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REVIEW ARTICLE

Dynamic relationships among tumor, immune response, and microbiota

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ABSTRACT

Recently, the analysis of microbiota has been of interest not only for the clarification of the molecular mechanisms of disease etiology, but also the discovery of novel strategies for treatment. Following the development of "next-generation" sequencing, novel areas have been discovered in microbiota; however, in oncology, the relationships between microbiota and cancer have not been fully clarified. In recent literature, surprisingly, detection of gut microbiota in tumor issue itself has been reported. Microbiota might play an important role in carcinogenesis. However, this phenomenon is not well understood, and research in this area has just begun. In the past five years, a paradigm shift has occurred in cancer treatment due to immunotherapy. Immunotherapy has made cure possible even in advanced cancer patients with not only melanoma but also non-small cell lung cancer and others. In this review, we discuss the mechanisms of novel immunotherapies, checkpoint inhibitors, and the relationship between microbiota and immunotherapy. It is of significance to clarify this relationship because it may lead to the discovery of predictive markers for immunotherapy and promote clinical efficacy. Finally, we also mention our activities in the construction of a big database for information on immunotherapy and microbiota, which may lead to excellent possibilities of discovering novel strategies for more effective cancer treatments, and may accelerate the alteration of cancers to the classification of chronic nonfatal disease.

Keywords: *gut microbiota; immunotherapy; checkpoint inhibitors; cancer treatment; immunoresponse*

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Introduction

In this review, an overview of the relationships of gut microbiota among tumor, immunological response, and therapeutic effect is presented. Gut microbiota are mainly located in the human gastrointestinal tract, and the microbes number in the hundreds of trillions in healthy subjects[1,2]. It has been reported that thousands of kinds of gut microbiota are in the body, which is a few times more than the kinds of adult human cells[3]. Before the advent of next-generation sequencing methods (NGS), our understanding of gut microbiota was not clear, due to technological limitations. For example, *Escherichia* (*E.*) *coli* had seemed to be the most common bacteria in gut because of its high frequency of detection by the previous methods. However, after NGS, *E. coli* has been found to be not so common in the human gut—anaerobic bacteria are more frequent. It is said that microbes become established in the gastrointestinal tract until the host reaches three years of age, and then the microbes reach an adult state as gut microbiota[4]. Gut microbiota and human beings are co-evolutionary, in a win-win symbiosis[5–7]. Recently, there have been many studies and much research on the relationships between the gut microbiota and human disease[8–11]. Gut microbiota seem to be strongly correlated with various kinds of disease, such as inflammatory bowel disease (IBD)[12–15], diabetes mellitus (DM)[16–18], central nervous disorder (CND)[19–24], allergic diseases[25–29], and infectious diseases[30]. On the other hand,

the relationship between tumor and microbiota is not well clarified, especially from the viewpoint of immunology. Here, we focus on the relationship between gut microbiota and cancer, which recently has been noted. Interestingly, the efficacy of a checkpoint inhibitor as tumor immunotherapy has also been reported to be related with the host gut microbiota[31–33].

Detection of microbiota

In general, most published analyses of gut microbiota have used a statistical analysis system (SAS). If the homology of the genetic information of microbes in different tests is over 96%, the species are defined to be the same because of the error in polymerase chain reaction (PCR). Before the appearance of the next-generation sequencer, many genetic analysis reports were made and the results were pooled into a database. In the genome of a bacterium composed of several million base pairs, there is a highly polymorphic region of approximately 1,500 base pairs, the "16S ribosomal RNA region." In the 16S region, there are nine hypervariable regions consisting of several hundred base pairs, with a characteristic arrangement depending on the kind of bacteria. Also, it is known that this hypervariable region is conserved within a bacterial species, and therefore, to identify a kind of species, it is good enough to sequence the 16S region.

Hattori *et al.* have reported that bacterial deoxyribonucleic acid (DNA) encoded in V1 and V2 region of 16S ribosomal ribonucleic acid (rRNA), which contains about 300 genes, is sufficient as the target for determination of the species of a microbe[34,35] because of the high species-specificity of the 16S rRNA. It is technically of importance to break the hard cell wall of bacteria in the extraction of DNA from samples. After creating a library based on PCR data using specific primers we had available, we used a PGM system (Thermo Fisher Scientific K.K., Yokohama, Japan) to read the sequences from the library. There are several other methods for determining gut microbiota as well; however, so far, there is insufficient data to evaluate these methods, and further studies or experiments are needed to clarify them. Special care must still be taken when determining the bacterial species until the detection methods are appropriately evaluated.

