Microbiota Modulate Tumoral Immune Surveillance in Lung through a γδT17 Immune Cell-Dependent Mechanism

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Abstract

Commensal bacteria are crucial to maintain immune homeostasis in mucosal tissues and disturbances in their ecology can affect disease susceptibility. Here, we report evidence that commensal bacteria shape the efficiency of immune surveillance in mucosal tissues. Antibiotic-treated (Abt) mice were more susceptible to development of engrafted B16/F10 melanoma and Lewis lung carcinoma, exhibiting a shortened mean survival time with more numerous and larger tumor foci in the lungs. The defective antitumor response of Abt mice was independent of dehydration caused by antibiotics. Host defenses relied upon intact commensal bacteria with no class specificity. Mechanistic investigations revealed a defective induction of the γδT17 cell response in lungs of Abt mice; here, more aggressive tumor development was observed, possibly related to a reduction in IL6 and IL23 expression there. Adding normal γδT cells or supplementing IL17 restored the impaired immune surveillance phenotype in Abt mice. Overall, our results demonstrated the importance of commensal bacteria in supporting the host immune response against cancer, defined an important role for γδT17 responses in the mechanism, and suggested deleterious effects of antibiotic treatment on cancer susceptibility and progression. Cancer Res 74(15): 4030–41. ©2014 AACR.

Introduction

Commensal bacteria colonize many parts of the body, including the gastrointestinal tract, oral cavity, respiratory tract, urogenital tract, and skin. Previous research has demonstrated that the absence of commensal bacteria in germ-free mice caused developmental defects in the immune system, resulting in hypoplastic Peyer patches, relatively structureless spleen and lymph nodes, and hypogammaglobulinemic serum. These abnormalities in germ-free mice could be restored within several weeks by commensal bacteria colonization (1). Any disturbance in the composition of commensal bacteria may result in an imbalanced immune response and could affect susceptibility of an individual to various diseases, including inflammatory (inflammatory bowel disease, Crohn disease, and colon cancer), autoimmune (e.g., celiac disease, arthritis, and multiple sclerosis), allergic (e.g., asthma and atopy), metabolic (e.g., diabetes, obesity, metabolic syndrome, and kwashiorkor), and psychologic/neurologic (e.g., autism) diseases (2).

Recent data have shown that commensal bacterial provide immunomodulatory signals during the development of immune tissues and the activation of immune cells, which are important for the maintenance of immune homeostasis in mucosal tissues, such as the intestine, lungs and skin (3–7). Commensal bacteria play a key role in controlling adaptive immunity against respiratory tract influenza A virus infection. This role is associated with defective activation of the inflammasome (4). Furthermore, commensal bacteria have been shown to provide tonic signals that establish the activation threshold and sensitivity of the innate immune system required for optimal antiviral immunity after either systemic lymphocytic choriomeningitis virus, LCMV) or mucosal (influenza virus) infection (8). However, whether there is a role for commensal bacteria in shaping the immune surveillance of tumor cells in mucosal tissues, such as the lung, remains unclear.

γδT cells act as a bridge between the innate and adaptive immune systems. These cells are functionally specialized into two subsets: IFNγ-producing γδT cells (γδT1) and IL17-producing γδT cells (γδT17). γδT17 cells were first described in a Mycobacterium tuberculosis model of lung infection. In this model, IL17 was predominantly produced by γδT cells but not CD4+ T cells (9). As an important innate immune source of IL17, γδT17 cells are found in the epithelial linings of the lung, tongue, and genital tract, and in the liver and peritoneal cavity. γδT17 cells play a role in various diseases, including infectious, autoimmunity, and inflammatory disorders (10–13). Recently, an important role of γδT17 cells was demonstrated in chemotherapy-induced anticancer immune responses (14). γδT cells are speculated to differentiate early...
during the development of the immune system in response to environmental or inflammatory stimuli, γδT cells could be induced to produce high levels of IL17 after bacterial invasion by using their T-cell receptors (TCR) as pattern recognition receptors and several types of Toll-like receptors (TLR), such as TLR2, TLR1, and dectin-1 (15). The microbial colonization of germ-free mice drives the expansion of CD62L− and IL1R1+ γδT cells that are quickly activated by microbes to produce IL17. In addition, antibiotic treatment in specific pathogen-free (SPF) mice further demonstrated that specific commensal bacteria are required for the maintenance of IL1R1+ γδT cells (16). Dynamic and reciprocal cross-talk between the intestinal microbiota and γδT cells has been revealed, and this cross-talk demonstrates that commensal bacteria direct antibacterial and proinflammatory responses in γδ intestinal intraepithelial lymphocytes following mucosal injury. Furthermore, γδT cells limit the opportunistic penetration of commensal bacteria following mucosal injury (17).

