint inhibitor treatment.

NCT	Study Type	Phase	Recruitment Information	Cancer type	ICIs	Intervention/purpose
NCT04107168	Observational	N/A	Not yet recruiting	Melanoma, Renal, Lung	anti-CTLA-4, anti-PD-L1 or Anti-PD-1	Assessment of the relation between GM and ICI therapy success in late-stage cancer.
NCT03643289	Observational	N/A	Unknown status	Melanoma	Not specified	Exploring response rate and side effects of immunotherapy in combination with GM.
NCT04636775	Observational	N/A	Recruiting	NSCLC	Anti-PD-1	Association between GM and prediction of the effectiveness of immunotherapy treatment.
NCT04957511	Observational	N/A	Recruiting	Gynecologic	Not specified	Inter- and intra-patient microbiota variations correlated to immunotherapy.
NCT04136470	Observational	N/A	Unknown status	NSCLC Melanoma	Anti-PD-1, anti-PD-L1 or anti-CTLA-4	Uncovering GM variances between responder patients and non-responder ones to ICI agents.
NCT04913311	Observational	N/A	Recruiting	NSCLC	Not specified	Modeling a network to predict the result and risks of treatment by correlating saliva, stool, and blood biomarkers, and obtained data from lung function tests.
NCT04204434	Observational	N/A	Recruiting	Advanced-stage cancer	Not specified	Characterization of serum and microbial factors influencing the efficacy of immune checkpoint inhibitors (ICIs).
NCT04243720	Observational	N/A	Recruiting	Solid tumors, Metastatic cancers	Not specified	Analysis of immunophenotypic, genomic, epigenetic, and transcriptomic profiles of irresponsive patients to IC inhibitors.
NCT04435964	Observational	N/A	Completed	Head and Neck, Breast, Urogenital and Lung Neoplasms, melanoma	Not specified	Studying gender impact factor on the correlation between genomic, immunological, and hormonal profiles and irAEs.
NCT04579978	Observational	N/A	Recruiting	Advanced solid tumor	Not specified	Inspecting the gut bacteria composition and identifying the probable mechanisms employed to modify immune responses.
NCT04954885	Observational	N/A	Recruiting	Lung, NSCLC	Anti-PD-1	Evaluating the future view of gut microbiome manipulation and its influence on treatment outcome.
NCT05037825	Observational	N/A	Recruiting	NSCLC, Melanoma, RCC, Triple-Negative Breast	Aanti-PD-1, anti-PD-L1 or anti-CTLA-4	Interrelation between the individual immune system, the gut microbiome variation, and ICI medicines efficiency.
NCT04009122	Interventional	N/A	Completed	NSCLC	Not specified	Evaluation of the effects of dietary supplement: IGEN0206, on the flora modifications, cytokines levels, and general life quality.
NCT05083416	Interventional	N/A	Active, not recruiting	Head and neck	Not specified	Assessing the connection between Prolonged Nightly Fasting (PNF) and sensitivity to Immunotherapy measures in the case of Advanced Head and Neck Cancer (HNSCC)considering the gut microbes' impact.
NCT04636775	Interventional	Phase IV	Recruiting	NSCLC	Anti-PD-1, anti-PD-L1	Determination of the correlation of microbiome to adverse events (AEs), PD-L1 expression, and diet immunotherapy naïve NSCLC individuals subjecting to PD-1/L1 inhibitors.
NCT05384873	Interventional	phase II	Not yet recruiting	NSCLC	Not specified	Investigating the outcome of adding probiotic supplements to the diet of NSCLC patients who received immunotherapy.

[DOI: 10.61186/ijbc.16.3.70]