Tumors and microbiota

Interestingly, our group and others have successfully detected gut microbiota in tumor itself, even in tumors derived from outside of the gastrointestinal tract. Cluster analysis methods have demonstrated totally different patterns in comparison between microbiota in tumor tissue and the adjacent normal tissue, clearly indicating that bacteria in gut move from gut or elsewhere in the body to the tumor via blood or lymphatic vessels, and the kinds of bacteria in cancer tissues are different from those in normal tissues. The biology of tumor microbiota is not yet fully clarified. Below, we discuss some types of cancer from the viewpoint of microbiota.

Breast cancer

Although the molecular biological etiology of breast cancer is not yet fully known, it has been reported to involve a combination of genetic and environmental elements. Along with genetics, environmental factors contribute to breast cancer development, but what these exact environmental factors remain unknown. Although results of their analysis were different from ours, some interesting studies may offer support for certain environmental factors being associated with an increased incidence of breast cancer[36,37]. Urbaniak *et al*. demonstrated that breast tissue contains a diverse population of bacteria[38,39]. Using the analysis of 16S rRNA, they showed that the pattern of microbiome is completely different between the adjacent normal breast tissue from women with breast cancer and breast tissue from healthy controls. Furthermore, they also observed that the pattern of bacteria is almost similar between adjacent normal breast tissue and breast tissue sampled directly from breast cancer. This might indicate that the development of breast cancer is affected by patients' microbiome as one of the environmental elements. In their publications, patients with breast cancer had higher relative abundances of *Bacillus*, *Enterobacteriaceae,* and *Staphylococcus* in comparison with those in healthy controls. These bacteria species induce DNA damage, possibly double-stranded DNA breaks. Bacteria that have a function of causing DNA damage were detected in breast cancer patients; on the other hand, there were lower levels of some lactic acid bacteria, known for their beneficial health effects, including anticarcinogenic properties. *Bacillus* is more frequently detected in breast cancer patients compared with healthy controls. Although *Bacillus* does not induce DNA damage as do *E. coli* and *S. epidermidis*, it may have other carcinogenic effects, such as metabolization of hormone and/or stimulation of cell proliferation. On the other hand, in a role of prevention, *Lactococcus* and *Streptococcus*, which are higher in healthy women than in breast cancer patients, may show anticarcinogenic properties. However, it is very difficult to conclude that, in terms of mechanism, some bacteria by themselves cause a high incidence of breast carcinoma because patients

have the prevention pathway of immunosurveillance, which will be discussed later.

Colorectal cancer

Recent reports have clearly demonstrated that *Fusobacterium* (*F.*) *nucleatum* is one of the major risk factors for colorectal cancer[40–42]. Some studies suggested that *F. nucleatum* showed immunosuppressive activities of T cell response in the tumor microenvironment. This indicated that microbiome and immunoresponse are strongly associated with carcinogenesis. Nosho *et al.* and Mima *et al.* demonstrated that *F. nucleatum* in colorectal cancer activates and proliferates myeloid-derived immune cells, typically myeloid-derived dendritic cells (DCs) and M2 macrophages, which strongly induce immunosuppressive action of T cells in the tumor microenvironment[43,44]. These cells produce a number of reactive oxygen species (ROS) and inflammatory cytokines, such as Interleukin (IL)-10. There may be some possibility that *F. nucleatum* stimulates and produces microRNA-21, which also stimulates the production of IL-10 and prostaglandin E2. These products strongly suppress antitumor T cell-mediated adaptive immunity via regulatory T cell in the tumor microenvironment. ROS also causes epigenetic silencing of the mismatch repair protein MLH1 to induce microsatellite instability. The mechanisms regarding the association between immune cells and molecular alterations caused by *F. nucleatum* have not been well clarified. However, it is clear that gut microbiota in tumor affect the host's immune response in the tumor microenvironment.

