

The role of extratumoral and intratumoral microorganisms in cancer immunotherapy

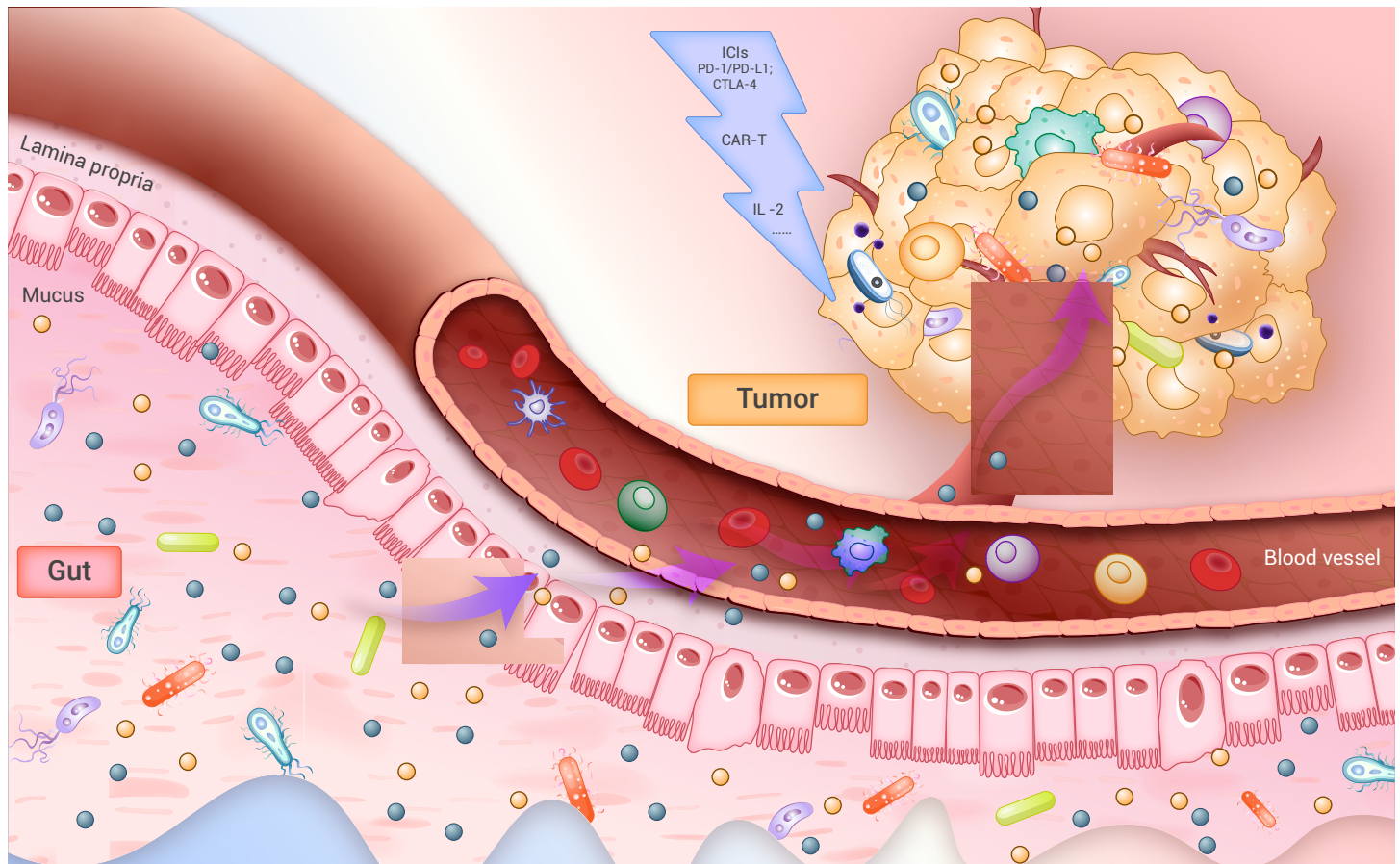
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Human microorganisms are distributed inside and outside tumors.
- Gut microbiome regulates immune cells and affects anti-tumor immunotherapy.
- Intratumoral bacteria promote tumor development.
- Intratumoral bacteria activate immune cells to enhance immunotherapy efficacy.
- Engineered bacteria accurately target tumor sites to deliver therapeutic vectors.



The role of extratumoral and intratumoral microorganisms in cancer immunotherapy

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Microbiome is ubiquitous in human and distributed in not only normal organs such as gut, but also in tumor tissues of the host. Numerous studies have proven that the extratumoral microbiota (mainly gut microbiota) has a close relationship with the local and systemic immune systems of the host. The bacteria, viruses and fungi in gut can influence the activity of innate and adaptive immune cells, affecting the outcomes of immunotherapy. In addition to microbiota in the gut, special microbiota (intratumoral microbiota) exists in the tumor microenvironment (TME), which provides a critical niche for anaerobic or facultative anaerobic bacteria to colonize and proliferate. Intratumoral microorganisms or their metabolites can substantially improve the immunosuppressive of the TME, reactivate immune cells, or recruit activated immune cells, indicating a potential effect on immunotherapy. Furthermore, with the development of synthetic biology, some tumor-targeting bacteria can be used as a biological chassis for the accurate delivery of different immunotherapeutic agents to tumor core through genetic programming technologies, enriching immunotherapy paradigms. In this review, we summarize the recent developments in effect of human microbiota, especially microorganisms in the TME, on immunoregulation, and discuss their potential application in the field of cancer immunotherapy. We also describe the ways to take advantage of genetically engineered bacteria targeting the TME to strengthen the efficacy of immunotherapy against cancer. Additionally, the remaining questions and further directions for microbiota application in immunotherapy are also discussed.

INTRODUCTION

The International Agency for Research on Cancer, using data on the global cancer burden, estimated 19.29 million new cancer cases worldwide and 9.96 million cancer deaths worldwide in 2020.¹ Despite the technical breakthroughs in cancer treatments, cancer-related mortality has maintained an overall upward trend. Tumor immunotherapy has emerged as a breakthrough in cancer therapy that dramatically improves the outcomes for patients with cancer, including patients who have experienced failure of traditional chemotherapies and molecular targeted therapy. Immunotherapeutic agents, such as immune checkpoint inhibitors (ICIs) and chimeric antigen receptor (CAR) T-cell therapy, can stimulate the immune system of the host and enhance the immune response to the tumor. Various experimental and clinical studies have shown that immunotherapy has unmatched advantages compared with conventional antitumor therapy, extending progression-free survival (PFS) and overall survival (OS).² However, immunotherapy is far from perfect. The response of patients with cancer to immunotherapies is heterogeneous. Some patients benefit from cancer immunotherapy, whereas a large percentage of patients (at least 50%) exhibit primary or acquired drug resistance during treatment and experience no benefit at all.^{3,4} Additionally, immunotherapies can result in adverse effects related to their mechanisms of action. For example, ICIs promote T-cell antitumor function, leading to organ-specific inflammatory responses or immune-related adverse events.⁵ The most common adverse effects of CAR-T cell therapy for hematologic malignancies are cytokine release syndrome and immune effector cell-asso-

ciated neurotoxicity syndrome.⁶ These adverse reactions are sometimes fatal. Therefore, it becomes essential to develop auxiliary treatments that achieve better responses to immunotherapy with reduced adverse events or to develop novel cancer therapies that directly treat cancer.