NCT04866810	Interventional	N/A	Recruiting	Melanoma	Anti-PD-1/PD-L1 monotherapy	Exploring exercise and diet impact on immunotherapy and structure of microbiome in melanoma patients receiving checkpoint inhibitor treatment.
NCT04645680	Interventional	Phase II	Recruiting	Cutaneous melanoma MM	Anti-PD-1	Scrutiny of the changes in microbiota following insertion of fiber into melanoma patients' diet during receiving Pembrolizumab or Nivolumab.
NCT04163289	Interventional	Phase I	Recruiting	RCC	Anti-PD-1	Evaluation of mucosa-associated invariant T (MAIT) cells before and seven days after FMT in patients with metastatic renal cell carcinoma(mRCC).
NCT05122546	Interventional	Phase I	Active, not recruiting	mRCC	Not specified	Exploring the effects of ketogenic diets enriched with microbiome during antineoplastic treatment.
NCT05220124	Interventional	Phase IV	Recruiting	Bladder Urothelial Carcinoma	Not specified	Assessing the effects of compounding live probiotic supplements and immunotherapy in individuals suffering platinum-ineligible metastatic urothelial cancer.
NCT03829111	Interventional	Phase I	Active, not recruiting	RCC	Anti-PD-1/anti-CTLA-4	Monitoring patients in late-stage of renal cell carcinoma taking a cocktail of ipilimumab and nivolumab accompanied with Clostridium butyricum (CBM 588).
NCT04699721	Interventional	Phase I	Active, not recruiting	NSCLC	Anti-PD-1 and chemotherapy	Assessment of CBN588 addition to drug regimen of patients who received ipilimumab plus nivolumab.
NCT05094167	Interventional	N/A	Recruiting	NSCLC	anti-PD-1	Investigating administration of probiotic strains of Lactobacillus Bifidobacterium V9 (Kex02) in combination with Carlizumab with platinum.
NCT05032014	Interventional	N/A	Recruiting	Liver	anti-PD-1	Evaluating probiotics (Probio-49) effect on PD-1 inhibition therapy outcome in case of liver neoplasia.
NCT03772899	Interventional	Phase I	Active, not recruiting	Melanoma	anti-PD-1	Examination the safety of combining FMT with the approved immunotherapy drugs pembrolizumab or nivolumab and the effect of this combination on the immune system and microbial ecosystem of the gut.
NCT03341143	Interventional	Phase I	Active, not recruiting	Melanoma	anti-PD-1	Examining the FMT procedure to restore PD-1 suppression efficiency in irresponsive melanoma patients.
NCT04577729	Interventional	N/A	Terminated	Melanoma	Not specified	Testing FMT as an option to overcome refractoriness in ICI-insensitive patients.
NCT04521075	Interventional	Phase I/II	Unknown status	MM Melanoma NSCLC	anti-PD-1	Evaluating the effects of FMT in Nivolumab-resistant patients.
NCT03353402	Interventional	Phase I	Unknown status	MM Melanoma	Not specified	Inspecting the influence of microbiota manipulation through FMT on patients did not respond to immunotherapy.
NCT04264975	Interventional	N/A	Unknown status	Solid Carcinoma	Not specified	Evaluating the practicability of using FMT as a strategy For prevailing the resistance in individuals with no response to IC inhibitors. Also, potentially beneficial transplanted bacteria will be characterized.
NCT05273255	Interventional	N/A	Recruiting	Any Solid Tumor	Not specified	Assessing the possibility of using FMT to ignite the extinguished flame of ICI in non-responder patients.

[DOI: 10.61186/ijbc.16.3.70]

NCT03686202	Interventional	Phase I/III	Active, not recruiting	Any Solid Tumor	anti-PD-1/PD-L1 and/or anti-CTLA4	To assess the feasibility of administering MET-4 strains concurrent with ICIs.
NCT04758507	Interventional	Phase I/II	Recruiting	RCC	Not specified	Evaluating the competence of FMT procedure to reduce drug-related toxicity or improve its efficiency.
NCT04729322	Interventional	Phase II	Recruiting	Small intestinal adenocarcinoma, Metastatic small Intestinal adenocarcinoma, CRC, Metastatic CRC	Anti-PD-1	Investigation of FMT capability to excite response to PD-1 suppressors in non-responder individuals suffering metastatic colorectal cancer.
NCT04924374	Interventional	N/A	Recruiting	NSCLC	Anti-PD-1	Transferring the gut microbes from healthy individuals or long-term survivors to people diagnosed with lung neoplasia.
NCT05251389	Interventional	Phase I/II	Recruiting	Melanoma	Not specified	Scrutinizing the impact of FMT that is carried out by endoscopy on ICI refractory melanoma patients who did not show any response to ICI therapy.
NCT05008861	Interventional	Phase I	Unknown status	Advanced or Metastatic NSCLC	Anti-PD-1/PD-L1	Utilization of FMT to amend NSCLC patients' damaged microbiota during immunotherapy.
NCT05286294	Interventional	Phase I	Recruiting	Melanoma Head and Neck RCC	Not specified	Elucidating the outcome of microbiota relocation from immunotherapy-sensitive patients who harbor solid tumors to non-responder ones and identifying intervening parameters.
NCT05502913	Interventional	Phase II	Recruiting	Metastatic Lung Cancer	Anti-PD-1	Studying FMT for reinforcing ICI effects with a special focus on progressive lung cancer.
NCT04951583	Interventional	Phase II	Recruiting	NSCLC Advanced Melanoma	Aanti-PD-1	Evaluation of putting FMT capsules in the standard immunotherapy procedure.
NCT04988841	Interventional	Phase II	Recruiting	Melanoma	anti-PD-1 or anti-CTLA-4	Assessing the advantages and disadvantages of performing FMT on patients with melanoma.
NCT04116775	Interventional	Phase II	Recruiting	mCRPC	Anti-PD-1	Transplantation of fecal microbiota from pembrolizumab-responders to non-responders using endoscopy to strengthen recipients' immunity against tumor and improve their sensitivity to ICI.

