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The gut microbiota positively regulates anti-tumor immune responses through the activation of CD8$^+$ T cells

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Abstract

Inhibitors of the immune checkpoint molecules PD1 and CTLA4 have been used to enhance the T cell anti-tumor immune response, leading to successful treatment for some tumor types. However, not all patients benefit from treatment with these inhibitors. Recently, it was reported that the gut microbiota boosts the anti-tumor immune response to immune checkpoint therapy. However, the mechanism(s) by which the gut microbiota enhance tumor immunity are not fully understood. Here, by depletion of the gut microbiota, we asked how the microbiota influences the anti-tumor immune response, in particular examining the activation of tumor-specific CD8$^+$ T cells. We found that the number of tumor antigen-specific CD8$^+$ T cells in spleen, and the number of activated CD8$^+$ T cells in draining lymph node and tumor tissue in the absence of the gut microbiota were reduced. Furthermore, tumor-infiltrating CD8$^+$ T cells were impaired in their ability to produce IFNγ. These findings suggest that the gut microbiota contributes to the prevention of exhaustion of tumor-infiltrating CD8$^+$ T cells and to the activation of systemic CD8$^+$ T cells.

Introduction

The gut microbiota plays a crucial role in homeostasis of the normal intestinal environment[1]. On the other hand, certain gut microorganisms are associated with the pathogenesis of inflammatory bowel diseases and colon tumorigenesis[2,3]. These observations suggest that the composition of the gut microbiota must be tightly controlled to maintain a healthy intestine. Recently, it was reported that gut microbiota dysbiosis influences the pathogenesis of not only intestinal diseases but also several systemic diseases such as autoimmune diseases, metabolic syndrome, cancer and allergy[4,5]. It has been known for decades that oral administration of antigens tends to induce immune tolerance rather than activation, leading to suppression of allergic and experimental autoimmune diseases[6,7]. It has also been reported that short chain fatty acids such as butyrate, which are produced by certain members of the gut microbiota as metabolites of a high-fiber diet, ameliorated autoimmune and allergic diseases[8,9,10]. These reports indicate that gut microbiota-induced immune responses and/or the gut microbiota itself are strongly associated with immune homeostasis, not only in the intestines but also throughout the body.

Developing tumor cells are eliminated by the immune surveillance system before showing clinical presentation[11]. However, once tumor cells make the transition to the progression phase, they establish a tumor microenvironment that is immunosuppressive, leading to escape from immune surveillance. Several mechanisms for this escape have been identified. Tumor cells can express immune inhibitory molecules such as PD-L1, which upon binding to PD-1 on tumor-specific CD8$^+$ T cells inhibits their proliferation and may induce apoptosis. Tumor-specific CD8 T cells in the lymph nodes draining the tumor are chronically antigen stimulated, leading to a state of "exhaustion" characterized by upregulation of the CD80/86 inhibitory receptor CTLA4[12]. Recently, neutralizing antibodies to the immune checkpoint molecules PD1, PD-L1 and CTLA4 have been developed and used therapeutically to break the exhausted status of tumor-specific CD8$^+$ T cells, and have shown good efficacy in some patients and with several different tumors[13]. However, this immunotherapy has been successful in some patients but not others, even for treatment of the same tumor type, therefore many researchers have explored ways enhance the efficacy of immune checkpoint therapy. Gajewski's group found that the particular member of the gut microbiota, *Bifidobacterium*, enhances immune response to anti-PD1 therapy in a murine tumor model[14]. It has also been reported that *Akkermansia muciniphilia, Faecalidacterum* and *Bacteroidales* are abundant in the gut microbiota of patients who responded well to PD1 immunotherapy[15,16], suggesting that the gut microbiota has the potential to promote the immune response to tumors and enhance the effectiveness of immune checkpoint inhibitors. However, the mechanism by which the gut microbiota enhances tumor immunity is not fully understood.