Gastric cancer

Helicobacter (*H.*) *pylori* selectively colonize the gastric epithelium, and are believed to be a major player in the etiology of gastric cancer. *H. pylori* infection begins in childhood and persists for the whole life of the host. In Japan, it is common to have *H. pylori,* which is thought to be a main reason that gastric cancer is one of the major cancers in Japan. Between approximately 1% and 3% of *H. pylori*-infected persons suffer gastric adenocarcinoma.

Tumor immunotherapy and microbiota

Tumor immunology and immunotherapy

Many recent publications have clearly demonstrated that microbiota are among the key elements for determination of antitumor effects. The efficacies of tumor immunotherapy of programmed death (PD) 1 and PD-ligand (L) 1 and cytotoxic T lymphocyte antigen (CTLA)-4 antagonist, known as immune checkpoint inhibitors, are determined by genetic and environmental factors. Microbiota may affect the drug efficacy, abrogation of anticancer effects, and mediation of toxicity of chemotherapeutic drugs[45–49]. This has not yet been completely clarified from the viewpoint of molecular biology, but some metabolites from microbiota may significantly affect these phenomena. However, it is difficult to explain how only a few kinds of microbiome could determine the phenomenon and antitumor activity *in vivo*.

Interesting studies in this regard have been published recently. Antitumor effect of immunomodulatory drugs, anti-CTLA-4 antibody, and anti-PD-L1 antibody, has been reported to strongly correlate with the gut microbiota in a murine model[31,32]. Recently, tumor immunotherapy using these types of drugs has demonstrated extremely strong antitumor effect in the clinical setting. Many clinical trials have revealed significant effect, not only in progression-free survival, but also in overall survival.

In terms of T cell activation, the first signal is the binding with major histocompatibility complex (MHC)-peptide complex and T cell receptor (TCR). After antigen-presenting cells (APC), such as DCs or macrophages, present tumor antigens onto their cell surface as the complex of MHC-tumor-derived peptide, the T cell, as a first signal, recognizes this complex by the TCR. Cluster differentiation (CD)28 on the T cell is a costimulatory molecule on activated T cell binding to B7.1 molecule as a second signal of the activated T cells. The third signal of the activated T cell is a cytokine release, such as IL-2, for maintenance to activate T cell. To continue the activation of the T cell, CD28 on the T cell binds to B7.1 molecule, and then cytokine is released from the activated T cell to continue proliferation and activation of T cells as a third signal. CTLA-4 is important for the inactivation of T cells[50–52]. To shut down the activation of T cells, CTLA-4 is up-regulated to the activated T cell surface. CTLA-4 has 100-times higher binding affinity to B7.1, and therefore CD28 does not bind to B7.1. This induces the activated T cell to be inactive, and therefore the T cell tones down to form a kind of resistance. Anti-CTLA-4 antibody strongly blocks the binding CTLA-4 molecule and B7.1 molecule, enabling the T cells to continue to activate and produce the lymphocyte-stimulatory cytokines. *In vivo*, anti-CTLA-4 antibody is thought to be involved at the lymph nodes for the activation of T cells. It has been known that CTLA-4 molecule is also highly expressed to regulate T cells and block the activation of regulatory T cells, inducing the continuation of the T cell activation.

Anti-CTLA-4 antibodies, ipilimumab and tremelimumab, showed strong antitumor effect and revealed excellent clinical efficacy against melanoma. An important characteristic of anti-CTLA-4 antibodies is the duration of their antitumor effect [53,54]. The overall survival of 4,846 melanoma patients treated with ipilimumab was statistically analyzed using Kaplan-Meier methods[53]. Surprisingly, almost 20% of patients who were treated with ipilimumab showed prolonged survival: patients who were expected to live for 3 years have lived for 10 years. This response of overall survival is said to form a "kangaroo-tail phenomenon" on the Kaplan-Meier curve[55].

On the other hand, in the periphery of tumor site, the axis of PD-1 and PD-L1 is important for T cell peripheral dysfunction[56–59]. In contrast to CTLA-4 blockade, PD-1 binds to PD-L1, which is expressed on the tumor and/or immune suppressor cells in the tumor microenvironment. The activated T cells express PD-1, these T cells bind to its ligand PD-L1, and then the activated T cells become inactive. As a result, the T cells neither proliferate nor kill the tumor.