In recent years, the ongoing exploration of biotherapy has revealed that the human microbiota plays a pivotal role in a range of cancer therapies, including immunotherapeutic treatment. Bacteria, viruses and fungi are the main microorganisms that regulate human immunity and the occurrence and development of tumors directly or indirectly, colonizing intestinal tract or tumor. As the most abundant microbes in the body, gut bacteria have profound effects on regulating the systemic immune system, indicating the close associations between gut bacteria and immune-based cancer therapies, such as immune checkpoint blockade. Data from animal and clinical studies have verified the dependence of immunotherapy on gut bacteria.⁷⁻¹⁰ It is well known that the vast majority of microbes colonize the gut, but recent studies have found that symbiotic microorganisms, such as bacteria and fungi, also exist in tumors and are involved in immune regulation.¹¹⁻¹³ Emerging evidence from modern technologies, such as sequencing methods, cultivation techniques, immunohistochemistry, immunofluorescence, and electron microscopy, have comprehensively revealed that the local bacteria of tumor-bearing organs constitute an essential part of the tumor microenvironment (TME). The bacteria in tumor tissue can promote tumor development and progression via DNA damage, oncogene activation, oncogenic pathway upregulation, and disturbance of the immune system in the TME.¹⁴ Conversely, some intratumoral bacteria directly suppress cancer cells or recruit immune cells and elicit the reactivity of immune cells to improve immunological surveillance and cytotoxicity to tumor cells. Furthermore, the development of synthetic biology promotes the application of bacteria therapy in cancer treatment. Bacteria can be used as an efficient chassis that can be genetically reprogrammed to produce or deliver antitumor agents according to clinical needs. There is potential for manipulating the human microbiota with defined bacteria strain(s) for tumor treatment.

In this review, we summarize the influence of microorganisms inside and outside the tumor (mainly gut bacteria) on cancer immunotherapy and explore the role of microorganisms inside the tumor (mainly intratumoral bacteria) in the immune shaping of the TME. We also discuss the possible mechanisms of intratumoral bacteria in the occurrence, development, and treatment of cancer. In addition, we highlight the application and the prospect of tumor-targeting bacteria in cancer immunotherapy to provide new therapeutic possibilities against cancer.

EXTRATUMORAL MICROORGANISMS AND TUMOR IMMUNOTHERAPY

Gut commensal microorganisms and immunity

The human body is colonized by numerous microorganisms (approximately 4×10^{13} microbial cells) residing on and inside humans.⁷ The intestinal tract is the primary location of these microorganisms and harbors the most abundant microbiota. During thousands of years of co-evolution, microbes are one of the four components of the gut barrier as a biological barrier, the gut and the microbiota together have formed a unique, symbiotic microecosystem.

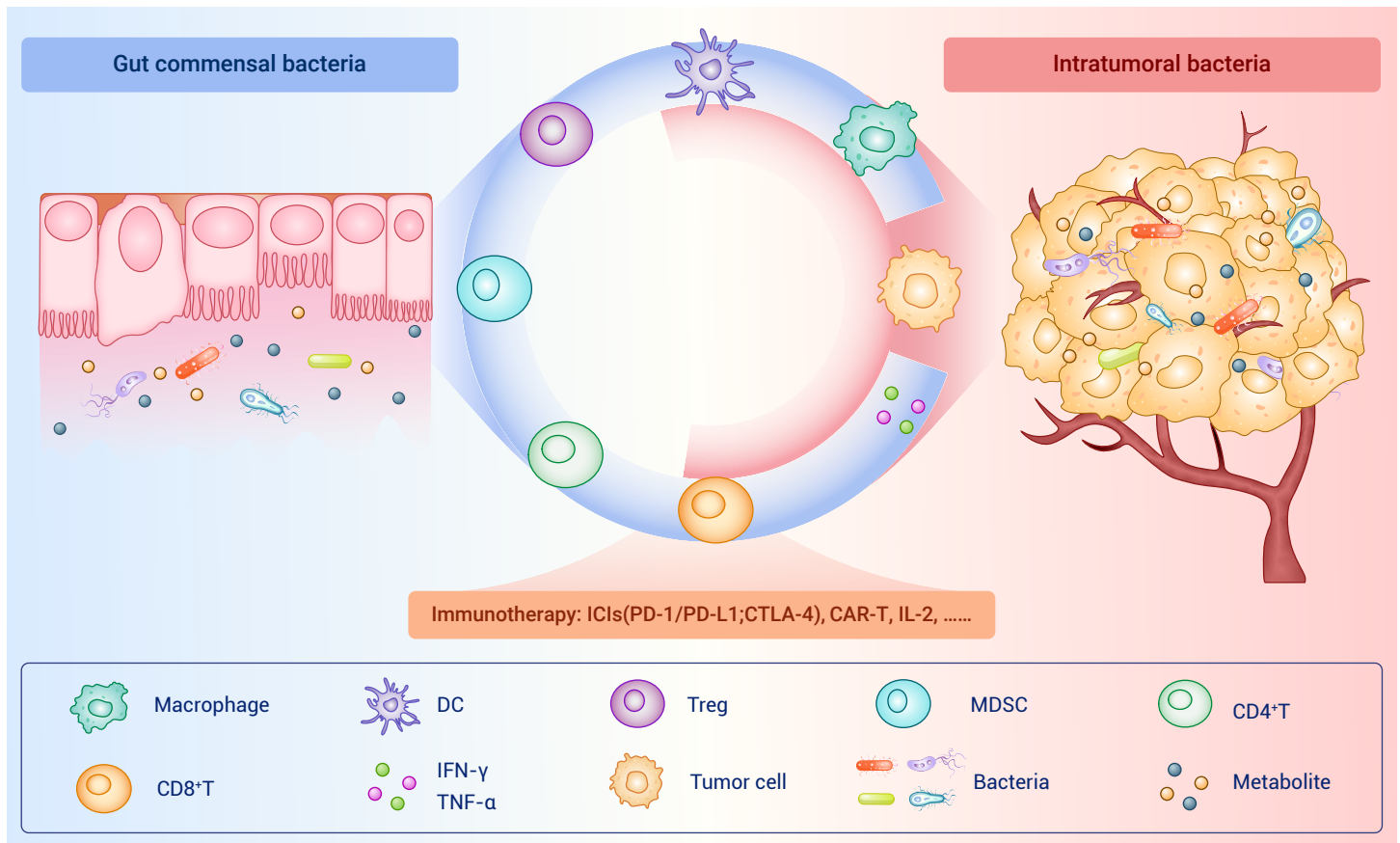


Figure 1. The role of extratumoral and intratumoral microbe in tumor and immunotherapy Extratumoral microbe can influence the immune systems thus affecting the effect of immunotherapy. Intratumoral microbe can improve the immunosuppressive microenvironment of the TME, enhancing the response to immunotherapy.

