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 impact 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 anti-tumor 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 IL-6 and IL-23 expression there. Adding normal γδT cells or supplementing IL-17 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.
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’s 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 an individual’s susceptibility to various diseases, including inflammatory (inflammatory bowel disease, Crohn’s 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 psychological/neurological (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: IFN-γ-producing γδT cells (γδT1) and IL-17-producing γδT cells (γδT17). γδT17 cells were first described in a Mycobacterium tuberculosis model of lung infection. In this model, IL-17 was predominantly produced by γδT cells but not CD4+ T cells (9).
As an important innate immune source of IL-17, 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, autoimmune, 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 IL-17 after bacterial invasion by using their T cell receptors as pattern recognition receptors and several types of Toll like receptors (TLRs), such as TLR2, TLR1, and dectin-1 (15). The microbial colonization of germ-free mice drives the expansion of CD62L- and IL-1R1+ γδT cells that are quickly activated by microbes to produce IL-17. Additionally antibiotic treatment in specific-pathogen-free (SPF) mice further demonstrated that specific commensal bacteria are required for the maintenance of IL-1R1+ 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 (γδ IELs) 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 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 anti-tumor 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 IL-17 supplementation was able to restore the impaired anti-tumor 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 cells (mouse melanoma cell line) and LLC cells (Lewis lung carcinoma cell line) were obtained from the American Type Culture Collection (ATCC, USA) and maintained in DMEM (Gibco BRL, USA) with 10% heat-inactivated fetal bovine serum (FBS) (ExCell Biology, Shanghai, China).

Mice and antibiotics treatment

Four- to five-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 h 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, 18). 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 Lewis lung carcinoma

Mice were injected intravenously (i.v.) with $1 \times 10^5$ 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 4μm thickness were stained with hematoxylin and eosin using routine methods. The sections were photographed using Olympus IX73 microscope. The same volume of phosphate-buffered saline (PBS) alone (250 μl) was used as a control. The Abt
mice challenged with B16/F10 cells were observed and compared with the controls. Additionally, the mice were injected i.v. with $2 \times 10^6$ LLC cells, and tumor assessment was performed as described above on day 21 after the challenge.

**Bacterial diversity analysis**

Stool samples were freshly collected and were stored at -80 °C before use. DNA was extracted from 0.18-0.22 g of stool using a QIAamp DNA Stool Mini Kit (Qiagen). The DNA was recovered with 30 µL of AE buffer (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 (19). 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 (San Diego, CA, USA) and used in this study: FITC-conjugated anti-NK1.1 (clone PK136), PE-CY7-conjugated anti-NK1.1 (clone PK136), PE-conjugated anti-IL17A (clone TC11-18H10), PerCP-CY5.5-conjugated anti-CD3e (clone 145-2C11), PE-conjugated anti-CD8α (clone 53-6.7), APC-conjugated anti-TCRβ (clone H57-597), APC-CY7-conjugated anti-CD4 (clone GK1.5), and PE-CY7-conjugated anti-IFN-γ (clone XMG1.2). FITC-conjugated anti-γδTCR (clone GL3), PE-conjugated anti-γδTCR (clone GL3), and APC-conjugated anti-γδTCR (clone GL3) were obtained from eBioscience (San Diego, CA, USA). PE-conjugated anti-biotin was obtained from Biolegend (San Diego, CA). For the surface phenotype assays, $1 \times 10^6$ cells were blocked with 10 µl
rat serum for 30 min at 4°C and then stained with the indicated antibody for 30 min at 4°C in the dark. For the intracellular cytokine assay, the cells were stimulated with PMA (Sigma, St Louis, MO), monensin (Sigma, St Louis, MO) and ionomycin (Calbiochem, San Diego, CA, USA) for 4 h. The cells were labeled with surface markers, fixed, permeabilized, and then labeled with the indicated intracellular antibody for 30 min at 4°C in the dark. All data were acquired using a FACS-Verse flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) 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 oligdT, 0.5 mmol/L dNTPs, 8 μl of 5× buffer, 10 mmol/L DTT, 56 units of RNase inhibitor, 400 units of M-MLV and distilled water (ultrapure, DNase and RNase free). The RT 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 supplemental materials and methods. The gene expression levels were quantified by 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, Bergisch Gladbach, Germany). γδT cells (1.5×10⁵ cells/mouse in 250 μl PBS) were adoptively transferred intravenously (i.v.) into the Abt mice. The γδT cells were transferred one day before the B16/F10 challenge, and additional transfers were performed every week. The same volume of PBS alone was used as the control.
**IL-17A treatment**