PD-1 was discovered by Ishida *et al*. in 1992 [60]. At first, PD-1 was thought to be one of the programmed death factors. In a genetically modified murine model, CTLA-4 knock-out is fatal at very young age or as a fetus, whereas PD-1 knock-out mouse is healthy until some weeks of age. The activated T cell highly expresses PD-1 continuously. On the other hand, PD-L1 is known to show two types of expression pattern. One type is constitutive, as some types of tumor highly express PD-L1 as a characteristic. The other is an inducible pattern. The activated T cell produces IFN-γ at the recognition of tumor. IFN-γ induces the expression of PD-L1 on the tumor and on the cells in the tumor microenvironment via nuclear factor-kappa B (NF-κB) or interferon regulatory transcription factor (IRF)-1[61]. This may be one reason why PD-L1 is not a good biomarker for anti-PD-1/PD-L1 therapy, because the IFN-γ release and/or PD-L1 expression is not very constant in the tumor microenvironment in terms of timing and quantity.

Anti-PD-1 antibody and anti-PD-L1 antibody are registered by the US Food and Drug Administration (FDA), the European Medicines Agency (EMA), and the Japanese Pharmaceuticals and Medical Devices Agency (PMDA) against several kinds of tumor, including melanoma, non-small cell lung cancer (NSCLC), and so on. One anti-PD-1 antibody, pembrolizumab, showed strong antitumor effect and clinical efficacy[62,63]. It was demonstrated that pembrolizumab shows significant clinical benefit, namely improvement of overall survival (OS), compared with platinum doublet as a first-line chemotherapy against NSCLC. In advanced NSCLC, platinum doublet has been a first-line chemotherapy for 30 years; however, in this clinical trial, Keynote-024, OS is significantly longer with pembrolizumab than with platinum doublet. This clearly indicates that the immunological response against tumor cells in our body has a much stronger antitumor effect than chemotherapy when the breaks on the immune checkpoints are released.

Mechanism of tumor immunology in humans

The following clinical evidence has provided answers to several previous fundamental questions regarding tumor immunology in humans: First, immunological reactions that recognize and kill tumor do not exist or do not function in (advanced) cancer patients. If we have an immunological response against tumor, we do not suffer from cancer. It has been clarified that humans have immunological response against cancer, even in advanced stages. Due to some clinical trials of anti-PD-1 antibody immediately showing antitumor effect, it clearly demonstrates that antitumoral immunological response is already in the tumor microenvironment; otherwise, it would take time to newly establish acquired types of immunological response against tumor. Secondly, even if we have immunological response against cancer, it might not be very strong. For example, if antitumoral immunological response is observed in only a few cases out of 100, it is not sufficient in the clinical setting. This indicates that only a very small number of patients have a benefit from their own immunological response against cancer. Recent early clinical trials have indicated that response rate is significantly high even in advanced cancer, and demonstrated that a meaningful number of patients are possible candidates to benefit from their own immunological response to tumor. Lastly, for each type of cancer, some significant drugs have been registered and they show clinical benefit even if it is not very strong. Until a few years ago, most believed that drugs utilizing the immunological response against cancer might not be effective because immune reactions are not strong enough to show clinical efficacy comparable to the presently registered anticancer drugs. Recent clinical trials have demonstrated that the drugs that utilize immunological response against cancer are so potent as to show higher antitumor effect compared with previously existing antitumor treatment such as chemotherapy.

Predictive biomarkers for immunotherapy and microbiota

As shown in these clinical results, immunotherapy has demonstrated clinical efficacy for cancer patients. However, it has not for all cancer patients, and it is limited. In the Keynote-024 study[63], it was shown that only patients with >50% of PD-L1 expression benefit from clinical efficacy. The big questions raised are which patients will show clinical benefit, and what are the markers for prediction? Basically, immunotherapy is much different from chemotherapy and radiotherapy (**Figure 1**). Chemotherapy and radiotherapy already show antitumor effect before administration, as when anticancer drugs are co-cultured with tumor cells or tumor cells are bombarded with X-rays *in vitro*. On the other hand, immunotherapy itself does not show antitumor activity before administration, as it is activated in the patient's body after administration. The immune checkpoint inhibitor does not show antitumor effect *in vitro*. In other words, in immunotherapy, patients make immunological drugs become active in their own body. It is crucial to understand how to make immunotherapy more effective, such as with combination therapy and discovery of predictive biomarkers.