In this study, we examined the role of commensal bacteria in the host immune surveillance system using an antibiotic-treated (Abt) mouse model. Abt mice are more susceptible to B16/F10 melanoma and Lewis lung carcinoma (LLC) development and exhibit a shortened mean survival time. The Abt mice had larger tumors and more tumor foci in their lungs. In the host, the antitumor defense required intact commensal bacteria with no class specificity. A defective γδT17 cell response was observed in the Abt mice, and the addition of normal γδT cells or IL17 supplementation was able to restore the impaired antitumor responses. Commensal bacteria were essential for the function of γδT17 cells in the lung, and the absence of these cells increased the susceptibility to tumor development.

Materials and Methods

Cell lines and cell culture

B16/F10 (mouse melanoma cell line) and LLC cells (LLC cell line) were obtained from the American Type Culture Collection and maintained in DMEM (Gibco BRL) with 10% heat-inactivated FBS (ExCell Biology).

Mice and antibiotics treatment

Four- to 5-week-old female C57BL/6 mice were obtained from the Shanghai Experimental Center of the Chinese Science Academy (Shanghai, China). All mice were maintained under SPF and controlled conditions (22°C, 55% humidity, and 12 hours day/night rhythm). The animal experiments were performed in compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. To generate the Abt experimental groups, the mice were treated for 4 to 6 weeks with ampicillin (1 g/L), vancomycin (0.5 g/L), neomycin sulfate (1 g/L), and metronidazole (1 g/L) in their drinking water as previously described (4, 8). The antibiotic water was changed twice a week. If the body weight of a treated mouse declined to 70% of that of the control mice, the mouse was removed. The antibiotic treatment continued for the entire experimental period.

Induction and assessment of B16/F10 lung melanoma and LLC

Mice were injected i.v. with 1 × 10⁷ B16/F10 cells. On day 17 after tumor introduction, the mice were euthanized, and the number of metastatic lung foci was counted. All lung lobes were evaluated. Lung tissue sections of 4 μm thickness were stained with hematoxylin and eosin using routine methods.

Bacterial diversity analysis

Stool samples were freshly collected and were stored at −80°C before use. DNA was extracted from 0.18 to 0.22 g of stool using a QIAamp DNA Stool Mini Kit (Qiagen). The DNA was recovered with 30 μL of AE buffer (10 mM Tris-cl, 0.5 mM EDTA, pH 9.0, Qiagen). The 16S ribosomal RNA (rRNA) gene was analyzed to evaluate the bacterial diversity by using Illumina MiSeq (Novogene Bioinformatics Technology Co., Ltd.).

Isolation of lymphocytes

Lymphocytes from the lung, liver, and spleen were isolated as previously described (18). The lymphocytes from the liver and lung were prepared by density gradient centrifugation with 40% and 70% Percoll.