Bacteria. The gut is considered the largest immune organ in the human body. Many immune-related compartments, such as Payer's patches, mesenteric lymph nodes, and isolated lymphatic follicles, are present in the intestine. The intimate crosstalk between the gut bacteria and the intestinal barrier is indispensable in shaping the homeostasis of local mucosal immunity, as demonstrated in germ-free mice. Germ-free mice lacking gut microbiota exhibited a flimsy gut barrier and were immune deficient.¹⁵ The microbiota in the gut could interact with the human immune system to promote the formation and maturation of immune cells. Previous studies have shown that *Akkermansia muciniphila* (AKK) has immunostimulatory and antitumor effects. AKK improved the proportion of GZMB⁺IFN- γ ⁺CD8⁺ T cells *in vitro* and recruited tumor-killing M1 macrophages to inhibit the proliferation and invasion of prostate cells.^{16–19} The signals sent by the intestinal microbiota to immune cells fall into two categories: bacteria-derived metabolites and bacterial components. Metabolites mainly include Short-chain fatty acids (SCFAs), bile acids (BAs), lactic acid and so on. As histone deacetylase (HDAC) inhibitors, SCFAs promoted histone H3 acetylation in *Foxp3* gene promoter to promote Foxp3⁺Tregs production, accelerate Foxp3⁺Treg differentiation, and promote its suppressive activity through GPR43 signaling pathway.^{20,21} SCFAs could also activate STAT3 and mTOR pathways in Th1 via GPR43 to up-regulate transcription factor Blimp-1, thus promoting the production of IL-10 by Th1 and alleviating colitis in mice.²² However, Wen et al. showed that SCFAs might also enhance the production of Th2 cytokines,²³ and further studies were needed on the effect of SCFA on Th2 differentiation and function. The interaction of BAs with TGR5 downregulated the production of proinflammatory cytokines by macrophages by reducing the activation of NLRP3 inflammasome,²⁴ inhibiting NF- κ B signaling,²⁵ or inducing CREB-mediated production of IL-10.²⁶ Lactate exerted its immunomodulatory activity by inducing dendritic protrusion of CX₃CR1⁺ phagocytes by activating GPR31 signal transduction.²⁷ Bacterial components mainly include lipopolysaccharide, flagellin, peptidoglycan, formyl peptides, and unique nucleic acid structures. They instructed host immune responses by activating the TLR pathway in the intestine.²⁸

ing the TLR pathway in the intestine.²⁸

Systemic immunity is also shaped by gut commensal bacteria. The commensal bacteria can prime immune cells under the antigens presented by intestinal mucosal dendritic cells, and the primed immune cells can migrate to distal organs from the intestinal mucosal layer to display immune responses. Interestingly, gut microbiota was related to the immune cytodynamics of the human body. Joao B Xavier et al. collected daily data from 2335 patients for more than 100 days to compare immune system reconstruction and intestinal microbiota before and after hematopoietic cell transplantation (HCT). In this study, it was found that *Faecalibacterium*, *Ruminococcus* and *Akkermansia* had the strongest positive correlation with immune cell dynamics, and enrichment of these bacteria communities could accelerate immune reestablishment.²⁹ These results reveal the interaction between bacteria and immunity and lay the foundation for further research on the mechanism.

Viruses. Metagenomics sequencing analysis showed that a large number of DNA and RNA viruses colonized the intestines of both healthy humans and animals.³⁰ In intestinal immune system, intestinal intraepithelial lymphocytes (IELs) are located in intestinal epithelium and interact directly with intestinal epithelial cells. As the first line of defense of intestinal mucosal immunity, IELs play an important role in maintaining intestinal mucosal immune homeostasis. Zhou et al. found that intestinal commensal viruses bounded to RIG-I and promoted IL-15 generation through the MAVS-IRF-1 pathway in a manner independent of IFN-I, to maintain the proliferation and survival of IELs. The IELs of mice treated with antiviral cocktail were significantly reduced.³¹

Fungi. Although fungi are less abundant than bacteria, they are widely distributed in skin, brain, lung, liver, intestine and so on. Fungi exhibit immunosuppressive and carcinogenic effects. In the absence of CARD9 (an adaptor protein), increased *Candida tropicalis* could promote the differentiation of myeloid cells into myeloid-derived suppressor cells (MDSCs) and activate the immunosuppressive activity of MDSCs, thus promoting the occur-

Table 1. Studies linking the gut microbiome composition to efficacy of cancer immunotherapy

Cancer	Microbe/ Metabolites	Immunotherapy	Effectors/Targets/Pathway	Mouse or Human data	Refs
Colon cancer	11-strain mixture*	PD-1, CTLA-4	IFN γ +CD8 $^{+}$ T cell, innate immune pathways	Mouse	131
	<i>Bifidobacterium pseudolongum</i> , <i>Lactobacillus johnsonii</i> , <i>Olsenella</i> spp. (inosine)	PD-L1, CTLA-4	A $_{2A}$ R signaling	Mouse	40
	<i>Bifidobacterium longum</i> , <i>Collinsella aerofaciens</i> , <i>Enterococcus faecium</i>	PD-1/PD-L1	DC, CD8 $^{+}$ T cell, Treg	Mouse, Human	34,35
Melanoma	<i>B. caccae</i> , <i>B. thetaiotamicron</i> , <i>Faecalibacterium prauznitzii</i> , <i>Holdemania filiformis</i> , <i>D. formicigenerans</i>	PD-1, CTLA-4	DC, Th1	Mouse, Human	42
	<i>Clostridiales/Ruminococcaceae</i>	PD-1	CD8 $^{+}$ T cell	Mouse, Human	132
	<i>Bacillus fragilis</i>	CTLA-4	Th1, intratumoral DC	Mouse, Human	43
	Indole-3-aldehyde (I3A)	PD-1, IFN	NA	Human	41
Colorectal tumor, melanoma	<i>Akkermansia muciniphila</i>	IL-2	TLR2	Mouse	38
	<i>Lactobacillus rhamnosus</i> GG (LGG)	PD-1	DC, cGAS/STING, IFN-1, CD8 $^{+}$ T cell	Mouse	133
Lung cancer, Renal cell carcinoma, Bladder cancer	<i>Akkermansia muciniphila</i>	PD-1, CTLA-4	CCR9 $^{+}$ CXCR3 $^{+}$ CD4 $^{+}$ T cell, IL-2, IL-12, IFN- γ	Mouse, Human	36,37,39
Prostate cancer	<i>Akkermansia muciniphila</i>	PD-1	GZMB $^{+}$ CD8 $^{+}$ T cell, IFN- γ +CD8 $^{+}$ T cell, M1-like macrophage	Mouse	19
Pancreatic cancer	Short chain fatty acids (SCFA)	CAR-T	CD25, IFN- γ , TNF- α , ROR1	Mouse	50
Breast cancer	Staphylococcal enterotoxin-B(SEB)	CAR-T	CAR-T cell	Mouse	49