In this study, we asked a simple question of whether the global gut microbiota, but not a particular genus, influences the anti-tumor immune response, especially the activation of tumor-specific CD8$^+$ T cells, and find that the gut microbiota is required to suppress the exhaustion of tumor-infiltrating CD8$^+$ T cells. We also find that depletion of the gut microbiota reduces the number of tumor antigen-specific CD8$^+$ T cells in the spleen. These findings suggest that the global gut microbiota contributes to the escape from exhaustion by tumor-infiltrating CD8$^+$ T cells and the activation of systemic CD8$^+$ T cells.

Materials and Methods

Mice

C57BL/6 (B6) mice were obtained from Sankyo Labo Services. All animals were maintained under specific pathogen-free conditions in our facility. All experiments were performed in accordance with protocols approved by the Animal Care and Use Committee of Tokyo University of Science.

Cell lines

The Lewis lung carcinoma cell line expressing LCMV gp33 from a minigene (LLC-gp33) was established previously[17] and maintained in high glucose DMEM supplemented with 10% FBS, 2mM l-glutamine, 100U/ml penicillin, streptomycin and 1mg/ml G418.

Antibodies

PE-anti-KLRG1 and efluor780-anti-CD44 monoclonal antibodies (mAbs) were purchased from eBioscience (San Diego, CA, USA). The LCMV gp33 tetramer was purchased from MBL (Nagoya, Japan). FITC-anti-CD8a, biotin-anti-

TCRβ, PE-anti-CD3ε and APC-anti-IFNγ mAbs were prepared in-house.

Flow cytometry analysis

Cells were treated with FcgRII/III mAb (laboratory prepared) to block nonspecific binding. 7AAD (BD) was used to exclude nonviable cells. A Canto II (BD) and Gallios (Beckman Coulter, Brea, California, United States) were used for analysis. Data were analyzed using FlowJo software (Tree Star, Ashland, Oregon, United States).

Intracellular staining of IFNγ

Isolated tumor-infiltrating cells were stimulated with LLC-gp33 cells which were stimulated by IFNγ to induce the expression of MHC class I or PMA and Ionomycin for 12 h *in vitro* in the presence of monensin (Sigma-Aldrich Inc.). After staining for surface antigens, the cells were fixed 4 % PFA, then permeabilized with 0.5% Triton X-100 and stained with anti-mouse IFNγ mAbs.

Inoculation of tumor cells into mice receiving antibiotics water

Mice were provided water containing ampicillin (1 mg/ml), metronidazole (1 mg/ml), neomycin (1 mg/ml) and vancomycin (0.5 mg/ml) *ad libitum*. At 1 month after treatment with antibiotics, LLC-gp33 cells ($1x10^5$ cells/ mouse) were intradermally inoculated into mice, and then tumor size was measured every 3 days.

Isolation of lymphocytes from tumor tissue

The tumor tissue was minced into small pieces. To isolate lymphocytes, the tissue was treated twice with 0.5 mg/ml type I collagenase (Roche, Basel, Switzerland), 0.02 mg/ml hyaluronidase and 0.01 mg/ml DNAse I (Sigma Aldrich, St. Louis, Missouri, United States) for 30 min at 37°C. After filtration, cells were suspended in RPMI with 10 % FBS.

Statistical analysis

Statistical analysis was performed by a two-tailed unpaired Student t-test and a Kaplan-Meier method in Prism6 (GraphPad Software, San Diego, California, United States). The *p*-values < 0.05 were considered significant.

Results

The depletion of gut flora increases tumor growth in mice

To investigate whether gut microbiota influenced the anti-tumor immune response, we intradermally inoculated LLC-gp33 cells into mice that had received antibiotic water (ampicillin, vancomycin, metronidazole and neomycin) for four weeks previously and continued to receive it for the course of the experiment (Fig. 1A). At first, to confirmed whether the depletion of the gut flora influenced T cells, we analyzed the frequency of $CD8^+$, $CD4^+$ T cells and $CD8^+$ $CD44^{high}$ population in the peripherally blood lymphocytes (PBLs). The frequencies of total $CD8^+$, total $CD4^+$ T cells and $CD8^+$ $CD44^{high}$ population in PBLs were not differences between control and antibiotics-treated mice (Fig. 1B-1D). Compared with controls, there was significantly increased tumor growth in mice that had received antibiotic water (Fig. 1E), and also a decreased survival rate (Fig. 1F). These data imply that the depletion of the gut flora attenuates anti-tumor immune response, resulting in increased tumor growth.