Recombinant murine IL-17A (catalog 210-17, Peprotech) was injected i.v. into the Abt mice (0.5 μg/mouse in 250 μl of PBS). IL-17A was injected one day before B16/F10 challenge, and additional injections were performed every three 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 Student’s t-test or analysis of variance (one-way ANOVA) when appropriate. Least significant difference tests (LSD, 0<α<1) were used for the post hoc tests. Mouse survival was analyzed using the Kaplan-Meier method. A P-value < 0.05 was considered statistically significant.

**Results**

1. Enhanced lung tumor induction in antibiotic-treated mice.

To determine whether commensal microbiota might influence the host’s defense against tumors, mice were treated with ampicillin, vancomycin, neomycin sulfate, and metronidazole (V/N/M/A) to deplete their microbiota as previously reported (4, 18). As shown in Figure 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 lung compared with the control mice as assessed by morphological and histopathological examinations (Figure 1B, C and D). The enhanced susceptibility to tumor induction in the lungs caused by antibiotics treatment was also observed using another lung tumor model with mouse Lewis lung carcinoma (LLC). The mean survival time was reduced from 36.90 days for the control mice to 26.50 days for the Abt mice after LLC challenge (Figure 1E). On day 21 after the challenge, the Abt mice exhibited larger tumors and an
increased number of tumor foci in the lungs (Figure 1F, G and 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, the tumor development did not change after we co-administrated sucralose in the antibiotic water. This result suggested that the defective antitumor effect in the Abt mice were independent of dehydration (data not shown).

2. Anti-tumor defense requires intact commensal bacteria with no class specificity.

To clearly demonstrate which class of bacterial 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 (Figure 2A and 2B). These results indicate that the commensal bacteria required for the host anti-tumor defense are not restricted to one type of bacteria. To explore whether the defective anti-tumor response in the Abt mice was associated with the overgrowth of antibiotics resistant bacteria induced by prolonged antibiotics treatment, the abundance and composition of the bacteria in the stool of these mice were evaluated by microbiological analyses. Antibiotics treatment resulted in a significant decrease in the aerobic bacterial stool load compared with the water control group (Figure 2C). Although more than 75% of bacteria were depleted after the antibiotics treatment, we considered the possibility that antibiotics resistant strain could appear. Thus, we used 16S rRNA assays to analyze the bacteria composition after antibiotics treatment. As shown in Figure 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 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.
3. Defective γδT17 cell responses in the lungs of antibiotic-treated mice