*11-strain mixture: *Parabacteroides distasonis*, *Parabacteroides gordonii*, *Alistipes senegalensis*, *Parabacteroides johnsonii*, *Paraprevotella xylaniphila*, *Bacteroides dorei*, *Bacteroides uniformis* JCM 5828, *Eubacterium limosum*, *Ruminococcaceae bacterium* cv2, *Phascolarctobacterium faecium*, *Fusobacterium ulcerans*. NA, not applicable.

rence and development of colon tumors.³² A common skin fungus, *Malassezia*, was abundant in ductal adenocarcinoma of the Pancreas (PDAC), which activated complement C3 by binding to mannose-binding lectin (MBL) secreted by the host, thereby accelerating tumor formation. The progression of PDAC in mice was inhibited by oral antifungal agents or by blocking the MBL-C3 axis.³³

Influence of gut bacteria on immunotherapy response

In the past decades, immunotherapies, especially ICIs, have gained momentum. Immunotherapies discussed here include (i) ICIs (i.e., programmed cell death 1 [PD-1]/programmed death ligand [PD-L1] monoclonal antibodies, cytotoxic T-lymphocyte antigen 4 [CTLA-4] monoclonal antibodies, and other types of monoclonal antibodies); (ii) cellular therapies (i.e., tumor-infiltrating lymphocytes, multiple cytokine-induced killer cells, lymphocyte-activated cytokines, CAR-T therapy, and other cellular therapies); (iii) cancer vaccines; (iv) bispecific antibodies, such as blinatumomab, which was approved by the U.S. Food and Drug Administration in 2014 for the treatment of acute lymphoblastic leukemia; (v) small molecule inhibitors; and (vi) immune system modulators, such as interleukin (IL)-2 and interferon (IFN). The effects of gut bacteria on immune cells and immunotherapy are shown in Figure 1.

ICIs. Numerous studies have verified that gut bacteria can influence the efficacy of the immunotherapeutic blockade (Table 1). Gajewski et al.^{34,35} found and confirmed for the first time that gut microbiota could promote the therapeutic effect of ICI based on PD-1/PD-L1. In 2015, they found that JAX and TAC mice with different gut microbiota showed differential responses to ICIs, and 16s RNA sequencing confirmed that *Bifidobacterium* was the genus that correlated positively with tumor immunity. The study showed that *Bifidobacterium* enhanced the function of dendritic cells (DCs), thus promoting the activation of CD8 $^{+}$ T cells and their accumulation in the tumor microenvironment, thus exerting anti-tumor immune response.³⁴ Four years later, similar results were observed in patients with melanoma: a positive correlation existed between the abundance of *Bifidobacterium longum* and the efficiency of ICIs.³⁵ This finding has also been confirmed by fecal microbiota transplantation (FMT). The transplantation of feces from responders

improved the response of antibiotic-treated mice or germ-free mice bearing tumors; conversely, transplantation of feces from nonresponders showed no effect.^{35,36} In a study by Derrien et al.³⁷ in the same year, more AKK were found in the gut of patients with lung cancer, renal cell carcinoma, or bladder cancer who experienced responses to PD-1 inhibitors than in patients without a response. A new potential strain comes into view. Although the role of AKK in tumor immunotherapy has attracted attention since Derrien's study, research has been extremely limited. A study in 2020 showed that AKK could significantly potentiate the therapeutic outcome of IL-2 *in vitro* and in mice with subcutaneous melanoma and colorectal tumors through the activation of the toll-like receptor 2 signaling pathway mediated by Amuc derived from the outer membrane protein of AKK.³⁸ Oral supplementation with AKK restored the efficacy of PD-1 blockade in an IL-12-dependent manner by increasing the recruitment of CCR9 $^{+}$ CXCR3 $^{+}$ CD4 $^{+}$ T cells.³⁶ A significant finding in clinical work was that the presence of AKK was associated with higher ORR and longer OS in NSCLC patients treated with ICIs, and oral supplementation of exogenous AKK could reverse resistance to PD-1 inhibitors by regulating intestinal microbiota.³⁹ Bacterial metabolites are also closely related to ICIs response. Inosine could effectively activate specific T cells to enhance the antitumor effect of ICIs.⁴⁰ Higher levels of Indole-3-aldehyde (I3A) were often detected in melanoma patients who responded to immune checkpoint therapy.⁴¹ They all hold promise as reliable biomarkers for treatment or prognosis in immunotherapy patients.

Tumor immunotherapy at the CTLA-4 target site has been similarly influenced by gut microbiota.^{36,42} *Bacillus fragilis* has reportedly reversed the nonresponse to CTLA-4 blockade by promoting CD4 $^{+}$ T cells to recover a Th1 phenotype and maturation of intratumoral dendritic cells, as demonstrated in both mice and patients.⁴³ On the contrary, some symbiotic bacteria may promote the differentiation of immune cells towards Tregs and MDSCs phenotypes, thus creating an immunosuppressive state and inhibiting the anti-tumor immunity.^{44,45} Therefore, it is a significant topic to study the effect of probiotics on immunotherapy and further study its mechanism of action.

CAR-T. Another crucial development of tumor immunotherapy is the advent of CAR-T cell therapy.⁴⁶⁻⁴⁸ CAR-T therapy was introduced into clinical medicine as a CD19-targeted therapy for the treatment of refractory and

recurrent B-cell malignancies. To date, few reports exist on the effect of intestinal microbiota on CAR-T therapy; however, several pieces of evidence suggest possible directions. In 2019, von Scheidt et al.⁴⁹ found that the bacterial enterotoxin staphylococcal enterotoxin-B (SEB) significantly enhanced the proliferation of CAR-T cells and inhibited the growth of solid tumors in mice. Another recently published study highlighted the role of short chain fatty acids (SCFA) in CAR-T therapy.⁵⁰ They found that SCFA enhanced the expression of CD25 and the production of IFN- γ and tumor necrosis factor alpha (TNF- α) in CAR-T cells targeting tyrosine kinase-like orphan receptor 1 (ROR1) in a murine model of pancreatic cancer, demonstrating great antitumor activity. Both studies reflect the role of bacterial metabolites in CAR-T therapy. Many studies have proven the converse: that the efficacy of CAR-T therapy is reduced after the application of antibiotics that interfere with intestinal microorganisms,⁵¹ which further illustrates the double-edged role of microbe in CAR-T therapy.