Abbreviations: N/A: not applicable; NSCLC: non-small cell lung cancer; GM: gut microbiota; irAEs: immune-related adverse events; RCC: renal cell carcinoma; mRCC: metastatic renal cell carcinoma; FMT: fecal microbiome transplant; MM: multiple myeloma; CRC: colorectal cancer.

[DOI: 10.61186/ijbc.16.3.70]

triggering pattern recognition receptors, molecular mimicry, and the influence of metabolites (60). **Table 1** summarizes the main findings from preclinical studies that have assessed the effect of microbiota on responses to ICI.

4.2. Clinical Studies

Despite the impressive initial responses observed in patients receiving ICIs, resistance to therapy may eventually be acquired by some patients (68). As previously mentioned, the gut flora can reinforce a patient's immunity against tumors and thus influence the clinical outcomes of patients undergoing immunotherapy (60). Given this understanding, manipulation of gut microbiota holds great potential as an adjunct treatment to current anti-cancer therapies (59). Recently, ongoing and planned clinical trials are underway to explore whether altering the composition of gut microbes in cancer patients improves sensitivity to medication and mitigates treatment-related toxicity. For instance, Chaput et al. reported a correlation between the response to ipilimumab therapy and the presence of Faecalibacterium spp. and Firmicutes - in patients with metastatic melanoma, leading to prolonged either overall survival (OS) and PFS (69). In another study, Routy et al. gathered stool samples of patients with either renal cell carcinoma (RCC) or NSCLC and studied their microbial spectrum. They reported a direct correlation between positive response to anti-PD-1 therapy and the existence of Enterococcus hirae and Akkermansia muciniphila (70). These bacteria could enhance the efficacy of anti-PD-1 medications in mice under treating antibiotics through the secretion of IL-12 by DCs (71, 72). In another study, the utilization of antibiotic therapy exhibited a noteworthy association with the OS, PFS, and objective response rate (ORR). This impact was particularly evident among patients diagnosed with NSCLC, RCC, urothelial carcinoma, and melanoma (73). Also, Ahmed et al. disclosed the adverse effect of broad-spectrum antibiotics, such as β lactams or quinolones, on the ORR and PFS. However, the administration of gram-positive specific antibiotics, such as vancomycin or linezolid, did not demonstrate any discernible influence on the response to immunotherapy (74). Contrarily, Pinato et al. discovered that in the case of solid tumors, patients treated with antibiotics 30 days before immunotherapy had meaningly lower OS compared to the simultaneous administration (75). Aligned with previous studies, Tinsley et al. perceived that antibiotic adverse effects on PFS and OS were time and dose-dependent (76). Gopalakrishnan et al. conducted a study in patients with melanoma, wherein they discovered that patients who experienced positive responses to PD-1 inhibition had a diverse variety of fecal beneficial microbes, particularly Ruminococcaceae and Clostridiales, in comparison to non-responders who predominantly harbored Bacteroidales spp. Also, the responder individuals have more active CD4 and CD8 T cells, along with increased infiltration of lymphocytes within the tumor microenvironment. Conversely, non-responders displayed elevated levels of Tregs (77). **Table 2** summarizes the clinical studies that have assessed the effect of microbiota on responses to ICIs.

5. IMPLICATIONS AND FUTURE DIRECTIONS

Due to the significant number of studies conducted on the impact of gut microbiome profiles on the effectiveness of ICIs, the clinical trials apply numerous therapeutic approaches. In addition, several strategies are currently being evaluated in preclinical studies before they can be made available in a clinical setting. For instance, FMT is a therapeutic technique in which the recipient receives donor fecal material via oral lyophilized capsule, thorough gastroscopy, or colonoscopy (60). This method was born during medical efforts for patients with recurrent or refractory *C. difficile colitis*, which improves clinical signs and symptoms (61). Recent experiments have shown that GF mice can restore the effects of anti-PD-1 therapy via FMT from responsive donors. However, fecal materials from non-responsive donors could not improve clinical outcomes (62). Also, fecal transplants showed immune cell infiltration in mice with pancreatic tumors (63). In phase I of a clinical trial, the ten patients with metastatic melanoma receive combinational therapy of FMT and PD-1 suppression. Among these patients, three patients achieved complete response (1/10) and partial responses (2/10), accompanied by upregulation of anti-cancer gene expression and more infiltrated immune cells in the tumor microenvironment (NCT03353402). In another trial tried out on 15 patients by Davar et al., a combination of FMT with pembrolizumab (a PD-1 inhibitor) improved clinical outcomes in 6 patients, 3 of whom showed a sustained response of 1 year or more. Median PFS and OS were three and seven months, respectively. Moreover, profound contemplation on this medical measure disclosed augmented infiltration of CD8+ T cells and diminution of IL-8-secreting myeloid cells (NCT03341143). In another ongoing clinical trial in patients with renal cell carcinoma (RCC), 50 patients suffering from RCC who had received a pembrolizumab-axitinib cocktail as the first choice for advanced RCC treatment, were randomized to donor microbiota and increasing regulatory T cells, consistent with those observed in the study by Wang et al. (64). However, these novel and engaging interventions also have adverse events. The most effective strategies to mitigate these adverse effects was using from FMT and probiotics. The