Activated $CD8^+$ T cells are decreased in lymphoid organs and the tumor of antibiotic-treated mice.

To dissect the influence of the gut microbiota on the anti-tumor immune response, we analyzed the activation status of $CD8^+$ T cells in spleen, draining lymph node (dLN) and tumor tissues using antibodies to CD44 and KLRG1 as activation markers and the tetramer of gp33 to detect tumor-specific $CD8^+$ T cells. Compared with control mice, the numbers of gp33-reactive T cells but not total $CD8^+$ T cells, $CD8^+$ $CD44^{high}$ cells, $KLRG1^+$ $CD8^+$ T cells and total $CD4^+$ T cells were decreased in the spleen of antibiotic-treated mice (Fig. 2A-2F), suggesting the possibility that the gut microbiota can influence systemic activation of tumor-specific $CD8^+$ T cells. Different from spleen, the number of total lymphocytes, total $CD8^+$ T cells and total $CD4^+$ T cells were reduced in the dLN of antibiotic-treated mice (Fig.2G, 2H, 2L). Also, $CD44^{high}$ and $KLRG1^+$ $CD8^+$ T cells in dLN were significantly decreased by the depletion of the gut microbiota (Fig. 2I and 2J), but the number of gp33-reactive cells was not difference between control and antibiotic-treated mice (Fig. 2K). Different from dLN, the frequency of total $CD8^+$ T cells in tumor-infiltrating lymphocytes was not difference between control and antibiotic-treated mice, and most of tumor-infiltrating $CD8^+$ cells express CD44 in both groups (Fig. 2M, 2N). Meanwhile, the frequency of $KLRG1^+$ $CD8^+$ T cells and gp33-reactive cells tended to decrease in antibiotic-treated mice (Fig. 2O, 2P). These data suggest the possibility that the gut microbiota also activate tumor-specific $CD8^+$ T cells in the local lesion.

Gut flora increases IFNγ-producing $CD8^+$ T cells in tumor tissue

In Figure 2, the frequency of $CD44^{high}$ $CD8^+$ T cells was similar between the control and antibiotics groups. It is reported that CD44 is highly expressed on exhausted $CD8^+$ T cells, which are hypo-responsive to antigen stimulation and have impaired production of cytokines such as IFNγ[18]. Therefore, we considered the possibility that $CD44^{high}$ cells in tumor tissues of antibiotics-treated mice were of the exhausted phenotype. To address this, we analyzed the ability of the in tumor-