Flow cytometry analysis

The following monoclonal antibodies were purchased from BD PharMingen and used in this study: FITC-conjugated anti-NK1.1 (clone PK136), PE (phycoerythrin)–CY7-conjugated anti-NK1.1 (clone PK136), PE-conjugated anti-IL17A (clone TC11-18H10), PerCP–CY5.5-conjugated anti-CD3ε (clone 145-2C11), PE-conjugated anti-CD8α (clone 53-67.6), allopheycoycin (APC)-conjugated anti-TCRβ (clone H57-597), APC–CY7-conjugated anti-CD4 (clone GK1.5), and PE–CY7-conjugated anti-IFNγ (clone XKMG1.2). FITC-conjugated anti-γδTCR (clone GL3), PE-conjugated anti-γδTCR (clone GL3), and APC-conjugated anti-γδTCR (clone GL3) were obtained from eBioscience. PE-conjugated antibiotin was obtained from BioLegend. For the surface phenotype assays, 1 × 10⁶ cells were blocked with 10 μL rat serum for 30 minutes at 4°C and then stained with the indicated antibody for 30 minutes at 4°C in the dark. For the intracellular cytokine assay, the cells were stimulated with phorbol 12-myristate 13-acetate (Sigma), monensin (Sigma), and ionomycin (Calbiochem) for 4 hours. The cells were labeled with surface markers, fixed, permeabilized, and then labeled with the indicated intracellular antibody for 30 minutes at 4°C in the dark. All data were acquired using a FACS-Verse flow cytometer (Becton-Dickinson) and analyzed using FlowJo 7.6.1 software.

RNA extraction and cDNA synthesis

Total cellular RNA was extracted using the TRIzol reagent (Invitrogen). mRNA was reverse transcribed into cDNA in a
40 μL reaction volume containing the following reagents: 4 μg of total mRNA, 5 μmol/L oligo(dT), 0.5 mmol/L dNTPs, 8 μL of 5 × buffer, 10 mmol/L DTT, 56 U of RNase inhibitor, 400 U of M-MLV, and distilled water (ultrapure, DNase and RNase free). The reverse transcription reaction was performed at 37°C for 50 minutes, followed by heating at 70°C for 15 minutes.

Real-time PCR

The standard reaction is described in the Supplementary Materials and Methods. The gene-expression levels were quantified by the ΔΔCt method.

Adoptive transfer of γδT cells

γδT cells were isolated and purified from the lungs of normal 9- to 10-week-old female C57BL/6 mice using the Mouse TCRγδ+ T Cell Isolation Kit (130-092-125; Miltenyi Biotec). γδT cells (1.5 × 10^6 cells/mouse in 250 μL PBS) were adoptively transferred i.v. into the Abt mice. The γδT cells were transferred 1 day before the B16/F10 challenge, and additional transfers were performed every week. The same volume of PBS alone was used as the control.

IL17A treatment

Recombinant murine IL17A (catalog 210-17; Peprotech) was injected i.v. into the Abt mice (0.5 μg/mouse in 250 μL of PBS). IL17A was injected 1 day before the B16/F10 challenge, and additional injections were performed every 3 days. The same volume of PBS alone was used as the control.

Statistical analysis

All data are shown as the mean ± SEM. The differences between the individual data were analyzed using the Student t test or one-way ANOVA when appropriate. Least significant difference tests (0<α<1) were used for the post hoc tests. Mouse survival was analyzed using the Kaplan–Meier method. A P value of <0.05 was considered statistically significant.

Results

Enhanced lung tumor induction in the Abt mice

To determine whether commensal microbiota might influence the defense of the host against tumors, mice were treated with ampicillin, vancomycin, neomycin sulfate, and metronidazole (V/N/M/A) to deplete their microbiota as previously reported (4, 8). As shown in Fig. 1A, the Abt mice were more susceptible to B16/F10 melanoma development and had a shortened mean survival time. The survival of the control mice was 29.70 days compared with 24.10 days for the Abt mice after the B16/F10 melanoma challenge. On day 17 after the challenge, the Abt mice exhibited larger tumors and an increased number of tumor foci in the lungs compared with the control mice as assessed by morphologic and histopathologic examinations (Fig. 1B–D). The enhanced susceptibility to tumor induction in the lungs caused by antibiotics treatment was also observed using another lung tumor model with mouse LLC. The mean survival time was reduced from 36.90 days for the control mice to 26.50 days for the Abt mice after the LLC challenge (Fig. 1E). On day 21 after the challenge, the Abt mice exhibited larger tumors and an increased number of tumor foci in the lungs (Fig. 1F–H).