Antibiotics treatment should not result in general immunodeficiency, and the mice showed normal peripheral immune responses to OVA and to respiratory infections with HSV-2 or *Legionella pneumophila* (4). After five 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 (Figure 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 NKT cells and CD8+ T cells in the lungs (Figure 3B and C). Additionally, a decrease in γδT cells was observed in the livers of Abt mice after B16/F10 melanoma challenge (Figure 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 Figure 4, γδT cells in the lungs of Abt mice expressed lower levels of CD44 and CD127 molecules compared with the control mice. This result indicates 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 IL-17A+ γδT cells in the lung, which were present in lower percentages and fewer numbers. However, the IFN-γ+ γδT cells in the lung were not affected in a similar manner (Figure 5 A, B, and C). There were no alterations in the IL-17A+ γδT cells in the lungs when the Abt mice were challenged with B16/F10 melanoma cells or LLC cells. Conversely, there were also more IL-17A+ γδT cells in the lungs of the control mice (Figure 5 A, B, and C). As shown in Figure 5, the γδT17 cell response to tumor cells was only found in the lung and did not occur in the spleen. The percentage of IL-17A+ 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 (Figure S1). Additionally, more IFN-γ+ 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 (Figure 5 A, B, and C). There was also no difference in the production of IFN-γ from NK cells observed in Abt mice (Figure S2). The expression of IL-17A in the γδT cells was enhanced by cytokines, such as TGF-β, IL-1β, IL-6 and IL-23. Our data indicated that the mRNA levels of IL-6 and IL-23 but not those of TGF-β and IL-1β 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 (Figure 6). These results suggested that the reduced IL-6 and IL-23 expression levels might be involved in the reduced IL-17A expression by γδT cells after antibiotics treatment.

4. γδT cells and IL-17 restore the anti-tumor immune response in antibiotic-treated mice

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

The functional cytokine IL-17A was reduced in the lung γδT cells of Abt mice. Thus, we tested whether it exerted effects similar to the normal γδT cell transfer as described in Figure 7A. We
observed that IL-17A supplementation also significantly restored the anti-tumor responses and resulted in smaller tumors and a decreased number of tumor foci in the lungs on day 17 after the challenge (Figure 7C). The mice also had a longer mean survival time (from 24.11 day to 31.89 day) (Figure 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 IL-17 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 Lewis lung carcinoma, 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 IL-17A could restore the anti-tumor immune responses of the Abt mice.

γδT cells have been shown to acquire the capacity to produce IL-17 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 (20). The mechanism of the commensal microbiota regulation of the γδT cells in the lung is an important issue. In the Abt mice, the total number of γδT cells in the lung was not significantly decreased, but the production of IL-17A was markedly impaired (Figure 3C and Figure 5). Recent research has indicated that PAMP-activated DC-produced IL-1β and IL-23 could subsequently induce innate IL-17 production from peripheral γδT cells in the absence of TCR activation (10). In this study, an enhanced expression of IL-6 and IL-23 in the lung was observed after tumor cell challenge. However, their expression was reduced in the Abt mice. The data suggested that IL-6 and IL-23 might account for the impaired IL-17A+ γδT cells during tumor development (Figure 6). In the lung, intact microbiota provided the signals
that led to the expression of pro-IL-1β and IL-18. 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 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* (21-24). 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 anti-tumor defense (Figure 2 A 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 (Figure 2 C, D and 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 acted as tumor-promoting cells by inducing angiogenesis (25). Consistently, IL-17 producing cells...
have been shown to facilitate cancer development by fostering angiogenesis and promoting VEGF production in cancer cells (26). Additionally, IL-17 is produced by γδT cells and enhances metastasis. These differences led to a reduced survival time in the Lewis lung carcinoma model due to the reduced ability of antigen-presenting cells to promote Th1 immunity (27). 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 (28). γδT17 cells also contributed to the efficacy of anticancer chemotherapy and are associated with the suppression of tumor growth (14, 29). 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 (Figure 5 and 7). Consistent with other reports, IL-1 induced IL-17 through the recruitment and activation of γδT cells in the microenvironment of lung tumors. This activation resulted in increased IL-17 production, with no involvement of Th17 cells (27) (Figure S1). 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 (Figure 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, Tregs, NKT cells, NK cells, and innate lymphoid cells (ILCs) (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 (Figure 3C); of the Abt mice, lung NKT cells were significantly inhibited after the B16/F10 melanoma challenge (Figure 3C). In the lung and mediastinal lymph nodes, there were higher percentages of Foxp3+CD4+ T cells detected in the antibiotic-treated mice (4). Whether the antibiotic-mediated impairment of
anti-tumor 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 nonmucosal 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 (30). 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 (Figure 3B and C). Furthermore, the production of IFN-\(\gamma\) from NK cells was not affected by the antibiotics treatment (Figure S2). After the B16/F10 melanoma cell or 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. Additionally, the production of IFN-\(\gamma\) by NK cells in the lungs and spleen were not different (Figure 3B and C, Figure 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 anti-tumor immune responses.
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Figure Legends