Influence of gut bacteria on side effects of immunotherapy

In addition to the ability of intestinal bacteria to mediate an improved response to immunotherapies, their ability to ameliorate adverse effects from immunotherapies has also been studied closely. Studies identified that intestinal bacteria could relieve diarrhea and intestinal inflammation, which are adverse reactions to ICIs. In a prospective study of patients with metastatic melanoma, the analysis showed that the patients with bacteria-enhanced treatment were resistant to ICI-induced colitis.⁵² Sun et al.⁵³ found that *Bifidobacterium* optimized the structure of intestinal symbiotic flora and enhances intestinal function and metabolism, thus alleviating intestinal inflammation induced by anti-CTLA-4 antibodies. With CAR-T therapy, antibiotics are often used to prevent infection, which can cause dysbacteriosis that manifests as the spread of enterococcus in the patient's stool. Probiotics or fecal microbiota transplantation restore the health of the gut microbiome and reduce the spread of potential disease-causing microbes.⁵⁴

Influence of viruses on immunotherapy response

About 12% of cancers worldwide are linked to viral infections. Although studies have shown differences in the immune system based on viral infection status, few studies have explored the interaction between viral infection and the therapeutic efficacy of ICI. In *Nature*, a team analyzed data from the CheckMate 459, Keynote-240, and IMbrave150 liver cancer clinical trials, and found that survival was superior to the control arm in patients with HBV-related HCC and HCV-related HCC, but not in patients with non-viral HCC.⁵⁵ However, a better prognosis was not evident in patients with human papillomavirus-positive HNSCC.⁵⁶ So far, there is no consensus on the effect of virus on immunotherapy.

INTRATUMORAL MICROORGANISMS AND TUMOR IMMUNE MICROENVIRONMENT

Presence and targeting of intratumoral bacteria

The presence of bacteria at the tumor site was first described more than a century ago,⁵⁷ however, the importance of intratumoral bacteria to the TME was not realized until recently. The necrotic tumor core is a hypoxic and immune-privileged area, which provides an ideal niche for the colonization of some obligate anaerobes and facultative anaerobes. Studies have found that these anaerobes can infiltrate through peripheral blood circulation and preferentially accumulate and proliferate in tumor, that is, tumor-targeting bacteria.⁵⁸⁻⁶² The rapid growth of tumor tissue and abnormal vascular network are essential reasons for the formation of anaerobic and necrotic areas in tumor. It is these anoxic environments that provide a place for bacteria to colonize and grow, which tends to hypoxic metabolism. In addition, local deficiency of the immune system results in a greatly reduced ability of the immune system to remove bacteria from the tumor with the hypoxic microenvironment. Furthermore, the tumor tissue with a high metabolism and apoptotic cell fragments in necrotic area provide abundant raw materials for bacterial reproduction. These factors allow certain bacteria to exhibit specific tumor-targeting properties. In 2020, Nejman et al.⁶³ reported that bacteria exist in seven cancer types: melanoma, pancreatic cancer, lung cancer, ovarian cancer, glioblastoma, bone cancer, and breast cancer. In their comparison of paracancerous tissues, the intratumoral microbial composition appeared

tumor-type dependent. Different subtypes of breast cancer also showed differences in microbial species, consistent with previous findings.^{64,65} The metabolic functions possessed by microorganisms enriched in different tumors also varied, and these metabolic functions also appeared to be tumor-type specific.

The source of intratumoral bacteria

The main sources of intratumoral microbiota are currently considered to include mucosal organs, normal adjacent tissues (NATs), and the circulatory system. In a 2020 study, researchers suggested that the bacteria in tumors might have come from NATs because the composition of bacteria in tumor tissue was highly similar to NATs.⁶³ Several studies have mentioned that intratumoral bacteria found in PDAC were transferred from the gut via pancreatic ducts,⁶⁶ and this adenocarcinoma microenvironment might increase susceptibility to bacterial translocation. In addition, probiotics were able to "run away" from the intestines of the melanoma mice to the tumor and stimulate immune cells at the tumor site.⁶⁷ Microbes have also been found in tumors at non-mucosal sites (as in breast cancer), suggesting that there may be other sources of microbes within the tumor. Given the multi-source nature of the microbiome within a tumor, comparing the microbiome composition within a tumor with that elsewhere in the body may help us identify key microbes present in different tumors, providing new insights into cancer prevention.

Intratumoral bacteria promote tumor development

The researchers found that intratumoral bacteria were predominantly present in cancer cells and immune cells.⁶³ The bacteria residing within tumor have the ability to influence the TME and promote the development of the tumor, which may be achieved by promoting DNA damage and increasing mutations, activating oncogenes and upregulating oncogenic signaling pathways, and disrupting the immune system of the TME.^{14,68} Bacterial toxins can directly cause DNA damage in the host or can indirectly mediate DNA damage through the production of reactive oxygen species (ROS) by the host. When the damage exceeds the body's ability to repair, cancer-causing mutations may develop.⁶⁸ Although almost no microorganisms directly cause cancer, certain microbial components may activate genes and molecular pathways, such as TP53, the ERK and phosphoinositide 3-kinase (PI3K) signaling pathway, and the poly (ADP-ribose) polymerase 1 (PARP1) pathway, associated with cancer cell transformation and carcinogenesis.⁶⁹⁻⁷¹ Intratumoral bacteria directly affect the differentiation and function of local immune cells in the TME, causing inflammation or local immunosuppression and leading to immune system disorders.⁷² Inflammation may lead to alterations of bacteria composition and promote bacterial translocation into tumor tissue, which then promotes the expression of inflammatory cytokines and leads to increased tumor growth.⁷³ Triner et al.⁷⁴ found that bacteria in colon tumors induced IL-17 production and promoted the influx of intratumoral B cells, thereby promoting tumor growth and progression.