The antibiotic treatment induced weight loss early during treatment, which raised the concern that dehydration may affect the ability of the mice to resist tumor growth. However, tumor development did not change after we coadministered sucralose in the antibiotic water. This result suggested that the defective antitumor effect in the Abt mice was independent of dehydration (data not shown).

Antitumor defense requires intact commensal bacteria with no class specificity

To clearly demonstrate which class of bacteria is responsible for the observed effect, we treated the mice with individual antibiotics. Remarkably, the individual treatments with ampicillin, vancomycin, neomycin sulfate or metronidazole recapitulated the effects of the combination antibiotics treatment on the body weight and susceptibility to tumor development (Fig. 2A and B). These results indicate that the commensal bacteria required for the host antitumor defense are not restricted to one type of bacteria. To explore whether the defective antitumor response in the Abt mice was associated with the overgrowth of antibiotic-resistant bacteria induced by prolonged antibiotics treatment, the abundance and composition of the bacteria in the stool of these mice were evaluated by microbiologic analyses. Antibiotics treatment resulted in a significant decrease in the aerobic bacterial stool load compared with the water control group (Fig. 2C). Although more than 75% of bacteria were depleted after the antibiotics treatment, we considered the possibility that antibiotic-resistant strain could appear. Thus, we used 16S rRNA assays to analyze the bacteria composition after antibiotics treatment. As shown in Fig. 2D and E, antibiotics treatment caused a change in the overall composition, and led to a low frequency of Firmicutes and Bacteroidetes and a high frequency of Proteobacteria in the Abt mice. However, we did not observe any new strains that were antibiotic resistant. These results indicated that the enhanced tumor induction in the Abt mice was not associated with the appearance of antibiotic-resistant bacteria and was instead related to the presence of symbiotic bacteria with no class specificity.

Defective γδT cell responses in the lungs of the Abt mice

Antibiotics treatment should not result in general immunodeficiency, and the mice showed normal peripheral immune responses to ovalbumin and to respiratory infections with HSV-2 or Legionella pneumophila (4). After 5 weeks of antibiotics treatment, the number of total lymphocytes significantly decreased in the lungs and liver, but not in the spleen of the Abt mice. After the challenge with B16/F10 melanoma cells or LLC cells, the number of total lymphocytes in the lungs of the Abt mice was still significantly less than in the control mice. However, the number of lymphocytes in the livers and spleens was not significantly different (Fig. 3A). The lymphocyte subsets were analyzed by flow cytometry. In the Abt group, only the percentage and number of γδT cells in the lungs were significantly lower than the control mice in both the B16/F10 melanoma and LLC models. Furthermore, there was a decrease...
in the number of natural killer (NK) T cells and CD8+ T cells in the lungs (Fig. 3B and C). In addition, a decrease in γδT cells was observed in the livers of the Abt mice after the B16/F10 melanoma challenge (Fig. 3B and C). These results indicated that commensal microbiota profoundly affected the γδT cell frequency in the presence of tumor cells.