Figure 1. Abt mice were more susceptible to tumor development in the lungs. C57BL/6 mice in the Abt group were given 1 g/L ampicillin, 0.5 g/L vancomycin, 1 g/L neomycin sulfate, and 1 g/L metronidazole in their drinking water. The mice were given antibiotics for five weeks or not and then challenged with B16/F10 cells (1×10^5 cells/mouse, i.v.) or with LLC cells (2×10^6 cells/mouse, i.v.). (A&E) The survival times of the mice. The mice were treated with antibiotics for the entire experimental period. (B-D) On day 17 after the B16/F10 challenge, the lungs were analyzed. The graphs show the total number of tumor colonies present in the lung lobes, and the number of tumor foci in the lung was calculated. Lung samples were collected for hematoxylin and eosin staining. (F-H) On day 21 after the LLC challenge, the lungs were analyzed as described above. The data are shown as the mean ± SEM. *P<0.05 compared with the control group.

Figure 2. Intact commensal bacterial played a key role in anti-tumor defense. Mice were given single or two to four combinatorial antibiotics in their drinking water. (A) Body weight was measured. (B) After five weeks of treatment, the mice were challenged with B16/F10 cells (1×10^5 cells/mouse, i.v.). The survival times of the mice are shown. The mice were treated with antibiotics for the entire experimental period. (C) On day 17 after the B16/F10 challenge, the bacteria load in the stool from the Abt mice (n=3 per condition) was measured by blood agar plate culture. (D&E) The relative abundance and clustering map for the bacteria in the stool was analyzed by 16S rRNA analysis. *P<0.05 compared with the control group.

Figure 3. Percentage and number of γδT cells were lower in the lungs of the Abt mice after B16/F10 melanoma and LLC challenge. The mice were given antibiotics for five weeks and then challenged with B16/F10 cells (1×10^5 cells/mouse, i.v.) or with LLC cells (2×10^6 cells/mouse, i.v.). (A) Total number of lymphocytes in the lung, liver, and spleen. On day 17 after the B16/F10 challenge or day 21 after the LLC challenge, the lymphocytes were isolated (n=6). (B&C) The
isolated lymphocytes were analyzed by FACS. The percentage of each lymphocyte subset in the lung, liver, and spleen are shown (n=6). The absolute number of each lymphocyte subset was calculated (n=6). The data are shown as the mean ± SEM. *P<0.05 compared with the control group.

**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 five weeks and then challenged with B16/F10 melanoma cells (1×10⁵ 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 are shown as the mean ± SEM. *P<0.05 compared with the control group.

**Figure 5. Impaired production of IL-17A by lung γδT cells in Abt mice.** The mice were given antibiotics for five weeks and then challenged with B16/F10 melanoma cells (1×10⁵ cells/mouse, i.v.) or with LLC cells (2×10⁶ cells/mouse, i.v.). On days 17 and 21 after the B16/F10 melanoma and LLC challenge, the γδT cells in the lung and spleen were analyzed by FACS to determine their cytokine production. (A&B) The CD3+ γδTCR+ cells were gated, and the percentages of IL-17A+ γδT cells and IFN-γ+ γδT cells are shown. (C) The number of IL-17A+ γδT cells and IFN-γ+ γδT cells in the lung and spleen are shown. There were six mice in each group. The data are shown as the mean ± SEM. *P<0.05 compared with the control group.