Influence of intratumoral bacteria on immune cells and immunotherapy

In the previous section, intratumoral bacteria can suppress the immune response and promote cancer progression, but on the other hand, intratumoral bacteria can also trigger anti-cancer immunity (Figure 1). The different immunomodulatory effects of intracellular bacteria may be species-specific and/or influenced by their intracellular/extracellular niche. Many recent studies have found that some native tumor bacteria can recruit and activate immune cells, causing the response of immune cells and thus enhancing the antitumor immune response (Table 2).^{14, 66, 75-78} Kalaora et al. found that protein fragments - peptide chains derived from intracellular bacteria could be presented on the surface of tumor cells and recognized by immune T cells.¹² There were more *Clostridium*,⁶³ *Saccharopolyspora*, *Pseudoxanthomonas* and *Streptomyces*⁶⁵ in the tumor tissues of patients who responded to ICI treatment and patients with longer survival, respectively. Higher numbers of CD3⁺CD8⁺T cells were positively correlated with enrichment of *Saccharopolyspora*, *Pseudoxanthomonas*, and *Streptomyces*. These findings suggest that tumor bacteria may be involved in tumor suppression by promoting the recruitment and activation of CD8⁺T cells, thereby affecting tumor growth and tumor immune infiltration. Not long ago, translatable

Table 2. An overview of the native microbiota in the cancerous tissue, and their role in immune regulation and immunotherapy

Cancer	Microbe or Metabolites	Immunotherapy	Effectors/Targets/Pathway	Mouse or Human data	Refs
Melanoma	<i>Clostridium</i>	ICI	NA	Human	63
	<i>Enterococcus spp.</i> , <i>Lactobacillus johnsonii</i> , <i>Enterobacteriaceae</i>	PD-1, CTLA-4	DC, IFN γ *CD8 $^{+}$ T	Mouse	67
	<i>Lactobacillus reuteri</i> (I3A)	PD-L1	IFN γ *CD8 $^{+}$ T, AhR	Mouse	41
Colorectal cancer	<i>Bifidobacterium</i>	Anti-CD47	IFN, STING	Mouse	81,134
Lung cancer	<i>Acidovorax</i> , <i>Streptococcus</i> , <i>Veillonella</i>	NA	PI3K, ERK	Human	69
Melanoma B16 lung metastases	<i>Lactobacillus rhamnosus</i>	NA	T cell, NK	Mouse	76
Pancreatic cancer	<i>Pseudoxanthomonas</i> , <i>Saccharopolyspora</i> , <i>Streptomyces spp.</i>	NA	CD3 $^{+}$ T cell, CD8 $^{+}$ T cell, MUC16	Human	66,77
	TMAO	PD-1, Tim-3	IFN	Mouse	80
Triple-negative breast cancer	TMAO	PD-1	PERK, GSDM	Mouse	79
Skin cancer	Native bacteria	NA	TLR-5	Mouse	78

NA, not applicable.

bacteria were shown to trigger T cell immune responses by activating DCs in melanoma.⁶⁷ With the deepening of the research, the influence of the metabolites of neoplastic bacteria on immunity and immunotherapy effect has been gradually discovered. Trimethylamine oxide (TMAO) promoted M1 macrophage infiltration and CD8 $^{+}$ T cell activation and enhanced anti-tumor immunity mediated by CD8 $^{+}$ T cell in tumor, thereby improving anti-PD-1 therapeutic response.⁷⁹ This process relied on GSDME-mediated pyroptosis due to ER stress⁷⁹ or enhancement of type I interferon (IFN) pathway.⁸⁰ Due to the tumor-targeting properties, *Lactobacillus reuteri* also translocated, colonized and persisted in melanoma after administration. Through the release of tryptophan catabolite I3A, *Lactobacillus reuteri* locally promoted the production of IFN- γ *CD8 $^{+}$ T cells, thereby enhancing the ICIs response in situ of tumor tissue.⁴¹ The efficacy of another ICI, anti-CD47 antibody, could also be enhanced by *Bifidobacterium* in a stimulator of interferon genes (STING)–and IFN-dependent manner.⁸¹

Intratumoral microbiota is an important part of TME which has been identified recently. Although the mechanism of how intratumoral bacteria participate in tumor immunotherapy response remains elusive and unclear, a better understanding of the close interaction between intratumoral bacteria and TME immunity will clarify the feasibility of manipulating the bacteria in TME to enhance immunotherapy response and help patients fight cancer. Cancer therapy is facing a huge shift: traditional treatments are gradually being replaced by more precise and sophisticated ones. Understanding the different contributions of the microbiome within a tumor to the occurrence and progression of cancer will aid in the development of cancer prevention and treatment strategies.

Intratumoral fungi

Commensal fungi and bacteria in tumor tissue play completely different roles in tumor therapy. The binding of *C. albicans* to dectin-1 receptor in mouse breast cancer could up-regulate tumor-promoting macrophages and down-regulate anti-tumor T cells, thereby inhibiting anti-tumor immune response after radiotherapy (RT). Symbiotic bacteria, on the other hand, enhanced the efficacy of radiotherapy.¹¹ A recent study demonstrated that intratumoral fungi drove the secretion of IL-33 in PDAC and promoted the infiltration of type 2 immune cells (T_H2 and group 2 innate lymphoid cells [ILC2s]) into the PDAC-TME, leading to tumor progression.¹³ However, how type 2 immune cells infiltrate PDAC-TME remains unknown. Symbiotic fungi, both in and out of the tumor, mostly exhibit immunosuppressive effects. These results imply that antifungal drugs and IL-33 targeting drugs may have adjuvant therapeutic effects on pancreatic cancer.

MICROBIOME-BASED CANCER IMMUNOTHERAPY

Wild-type bacteria used for cancer suppression

Currently, the bacteria used in cancer therapy are mainly obligate anaerobes and facultative anaerobes, such as *Salmonella* spp., *Listeria* spp., and

Clostridium spp., which prefer to colonize, proliferate, and metabolize in the hypoxic/necrotic core of the solid tumor after intravenous or subcutaneous injection. The bacteria in the tumor can directly kill tumor cells or induce the immune response against tumor cells through their components (e.g., lipopolysaccharide and flagellin) or via secreted antitumor agents (e.g., exotoxins).⁸² Studies on the mechanism of tumor cell death induced by tumor-targeting bacteria suggest three possibilities⁸³: (i) The excessive proliferation of *Salmonella* spp. can directly lead to the rupture of the invaded tumor cells. Bacteria kill tumor cells by inducing apoptosis or autophagy and directly inhibit tumor cell proliferation by toll-like receptor 5 signaling.^{84–86} (ii) *Listeria* spp. can modify a subpopulation of these cells to have an immune-stimulating phenotype by infection of immunosuppressive MDSCs and can directly kill tumor cells through NADPH oxidase-mediated production of reactive oxygen species and intracellular calcium mobilization.^{87,88} (iii) *Clostridium* spp. can kill tumor cells through a variety of exotoxins secreted by the colonizing bacteria.^{89,90} Although the potential of bacteria-based cancer therapy is highly anticipated, the progress of live bacteria therapy has been slow in clinical applications. To date, only *Bacillus Calmette-Guérin* (BCG) and attenuating *Mycobacterium bovis*, are clinically used as a bacteria-based cancer therapy in bladder cancer.^{91,92} In addition to the development of radiotherapy and chemotherapy, one reason that bacteria-based research has not thrived is the contradiction to bacterial therapy—that is, the direct killing ability of cancer cells and activation of immune responses against cancer cells are inseparable from the pathogenicity of wild bacteria. For example, approximately 5% of patients with cancer treated with BCG have adverse effects, such as tissue sepsis.^{91,93} In addition, the antitumor ability of natural bacteria is limited (e.g., incomplete elimination of tumor cells). Therefore, more research is needed to identify a solution for these shortcomings.