The phenotypes and functions of lung γδT cells were analyzed. As shown in Fig. 4, γδT cells in the lungs of the Abt mice expressed lower levels of CD44 and CD127 molecules compared with the control mice. This result indicates that the cells have inhibited activation after challenge with B16/F10 melanoma cells. We also examined the cytokine production of lung γδT cells. Antibiotics treatment resulted in the impaired production of IL17A+ γδT cells in the lung, which were present in lower percentages and fewer numbers. However, the IFNγ+ γδT cells in the lung were not affected in a similar manner (Fig. 5A–C). There were no alterations in the IL17A+ γδT cells in the lungs when the Abt mice were challenged with B16/F10 melanoma cells or LLC cells. Conversely, there were also more IL17A+ γδT cells in the lungs of the control mice (Fig. 5A–C). As shown in Fig. 5, the γδT17 cell response to tumor cells was only found in the lung and did not occur in the spleen. The percentage of IL17A+ γδT cells in the spleen was decreased by the antibiotics treatment. There was no significant induction of Th17 cells after the B16/F10 melanoma and LLC challenges (Supplementary Fig. S1). In addition, more IFNγ+ γδT cells accumulated in the lungs of the control mice after the B16/F10 melanoma and LLC challenges; however, there was
no accumulation in the Abt mice (Fig. 5A–C). There was also no difference in the production of IFN-γ from NK cells observed in Abt mice (Supplementary Fig. S2). The expression of IL17A in the gd T cells was enhanced by cytokines, such as TGFβ, IL1β, IL6 and IL23. Our data indicated that the mRNA levels of IL6 and IL23 but not those of TGFβ and IL1β increased in the lungs in tumor-bearing mice. However, the expression did not increase in the spleen after tumor induction, and the mRNA expression levels significantly decreased in Abt mice with tumors (Fig. 6). These results suggested that the reduced IL6 and IL23 expression levels might be involved in the reduced IL17A expression by gd T cells after antibiotics treatment.

**gd T cells and IL17 restore the antitumor immune response in the Abt mice**

The function of γδ T cells in the lung was impaired after antibiotics treatment, particularly the function of the γδT17 cell subset. This dysfunction might be related to the susceptibility to tumor development in the lungs of these mice. To examine this hypothesis, γδ T cells and IL17A rescue experiments were performed. We purified lung γδ T cells from normal mice and adoptively transferred them to the Abt mice with B16/F10 tumors as described in Fig. 7A and B. Remarkably, normal γδ T cells transferred into the Abt mice led to smaller tumors and a decreased number of tumor foci in the lungs on day 17 after the challenge with B16/F10 melanoma cells (Fig. 7C). In addition, these mice had a longer mean survival time (from 24.11 to 29.89 days; Fig. 7D). This result was similar to the PBS-treated water-drinking control mice. These data indicated that γδ T cells were sufficient to restore the immune responses to the B16/F10 melanoma cells in the Abt mice.

The functional cytokine IL17A was reduced in the lung γδ T cells of Abt mice. Thus, we tested whether it exerted effects similar to the normal γδ T cell transfer as described in...
in Fig. 7A. We observed that IL17A supplementation also significantly restored the antitumor responses and resulted in smaller tumors and a decreased number of tumor foci in the lungs on day 17 after the challenge (Fig. 7C). The mice also had a longer mean survival time (from 24.11 to 31.89 days; Fig. 7D). These results indicated that the alteration in the microbiota caused by antibiotics led to the failure of the γδT cell response and the impaired IL17 release. These data explain the susceptibility of these mice to B16/F10 melanoma development in the lung.
Discussion

In this study, we used two mouse tumor models, including metastatic B16 melanoma and LLC, to reveal that commensal microbiota could regulate the host immune surveillance of tumor cells in the lung. Oral antibiotics treatment led to defective γδT17 cell immunity, which resulted in the susceptibility of the mice to B16/F10 melanoma and LLC development with larger tumors and an increased number of tumor foci in the lungs. As a result, these mice had a shortened mean survival time. We found that supplementation with normal γδT cells or IL17A could restore the antitumor immune responses of the Abt mice.

γδT cells have been shown to acquire the capacity to produce IL17 in the embryonic thymus, and these cells are subsequently disseminated to peripheral organs, including the lymph nodes, spleen, liver, and lung, as self-renewing, long-lived cells (19). The mechanism of the commensal microbiota regulation of the γδT cells in the lung is an important issue. In

![Figure 4](image-url)