**Figure 6. Lower expression levels of IL-6 and IL-23 in the lungs of the Abt mice after the tumor cell challenge.** The mice were given antibiotics for five weeks and then challenged with B16/F10 melanoma cells (1×10⁵ cells/mouse, i.v.). On day 17 after the B16/F10 melanoma challenge, the lymphocytes were isolated by FACS. The percentage of each lymphocyte subset in the lung, liver, and spleen are shown (n=6). The absolute number of each lymphocyte subset was calculated (n=6). The data are shown as the mean ± SEM. *P<0.05 compared with the control group.
challenge, the mRNA expression levels of TGF-β, IL-1β, IL-6 and IL-23 in the lungs and spleen were measured by real-time PCR. *P<0.05 compared with the control group.

**Figure 7. Transfer of normal γδT cells or IL-17 supplementation rescues the immune impairment of the Abt mice.** The mice were given antibiotics for five weeks and then challenged with B16/F10 cells (1×10⁵ cells/mouse, i.v.). The mice were treated with antibiotics for the entire experimental period. (A) The procedures for the γδT cell transfer and IL-17A injection in the recipient antibiotic-treated mice. γδT cells were transferred one day prior to the B16/F10 challenge, followed by additional transfers every week. IL-17A was injected one day prior to B16/F10 challenge, and additional injections were performed every three days. (B) Purified γδT cells from the lungs of normal WT mice. (C) On day 17 after the B16/F10 melanoma challenge, the lungs were analyzed. The graphs show the total number of tumor colonies present in the lung lobes, and the number of tumor foci in the lung was calculated. There were six mice in each group. The data are shown as the mean ± SEM. (D) The survival times of the mice were compared among the four groups. *P<0.05 compared with the control Abt group.
Cheng M, Figure 1

A. Survival (%) of mice injected with B16/F10 cells.

B. Tumor foci (#) for Day 17 of B16/F10 challenge.

C. Day 17 of B16/F10 challenge images.

D. Day 17 of B16/F10 challenge images (H&E stain).

E. Survival (%) of mice injected with LLC cells.


G. Day 17 of LLC challenge images.

H. Day 21 of LLC challenge images (H&E stain).
Cheng M, Figure 4

### A

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<th>Lung</th>
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<td>Water+B16/F10</td>
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### B

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<th>CD44⁺γδT (%)</th>
<th>CD127⁺γδT (%)</th>
<th>CD27⁺γδT (%)</th>
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<tr>
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<td>90</td>
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<td>75</td>
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</tbody>
</table>

* indicates significant differences between groups.
Figure 5

(A) Water vs. LLC treatment in Lung and Spleen. (B) Abt inhibition of IL-17A+ γδ T cells. (C) Abt inhibition of IFN-γ+ γδ T cells.

* indicates p < 0.05 compared to corresponding control.
Cheng M, Figure 6

**A**  
**Lung**  

![Graph showing relative mRNA levels in Lung](image)

**B**  
**Spleen**  

![Graph showing relative mRNA levels in Spleen](image)

Author Manuscript Published OnlineFirst on June 19, 2014; DOI: 10.1158/0008-5472.CAN-13-2462
**A**

B16/F10 melanoma challenge (1×10⁵ cells/mouse, i.v.)

Recipient

Day -1

Adoptive transfer (i.v.)

Day 0

Day 7

Day 14

Day 21

Survival

Donor

Abt mice

Purified lung γδT cells (1.5×10⁵ cells/mouse)

Recipient

Abt mice

IL-17A (0.5μg/mouse)

**B**

Lymphocytes from lungs

γδTCR

CD3

0.200%

1.33%

57.3%

41.1%

0.714%

97.2%

0.676%

1.37%

**C**

Water+PBS

Abt+γδT cells

Abt+IL-17A

Abt+PBS

**D**

Survival (%)

day post-injection

Water+B16/F10+PBS (n=9)

Abt+B16/F10+γδT (n=9)

Abt+B16/F10+IL-17A (n=9)

Abt+B16/F10+PBS (n=9)
Microbiota modulate tumoral immune surveillance in lung through a $\gamma\delta T17$ immune cell-dependent mechanism

Min Cheng, Liting Qian, Guodong Shen, et al.

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