Engineered bacteria-based strategy

Tumor tropism of bacteria. The tumor tropism of bacteria also enables bacteria to act as a chassis to deliver a therapeutic payload. The development of synthetic biology provides technical support and a broader space for applying bacteria-based cancer therapies. Engineered bacteria with different functions can be designed according to clinical needs. In clinical applications, the safety of bacteria is the primary consideration. The safer attenuated bacteria can be obtained by deleting the major genes involved in synthesizing virulence factors, such as lethal exotoxin, lipopolysaccharide, and ppGpp.^{94–96} In addition, construction of auxotrophic mutants is another strategy to enhance safety. For example, *Salmonella* A1-R, which is auxotrophic for leucine and arginine, can only proliferate in the TME, because leucine and arginine are enriched in the tumor but not in normal tissues.⁹⁷ The auxotrophic mutants of *E. coli* Nissle (EcN) 1917 with deletion of the *dapA* and *thyA* genes has been used as a vector to deliver therapeutic agents.⁹⁸ Unlike strict anaerobes, facultative anaerobes, such as *Salmonella* spp. and *Listeria* spp., can multiply in normal tissues containing oxygen, causing

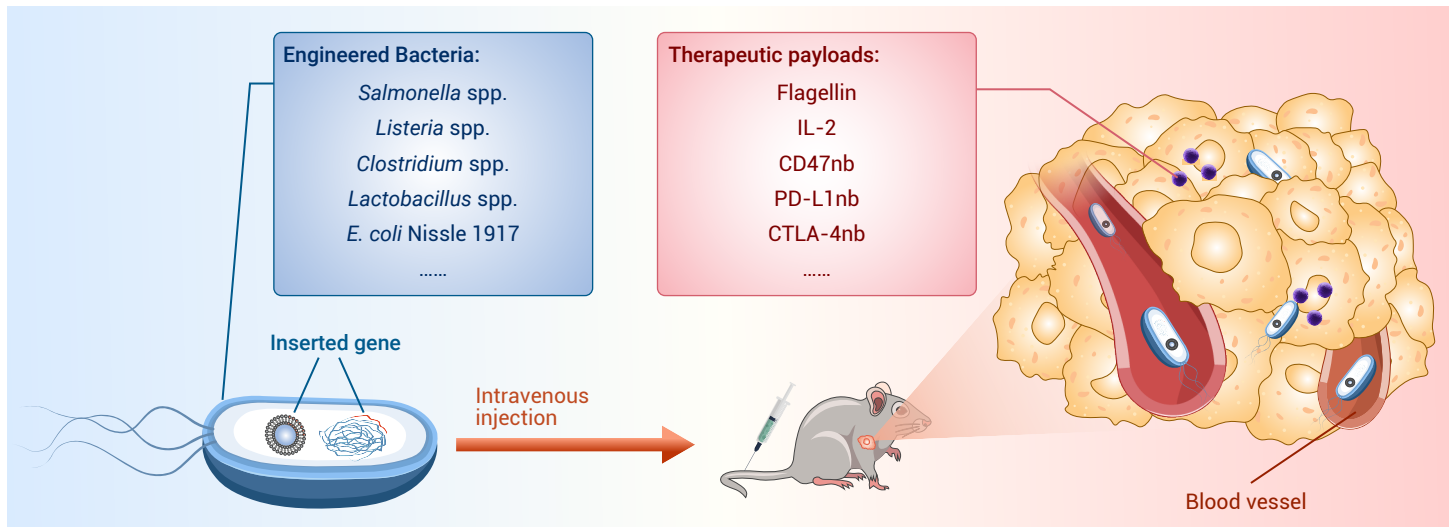


Figure 2. Engineered bacteria for cancer therapy Tumor-targeting bacteria are genetically engineered to deliver therapeutic payloads to tumor tissue by integrating specific gene sequences onto plasmids or DNA.

potential damage to the healthy cells. The tumor-targeting specificity of facultative anaerobes can be enhanced through genetic modification by displaying the tumor-specific binding proteins on the surface of bacteria. The resolution of off-target bacteria-expressing antitumor agents is also an effective way to reduce toxicity to normal organs.⁸³ In addition, the inducible promoters responding to unique TME signals, such as hypoxia and low pH, promote the release of anticancer agents carried by bacteria in tumors but not in normal tissues, which minimizes direct cytotoxicity to normal tissues.^{99,100} The genetic programming of bacteria on the lysis circuit with a quorum-sensing system can prevent bacteria from overgrowth in the body, including in tumors.¹⁰¹

Therapeutic payloads. The limitation of antitumor activity by a single species of wild-type bacteria is not negligible. In particular, although attenuated bacteria improve safety, the toxicity against tumor cells may also be reduced. Therefore, to enhance the anticancer effects, the tumor-targeting bacteria can be genetically engineered to deliver therapeutic payloads, including proteins, RNA, and DNA (Figure 2). Bacteria can express cytotoxic proteins, such as cytolysin A (ClyA), hemolysin E (HlyE), azurin, transforming growth factor alpha (TGF α), and *Pseudomonas* exotoxin A (PE38), which can directly kill tumor cells.^{102,103} The bacteria can also express prodrug-converting enzymes, such as cytosine deaminase and *Armoracia rusticana*-derived myrosinase, which can convert nontoxic compounds into toxic chemotherapeutic drugs in tumors, achieving tumor site treatment and reducing the toxicity to normal tissues.^{104,105} In 2021, our team used an engineered EcN 1917 that secrete catalase to alleviate intratumoral hypoxia by promoting the release of oxygen and ROS, and to enhance tumor radiosensitivity.¹⁰⁶ Genetically engineered bacteria with tumor orientation can express and release immune agents in tumors, which not only improves the delivery of the payload but also reduces the systemic side effects associated with off-target immune agents.