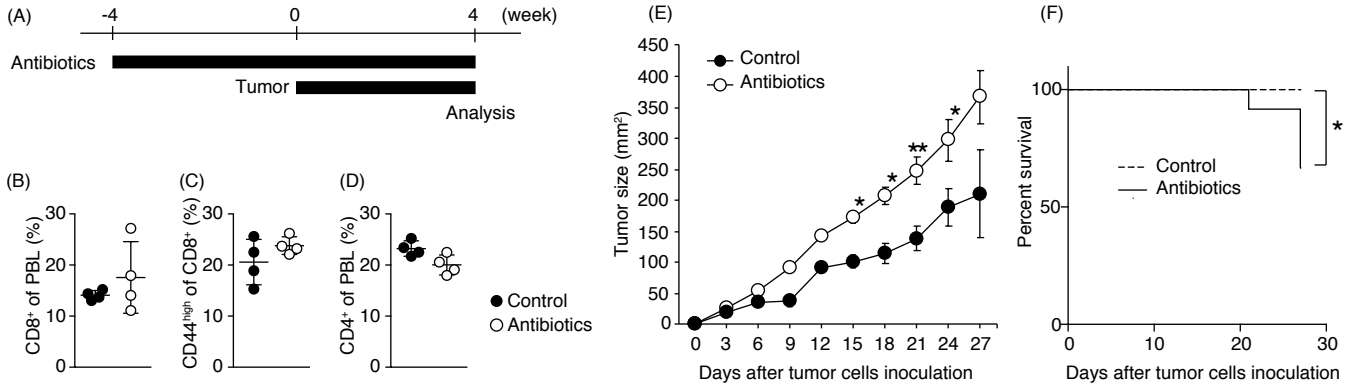

Figure 1. Depletion of the gut microbiota promotes the growth of tumor cells *in vivo*.
(A) Experimental design to analyze the influence of the gut microbiota on tumor growth and survival. Mice were given untreated water as control or water containing antibiotics (ampicillin, metronidazole, neomycin and vancomycin) for 1 month. LLC-gp33 cells were inoculated intradermally and antibiotic treatment was continued until the experiment was concluded 1 month later. (B-D) The peripheral blood lymphocytes (PBLs) at 4 weeks after the orally administration of antibiotics were analyzed by flow cytometry. The frequency of total $CD8^+$ T cells (A), $CD44^{high}$ in $CD8^+$ T cells (B) and total $CD4^+$ T cells (C) in PBLs. (E) Graph indicates mean ± SE of tumor size (mm^2). Mean ± SE was calculated every 3 days from 11 control mice and 7 antibiotic-treated mice, which survived until the endpoint. P-values from student's t-test: **p*<0.05, ***p*<0.01. (F) Survival rate shown by the Kaplan-Meier method from 11 control mice and 12 antibiotic-treated mice. **p*<0.05.