Chemotherapy and radiotherapy themselves are already active before treatment; however, immunotherapy is not active before administration. *In vivo*, our body makes immunological drugs active (to show antitumor effect), and therefore it is necessary to understand the mechanisms in terms of environmental elements such as microbiota.

It is believed that the efficacy of an anticancer drug is derived from genetics, the drug itself, and host factors. Immunological drugs seem to be strongly dependent on host factors. Recently, some literature had demonstrated that tumor-infiltrating lymphocytes are strongly correlated with the clinical efficacy of checkpoint inhibitors[64]. The amount of CD8-positive lymphocytes in tumor microenvironment is strongly correlated with the decrease in size of tumor after checkpoint inhibitor therapy. In theory, this phenomenon is understood clearly. CD8-positive cytotoxic T lymphocytes are recognized as elements from tumor cells, which recently brought focus on neoantigens, and are clonally expanded and would kill tumor cells. However, activated T cells also express PD-1, and tumor cells themselves or the tumor microenvironmental immune cells express PD-L1, which induces tolerance of activated T cells. After the shutting down of the PD-1 and PD-L1 pathway using anti-PD-1 antibody, T cells re-activate and kill tumor cells, and demonstrate strong antitumor activity.

A core question is raised as to which patients show CD8-positive T cell in tumor tissue, supposedly a responder for immunotherapy. This question is important for the discovery of predictive markers for immunotherapy. Recently, to answer this question, the relationships between immunotherapy and microbiota were focused on (**Table 1**). Two murine studies were published[31,32]. The French group[31] demonstrated that antitumor effect using anti-CTLA-4 antibody is strongly related to *B. fragilis* and *B. thetaiotaomicron*. At first, it is an interesting observation that specific pathogen-free (SPF) mice showed potent antitumor effect, while germ-free mice did not show significant antitumor effect. SPF mice still had microbiota in them. The group hypothesized that some kinds of microbiota affect the antitumor effects. As their conclusion, they found that *B. fragilis* and *B. thetaiotaomicron* are the key bacteria to show the antitumor effect. They thought that the mechanism for this phenomenon is that these bacteria stimulate DCs, and DCs activate immune response, including CD8 T cell in tumor microenvironment. On the other hand, the Chicago

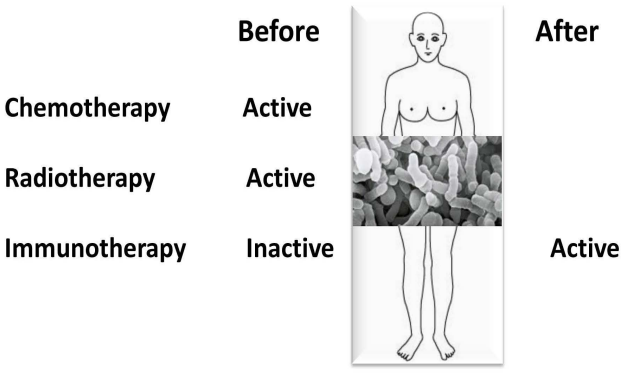

Figure 1. Differences among chemotherapy, radiotherapy, and immunotherapy

Table 1. Immunotherapy and microbiota

Immunotherapy	Disease	Microbiota	Human/Mouse	References
CTLA4	Melanoma	*B. fragilis/B. thetaiotaomicron*	Mouse → Human	Vétizou *et al*., 2015[31]
PD-L1	Melanoma	*Bifidobacterium*	Mouse → Human	Sivan *et al*., 2015[32]
PD-1	Melanoma	Diversity of microbiota/*Clostridia* → responder *Bacteroidia* → non-responder	Human	Gopalakrishnan *et al*., 2017[33]
Stem cell transplant	Allogeneic hematopoietic stem cell transplantation	Not particular, diversity of microbiota	Human	Taur *et al*., 2014[66]

group[32] clearly demonstrated that antitumor effect using anti-PD-L1 antibody is derived from commensal *Bifidobacterium*. In this publication, it was an interesting observation that the mice from one vendor showed strong antitumor effect, whereas the mice from another vendor did not show antitumor effect, even though these mice were of the same strain. When these mice were kept together in the same cage, antitumor effect transferred to the mice from the vendor that previously did not show antitumor effect. Therefore, the group hypothesized that the antitumor character might have been transferred via eaten stool, which contained various kinds of bacteria. Finally, they found *Bifidobacterium* is the key bacterium to determine the antitumor effect, using anti-PD-L1 antibody. As for the mechanism, it was also thought that *Bifidobacterium* stimulates DCs, and these DCs also stimulate antitumor immune response[65]. This evidence strongly suggested that microbiota are strongly correlated with antitumor effects. However, the limitation is that the evidence came from microbiota in mice.