Effects on immune system modulators. IL-2 is one of the first immune-active agents approved by the U.S. Food and Drug Administration for the immunotherapy of advanced tumors, such as melanoma and kidney cancer.¹⁰⁷ Patients with cancer receiving high-dose IL-2 treatment to suppress tumor cells must be closely monitored for the consequential side effects, such as skin rashes, short-term neurological disorders, and dangerous blood pressure decreases.¹⁰⁸ Up to approximately 4% of patients die from IL-2 therapy. These risks of IL-2 therapy have been greatly reduced by applying attenuated *Salmonella typhimurium* delivering IL-2 to the TME; the approach has been tested in murine, veterinary canine, and human (NCT01099631) clinical trials.¹⁰⁹ Similarly, other stimulatory cytokines or chemokines,¹¹⁰⁻¹¹⁶ including IL-2, IL-4, IL-12, IL-18, IFN- γ , chemokine (C-C motif) ligand 21 (CCL21), granulocyte-macrophage colony-stimulating factor (GM-CSF), FMS-like tyrosine kinase 3 ligand (FLT3L), and TNF superfamily member 14 (TNFSF14), have also been delivered by engineered bacteria in

preclinical studies. Specific bacterial components can also be used as immunoregulators. Expression and secretion of *Vibrio vulnificus* flagellin B by the attenuated mutant *S. typhimurium* Δ pGpp could increase the proportion of M1 macrophages and promote the secretion of stimulatory cytokines, such as IL-1 β and TNF.¹¹⁷ STING plays an important role in inducing the anti-tumor effect of host. Leventhal et al.⁹⁸ constructed a dual auxotrophic EcN mutant strain (SYNB1891) to activate the STING pathway and induce the production of cytokines including type I IFNs by expressing diadenylate cyclase (DacA) under the control of an anaerobic promoter (P_{fms}). Activation of STING pathway can generate more tumor-specific T cells and enhance host immunity.¹¹⁸

Effects on ICIs. The activation of systemic T cells throughout the body by ICIs is one of the main side effects that limit the application of ICIs. Tumor-colonizing bacteria are ideal vehicles for delivering ICIs to tumors, which can lock the immune activation against the tumor in the TME, greatly improving the effective concentration and safety. Nanobodies, derived from Camelidae, are composed of only the variable region of the heavy chain and have a small molecular weight. Compared with traditional antibodies, nanobodies without glycosylation are suitable for expression and preparation in microbial systems. Chowdhury et al.¹¹⁹ used an engineered nonpathogenic *E. coli* to encode and release a nanobody antagonist of CD47 (CD47nb) under the auxiliary of a synchronized lysis circuit (SLC). Data from animal experiments showed that this system could specifically lyse and release a sufficient amount of CD47nb in the TME, thereby activating tumor-infiltrating T cells, suppressing tumor growth and metastasis, and significantly increasing the long-term survival of mice. Soon afterward, Gurbatri et al.¹²⁰ used the same SLC system to genetically engineer EcN to release an antagonist of PD-L1 and a CTLA-4 nanobody. This engineered EcN can release the PD-L1nb and CTLA-4nb locally into the TME to increase the number of CD8 $^{+}$ T cells and conventional CD4 $^{+}$ T cells in the tumor while decreasing the number of regulatory T cells, thus promoting tumor regression. In addition, some mediators, such as L-arginine, may enhance the immune function of tumor-infiltrating T cells. Roger Geiger et al.¹²¹ developed an engineered probiotic strain of EcN 1917 that colonized tumors and continuously converted ammonia (a metabolic waste product that accumulates in tumors) to L-arginine, increasing the number of tumor-infiltrating T cells. Moreover, it had a significant synergistic effect with PD-L1 blocking antibody in tumor clearance.

Although engineered bacteria have shown surprising anti-cancer effects by virtue of their tumor-targeting properties, their safety is an important factor that restricts a wide range of clinical applications. The attenuated bacteria may still act as antigens to cause the body's immune response and even life-threatening bacteremia. Genetic modification may lead to genetic pollution, leading to genetic changes in the surrounding naturally occurring bacteria. For example, EcN synthesized genotoxic metabolite colibactin encoded by pks islands, which was a carcinogenic virulence factor.^{122,123} These issues

need to be handled properly.

Engineered viruses-based strategy

Novel oncolytic virus (OVs) is a class of tumor-killing virus with replication ability and one of the hot spots in tumor immunotherapy. OVs mainly function by direct oncolysis, induction of anticancer immune responses, and expression of exogenous effector genes.¹²⁴ The first and most successful oncolytic virus to be approved by the FDA is T-Vec, a genetically engineered HSV-1 virus for the treatment of metastatic melanoma.¹²⁵ There are also many OVs in the preclinical and clinical research stages, such as Newcastle Disease virus (NDV),¹²⁶ Herpes Simplex virus-1 (HSV-1),¹²⁷ reovirus,¹²⁸ oncolytic adenovirus,¹²⁹ etc. The combination of OVs and immune checkpoints by gene recombination technology and the rise and continuous progress of tumor immune combination therapy has made the application of OVs more widely, but there are still bottlenecks such as virus targeting, safety, and route of administration.

OUTLOOK

The regulatory role of human microorganisms in the immune system and tumor development makes microorganisms have a positive or negative impact on the effect of certain tumor immunotherapy, providing new ideas and therapeutic targets for immunotherapy. With the advent of the era of precision medicine, screening out individualized specific strains or mixed strains of intestinal bacteria that respond positively to immunotherapy can improve the precision and effectiveness of bacterial application in immunotherapy. Whether it is intratumoral or extratumoral microbes, although the effects are gradually clear, there is still a lot of room for understanding the interaction between bacteria and their metabolites and immune cells as well as a clearer regulatory mechanism, which will be an indispensable part of future research. Current advanced research methods, including metagenomics, metabolomics and the combined application of multiomics, will further reveal the functional characteristics of the microbiome and host cells in health and disease, and open up ideas for the use of small molecule metabolites to prevent or treat refractory diseases related to the microbiome. A recent report demonstrated that *Coprobacillus cateniformis* promoted anti-tumor immune response by down-regulating PD-L2 and RGMb.¹³⁰ This study provides a new strategy and a new cancer immunotherapy target for patients who do not respond to PD-1 cancer immunotherapy and has great prospects for future research.

The low microbial content of tumor samples limits the progress of the research on intratumoral microbes. At present, the research on intratumoral colonies is still in its infancy, and many research methods are not mature. It has not been clarified whether the specific changes of tumor microorganisms are the cause or the result of cancer development, or whether these changes have nothing to do with cancer. How to ensure that samples are not contaminated during testing? How to eliminate the effects of environmental microbial pollution? How to reduce the deviation of sequencing results of tumor samples? Many problems need to be solved. Manipulating the bacterial communities within these tumors to improve outcomes for cancer patients is a long way off in the foreseeable future.

Engineering bacteria therapy can directly induce apoptosis or autophagy of cancer cells, as well as induce anti-tumor immune response, and target to regulate tumor metabolism during tumor development. As an excellent drug delivery system, engineered bacteria can accurately deliver drugs to lesions. For engineering bacteria, the pathogenicity of microorganisms as pathogens has not been completely solved, how to control adverse reactions within the acceptable range of clinical applications is a difficult problem that must be faced in future research.

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The authors declare no competing interests.

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Not applicable.

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