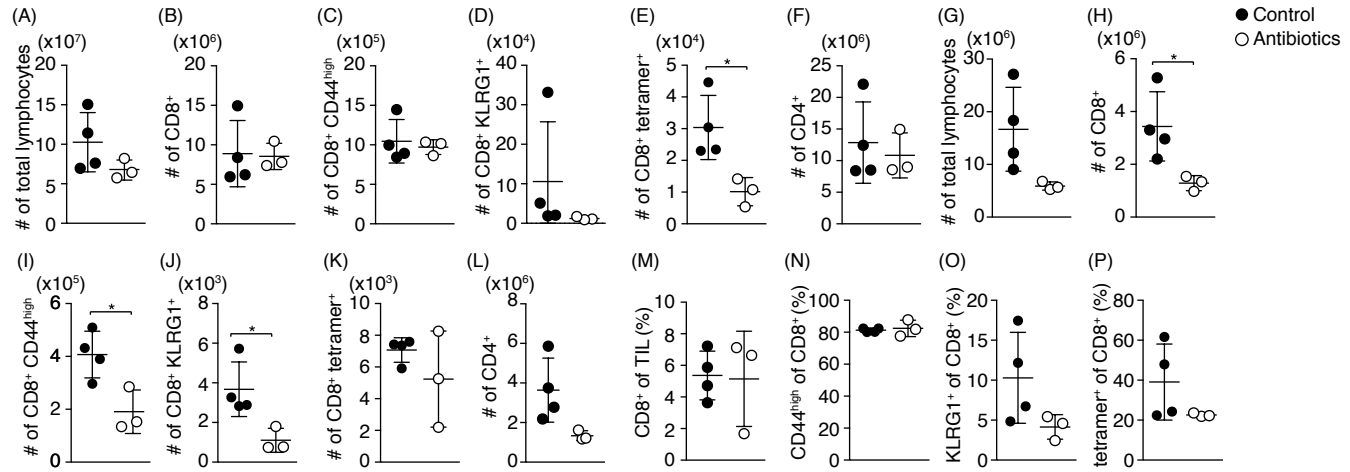

Figure 2. The gut microbiota influences local and systemic activation of CD8⁺ T cells.
CD44- and KLRG1-expressing CD8⁺ T cells and gp33 MHC tetramer-reactive CD8⁺ T cells in spleen, inguinal lymph node (ILN) and tumor tissues of tumor-bearing mice at 4 weeks after cell inoculation were analyzed flow cytometry. (A-F) The number of total lymphocytes (A), total CD8⁺ T cells (B), CD44^{high} (C), KLRG1⁺ (D), tetramer⁺ CD8⁺ T cells (E) and total CD4⁺ T cells in spleen. (G-L) The number of total lymphocytes (G), total CD8⁺ T cells (H), CD44^{high} (I), KLRG1⁺ (J), tetramer⁺ CD8⁺ T cells (K) and total CD4⁺ T cells (L) in ILN. (M-P) The frequency of total CD8⁺ T cells in tumor-infiltrating lymphocytes (M), CD44^{high} (N), KLRG1⁺ (O) and tetramer⁺ CD8⁺ T cells (P) in tumor tissues (n=4 for control, n=3 for antibiotics). P-values from student's t-test: *p<0.05.

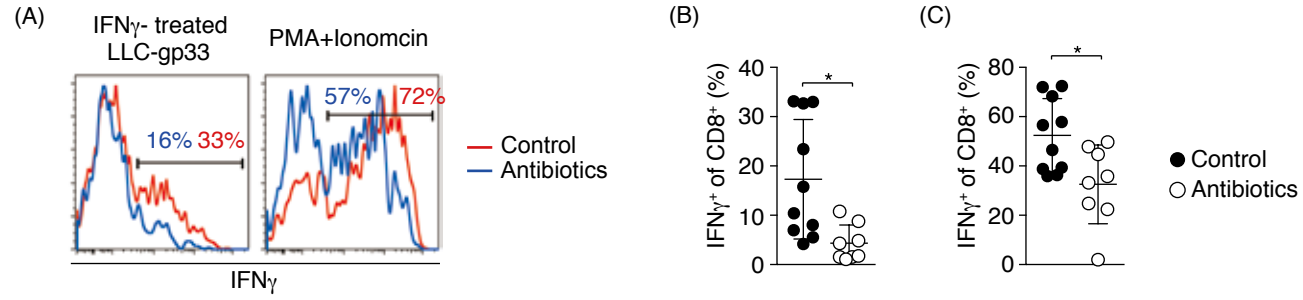

Figure 3. Depletion of the gut microbiota impairs IFNγ production by tumor-infiltrating CD8⁺ T cells.
Isolated tumor-infiltrating CD8⁺ T cells were stimulated with IFNγ-treated LLC-gp33 cells, or PMA and Ionomycin for 12h. The ability to produce IFNγ was analyzed by intracellular staining. (A) Representative histogram showing IFNγ⁺ CD8⁺ T cells. (B, C) Summary data for the frequency of IFNγ-producing CD8⁺ T cells stimulated with IFNγ-treated LLC-gp33 cells (B) and PMA+ Ionomycin (C). Data were pooled from three experiments (n=10 for control, n=8 for antibiotics). P-values from student's t-test: **p<0.01.

infiltrating CD8⁺ T cells to produce IFNγ. Consistent with the frequency of gp33-reactive cells, the tumor-infiltrating cells from mice receiving antibiotic water rarely had a significantly reduced IFNγ response when incubated with IFNγ-treated LLC-gp33 cells (Fig. 3A and 3B). The IFNγ response was similarly reduced in T cells from antibiotic-treated mice upon stimulation with PMA and ionomycin (Fig. 3A and 3C). These data indicate that the depletion of the gut flora induces CD8⁺ T cell hypo-responsiveness as well as a reduction in the number of tumor-reactive cells, consisted with the enhanced tumor growth and diminished survival of antibiotic-treated mice.