[DOI: 10.61186/ijbc.16.3.70]

results of previous studies showed that FMT can also alleviate immunotherapy adverse effects, such as colitis, particularly by restoring and remodeling the gut such as bacterial infection, bacteremia, or parasitemia, which are considered severe and life-threatening adverse events, and bloating, abdominal cramps, or sore throat as mild ones. There are several challenges associated with the use of fecal microbiota transplantation (FMT) as a therapeutic method. A primary concern is that the selection of patients and the preparation of fecal material must adhere to stringent laboratory quality control standards, which necessitates careful consideration (65). Furthermore, while animal studies have yielded encouraging results, these findings do not always translate effectively to human subjects. Research has indicated that approximately 85% to 90% of the gut microbiome in mice differs from that of humans. Additionally, various confounding and modifying factors can alter the composition of the human gut microbiome throughout an individual's life, including dietary fat, fiber intake, and the use of antibiotic medications (66).

Prebiotics are non-digestible chemicals that increase the growth of some specific and healthy microorganisms instead of using the macrobiotics directly. Usually, prebiotics are carbohydrates that convert to short-chain fatty acids (SCFA) through bacterial fermentation in colonic mucosa, and the SCFA can facilitate the growth of beneficial bacteria such as Bifidobacterium or *Lactobacillus* (69). Given that during FMT, a blend of beneficial and non-beneficial bacteria is relocated, it seems rational that probiotics are more effective for immune system rehabilitation because they contain specific targeted bacteria that may be useful to host immunity (67). In an ongoing clinical trial, the efficacy of the combination of Nivolumab and VE800, a combination of 11 nonpathogenic and commensal bacteria, was evaluated in patients with progressive malignancies (gastrointestinal or melanoma). The results are not yet released (NCT04208958). In another phase I clinical trial, 30 patients with metastatic renal cell carcinoma were selected randomly and received Nivolumab and Ipilimumab with or without daily oral CBM588, respectively. The results showed that PFS was statistically higher in patients who received CBM588 (12.7 months vs 2.5 months) than those who did not (NCT03829111). Also, several ongoing trials evaluate engineered microbes that can increase specific substrates that enhance the anti-tumor effect. Fernando P Canale et al. reported that an engineered strain of *E. coli* can increase the level of L-Arginine in tumoral tissue and expand tumor-infiltrating T cells, potentiating anti-PD-L1 immune reactions (68).

In 2005, Taper studied the incorporation of Inulin or oligofructose in the basal diet of animals. The addition of this composition led to a lower incidence of mammary tumors and other solid malignancy metastasis. Also, it can potentiate the anti-cancer efficacy of six chemotherapeutic drugs unexpectedly in sub-therapeutic doses (70). In NCT04552418, researchers studied the effectiveness and safety of adding starch as a dietary supplement to patients who received dual immune checkpoint inhibitors in solid cancer. The results are not published yet.

6. CONCLUSION

In conclusion, we reviewed potential mechanisms recruited by gut microbiota to optimize cancer immunotherapy and related preclinical clinical studies. As we know, microbiota has a crucial role in patients' sensitivity to immunotherapeutic drugs. They enhance the host immune system and manipulate the tumor microenvironment in favor of anti-tumor functions by interfering in immune cells and malignant cell interaction. Also, therapeutic approaches easily modify gut microbiota, including FMT, probiotics, prebiotics, and antibiotics. However, these data are still immature, and several randomized clinical studies are required to clarify the microbiota-driven mechanisms of response to immunotherapy and the potential benefit of these interventions, particularly in patients with treatment-refractory cancers.

Acknowledgment

The authors would like to express their gratitude to Shahid Beheshti University of Medical Sciences (Tehran, Iran) for supporting this study.

Conflict of interest

The authors declare that they have no conflict of interest.

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[DOI: 10.61186/ijbc.16.3.70]