Recently, human data were presented by MD Anderson Cancer Center at the ASCO-SITC 2017 Clinical Immuno Oncology Symposium[33]. Analysis was performed on melanoma patients treated with anti-PD-1 antibody. Comparison between responder group and non-responder group was made for microbiota identified using 16S rRNA. In the stool of responders, *Clostridia* was dominant, whereas in the stool of non-responders, *Bacteroidia* was dominant. On the other hand, one of the significant factors in the comparison between responders and non-responders was the diversity of microbiota. This phenomenon has also been reported in regard to the clinical efficacy of stem cell transplant[66]. It is of importance that diversity is strongly correlated to the CD8-positive cells infiltrated to tumor microenvironment. This means that microbiota are among the key factors for determining the clinical effect using immunotherapy, especially anti-PD-1 antibody.

It has been known that T cells play an important role in the showing of antitumor effect when using immunotherapy against cancer. We hypothesize that diversity of microbiota is significantly important for potent antitumor effect, because T cells are prepared for the pathogens. The number of pathogens present induce proportionate amounts of TCR, which are recognized by T cells. Gut microbiota somehow enter into human tissues from the gastrointestinal tract and travel via the blood. Innate immune responses react immediately and kill the bacteria in healthy subjects. If the immune response is weak in expelling the invading bacteria because of immunodeficient condition, live bacteria circulate in the blood: the condition of bacteremia. This is not very common in patients with normal immunity. Some of the bacteria are phagocyted by DCs or macrophages, and transfer their information as sequences of peptides restricted by MHC to adaptive immunity such as T cells and B cells. T cells are differentiated to cytotoxic T lymphocytes (CTL) against target cells, namely cellular immunity, and finally to memory T cells. B cells are differentiated to plasma cells against target molecules, namely humoral immunity, and finally to memory B cells. If a target pathogen invades the body, these memory T cells and memory B cells are immediately activated and get rid of the target. If microbiota are diverse, there are probably many pathogens in the body. This means that many kinds of TCRs are stimulated, and defend well against the invading targets. However, questions are raised because during that time, these targets are bacteria, not tumor. It has been well known that TCR is not sufficiently unique to recognize the target molecule, which is the complex binding target-derived peptides and MHC molecule. TCR often shows cross-reactivity, which is one of the major reasons autoimmune disease is induced etiologically. Type 1 DM sometimes occurs after infection with

common virus. It is thought that T cells against these viruses also may recognize the Langerhans cells, and afterwards these T cells attack and kill the pancreatic Langerhans cells. Recently, the most potent antigen against tumor is thought to be a neoantigen, which is derived from the mutations in the tumor. We hypothesize that T cells induced by microbiota might cross-recognize the tumor neoantigen. The presence of many pathogens provides a good opportunity for cross-recognition of the tumor antigen, including neoantigens *in vivo*.

Future directions

To clarify this hypothesis and the relationship between microbiota and clinical response, our group attempted to establish a microbiota database information bank system to accumulate information on microbiota. Big data might be needed for accurate evaluation and to utilize them in development of predictive markers, novel diagnoses, and modalities. There are many publications about immune competencies; however in clinical settings, no conclusive definition has yet been determined as to what the immune competencies by the host are. We believe that the tumor microenvironment, especially immune cell infiltration, is one of the significant immune competencies by the host, because it strongly correlated with the clinical effect of immunotherapy, immune checkpoint inhibitor therapy[67–71]. It is strongly expected that a predictive marker might be discovered from the big data on the relationships between immunotherapy and microbiota, and will provide us an opportunity to promote the clinical efficacy by altering the microbiota. Finally, we also expect the development of novel cancer vaccines targeting microbiota using meaningful databank information to induce potent CTLs with high diversity.

Conflict of interest

The authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of their article.

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