Discussion

Recently, it was reported that gut microbiota enhances the anti-tumor immune response induced by the blockade of immune checkpoint molecules[14,15,16]. But, how the gut microbiota enhances anti-tumor immune response, especially CTL activity, remains unclear. Here, we showed that depletion of the gut microbiota led to increased tumor growth and a reduction in the number of tumor-antigen specific CD8⁺ T cells in the spleen. In addition, tumor-infiltrating CD8⁺ T cells were impaired in their ability to produce IFNγ in the absence of the gut microbiota. Together, the gut microbiota might influence CD8⁺ T cell activation both systemically and locally, leading to the enhancement of anti-tumor immune response.

In this study, we demonstrated that depletion of the gut microbiota decreased the anti-tumor immune response. But the question remains, do most members of the gut microbiota enhance anti-tumor immune responses or only a few? Sivan et al. demonstrated that the anti-tumor immune response depended on the source of the mice, and thus was different between mice derived from Jackson Laboratory and Taconic Farms, and this difference was due to differences in the gut microbiota[14]. Also, recently, the transplantation of human feces to germ free mice showed that mice receiving a fecal transplant from a human responder to anti-PD1 immunotherapy had a significantly enhanced anti-tumor immune response, compared with mice receiving feces from a non-responder[15]. On the other hand, it is well-known that bacteria from the genus *Clostridium* strongly induce the development of regulatory T cells (Tregs) in the intestine, and it is thought that such Tregs influence immune tolerance systemically[19]. These reports indicate that there are several constituents of the gut microbiota that can contribute to either the activation or the suppression of systemic immune response. However, in our studies, the depletion of the gut microbiota reduced immune responses to a tumor. Therefore, it is possible that the majority of the gut microbiota might activate systemic immune response rather than promote immune tolerance.

CD8⁺ T cells infiltrate into tumor tissues to eliminate tumor cells. However, tumor cells and the tumor microenvironment gradually cause CTLs to become exhausted T cells, for example via PD1-PDL1 interaction. These exhausted T cells are hypo-responsive to TCR stimulation and impaired in cytokine production, and lose the capacity to kill tumor cells[12]. To break the exhausted state of CD8⁺ T cells, antibodies to PD1 and PDL1 are administrated into the patients with several types of tumors as immune checkpoint therapy. Recent reports showed a relationship between the therapeutic efficacy of antibodies to PD1/PDL1 and the gut microbiota, and suggested that particular members of the gut microbiota enhance anti-tumor immune responses via blockade of the PD1-PDL1 interaction[14,15,16]. However, the mechanism by which the gut microbiota enhances immune responses mediated by PD1 blockade is poorly understood. In this study, we observed that the ability of tumor infiltrating CD8⁺ T cells to produce IFNγ

was impaired in the absence of gut microbiota, suggesting the possibility that the gut microbiota suppresses the exhaustion of tumor infiltrating $CD8^+$ T cells. It is reported that $PD1^+$ $LAG3^+$ exhausted $CD8^+$ T cells are more dysfunctional than $PD1^+$ exhausted $CD8^+$ T cells[20]. Based on these observations, it is thought that the above identified species might suppress the terminal differentiation of PD1- and LAG3-coexpressing exhausted T cells, leading to good efficacy of immune checkpoint therapy. But, to properly address this issue, further experiments are required.

In this study, we have described that the depletion of the gut microbiota by orally administration of antibiotics reduces anti-tumor immune response. But, we have to consider the direct effect of antibiotics on immune cells, because it is reported that antibiotics broadly influence immune response such as the number of immune cell and cytokine production[21]. Here, we confirmed that the frequency of $CD8^+$ and $CD4^+$ in PBLs was not difference between control and antibiotics-treated group. However, we cannot exclude the possibility that antibiotics influence on cytokine production from these T cells, and the homeostasis and the function of other immune cells. Therefore, to clarify whether antibiotics directly influence on immune cells and suppress anti-tumor immune response, further experiments are required.

In conclusion, we demonstrated that gut flora activates local and systemic immune responses to tumor, leading to the inhibition of tumor growth. We believe that our observations will increase the understanding of anti-tumor immune response and immune evasion by tumor cells.

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