Figure 4. Activation of γδT cells was inhibited in the lungs of Abt mice after the B16/F10 melanoma challenge. The mice were given antibiotics for 5 weeks and then challenged with B16/F10 melanoma cells (1 × 10^5 cells/mouse, i.v.) or not. On day 17 after the B16/F10 melanoma challenge, the lymphocytes were isolated from the lung, liver, and spleen and analyzed by FACS. A, the percentages of each phenotypic marker on γδT cells (CD3^+ γδTCR^+) are shown by the representative dot plots. B, the percentages of each phenotypic marker on the γδT cells were statistically analyzed (n = 6). The data, the mean ± SEM; *, P < 0.05 compared with the control group.
Figure 5. Impaired production of IL17A by lung γδT cells in Abt mice. The mice were given antibiotics for 5 weeks and then challenged with B16/F10 melanoma cells (1 × 10^5 cells/mouse, i.v.) or with LLC cells (2 × 10^6 cells/mouse, i.v.). On days 17 and 21 after the B16/F10 melanoma and LLC challenges, the γδT cells in the lung and spleen were analyzed by FACS to determine their cytokine production. A and B, the CD3⁺ γδTCR⁺ cells were gated, and the percentages of IL17A⁺ γδT cells and IFNγ⁺ γδT cells are shown. C, the number of IL17A⁺ γδT cells and IFNγ⁺ γδT cells in the lung and spleen are shown. There were 6 mice in each group. The data, the mean ± SEM; *, P < 0.05 compared with the control group.
the Abt mice, the total number of γδT cells in the lung was not significantly decreased, but the production of IL17A was markedly impaired (Fig. 3C and Fig. 5). Recent research has indicated that PAMP-activated dendritic cell (DC)–produced IL1β and IL23 could subsequently induce innate IL17 production from peripheral γδT cells in the absence of TCR activation (10). In this study, an enhanced expression of IL6 and IL23 in the lung was observed after the tumor cell challenge. However, their expression was reduced in the Abt mice. The data suggested that IL6 and IL23 might account for the impaired IL17A+ γδT cells during tumor development (Fig. 6). In the lung, intact microbiota provided the signals that led to the expression of pro-II1β and II18. As a result, inflammasome-dependent cytokine activation led to the migration of DCs from the lung to the draining lymph node, and also caused T-cell priming. The impairment of γδT17 cells may have been related to DC function in the Abt mice, a hypothesis that requires further investigation.

TLR ligand stimulation could rescue the immune impairment in the Abt mice (4). However, γδT cells express several types of TLRs, such as TLR2, TLR1, and dectin-1, and their TCRs as pattern recognition receptors (15). Crucial roles for γδT17 cells have been demonstrated in response to bacterial infections, such as Staphylococcus aureus infection of the skin, S. enterica infection in the lung, Listeria monocytogenes infection in the liver, and intraperitoneal infection with Escherichia coli (20–23). Microbiota have been demonstrated to regulate the immune defense against respiratory tract influenza A virus infection. However, not all commensal bacteria contribute equally to the immunocompetence of the lung. Only neomycin-sensitive bacteria were associated with the induction of the productive immune responses in the lung (4). We found that treatment with ampicillin, vancomycin, neomycin sulfate, or metronidazole alone recapitulated the effects of the combination antibiotics treatment on the susceptibility to tumor development. These results demonstrated that intact commensal bacteria are required for the host antitumor defense (Fig. 2A and B). An important step will be defining the commensal bacterial species and signals responsible for conferring an immunogenic environment in the lung. The antibiotics treatment resulted in a significant decrease of total bacteria, and no new resistant strain appeared (Fig. 2C–E), which indicated that lung γδT17 maintenance was dependent on normal commensal bacterial. The potential direct interaction between the commensal bacteria and γδT17 cells deserves further investigation.

γδT cells play important roles in the establishment of the tumor microenvironment and the development of tumor immunity. Those roles are currently being explored as a target for tumor immunotherapy. Conversely, γδT17 cells generated in response to the tumor microenvironment act as tumor-promoting cells by inducing angiogenesis (24). Consistently, IL17-producing cells have been shown to facilitate cancer development by fostering angiogenesis and promoting VEGF production in cancer cells (25). In addition, IL17 is produced by γδT cells and enhances metastasis. These differences led to a reduced survival time in the LLC model due to the reduced ability of antigen-presenting cells to promote Th1 immunity (26). However, a beneficial role for γδT17 cells in the inhibition of tumor growth has also been demonstrated. In a mouse model of bladder cancer, γδT17 cells played a key role in the BCG-induced recruitment of neutrophils into the tumor and the reduction of tumor growth (27). γδT17 cells also contributed to the efficacy of anticancer chemotherapy and are associated with the suppression of tumor growth (14, 28). In this study, we demonstrated that the defective γδT17 cells in the Abt mice resulted in a susceptibility to B16/F10 melanoma and LLC development in the lung (Figs. 5 and 7). Consistent with other reports, IL1 induced IL17 through the recruitment and activation of γδT cells in the microenvironment of lung tumors. This activation resulted in increased IL17 production, with no involvement of Th17 cells (Supplementary Fig. S1; ref. 26). As described above, the role of γδT cells in tumor immunity was related to other immune cells such as neutrophils, APCs, CD4+ T cells, and CD8+ T cells. In this study, we observed that CD8+ T cells were significantly inhibited in the lung of Abt mice after the B16/F10 melanoma and LLC challenges (Fig. 3C). Further studies are needed to clarify the mechanism by which γδT17 cells affect tumor development and define their interactions with other immune cells.

In addition to γδT cells, commensal bacteria shape host immunity by interacting with epithelial barriers and stimulating IgA-secreting plasma cells, DCs, macrophages, regulatory T cells (Treg), NKT cells, NK cells, and innate lymphoid cells (2). In this study, we analyzed the percentages and numbers of lymphocyte subsets in the Abt mice compared with those in the
control mice. The antibiotics treatment led to a decrease in the number of CD4+T cells in the lung (Fig. 3C); of the Abt mice, lung NKT cells were significantly inhibited after the B16/F10 melanoma challenge (Fig. 3C). In the lung and mediastinal lymph nodes, there were higher percentages of Foxp3+CD4+T cells detected in the Abt mice (4). Whether the antibiotic-
mediated impairment of antitumor immunity was related to the higher number of Tregs or lower number of NKT cells requires further investigation.

NK cells are quite interesting in our experimental model, because NK cells were reported to control the lung foci after the B16/F10 cell injection. The antiviral immune response toward endogenous retrovirus rearrangements in B16 is very important. A previous report showed that, although commensal bacteria are not required for the development, differentiation, or maturation of splenic NK cells, NK cells residing in non-mucosal lymphoid organs of germ-free mice could not be primed to mount an effective antiviral immunity. This outcome is not an NK cell–intrinsic defect but rather a priming element by mononuclear phagocytes (29). This result is consistent with the results of our study, in which no significant difference in the percentage and number of NK cells in the lungs, liver, and spleen between the Abt and control mice was observed (Fig. 3B and C). Furthermore, the production of IFNγ from NK cells was not affected by the antibiotics treatment (Supplementary Fig. S2). After the B16/F10 melanoma cell or NK cells was not affected by the antibiotics treatment (Supplementary Fig. S2). After the B16/F10 melanoma cell or the LLC cell challenge, there were no marked responses of NK cells observed in the percentage and number of NK cells in the lungs, liver, and spleen. In addition, the production of IFNγ by NK cells in the lungs and spleen was not different (Fig. 3B and C and Supplementary Fig. S2). We speculate that NK cells might not be the critical responder during tumor development in this study.

In summary, our findings substantially expanded the contribution of commensal bacteria in maintaining host immune homeostasis in these mucosal tissues beyond their roles in infection and inflammation. Our results indicate a possible deleterious effect of antibiotics treatment in the initiation of antitumor immune responses.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: M. Cheng, G. Shen, S. Hu
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Cheng, L. Qian, G. Shen, G. Bian, T. Xu, G. Shen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M. Cheng, L. Qian, W. Xu, S. Hu
Writing, review, and/or revision of the manuscript: M. Cheng
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W. Xu, S. Hu
Study supervision: S. Hu

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