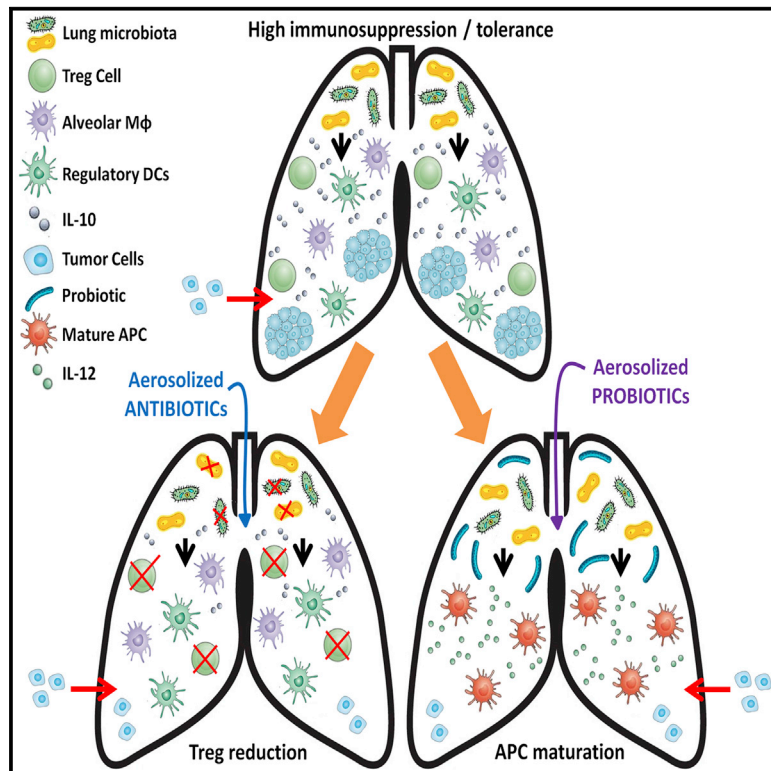# Modulation of Pulmonary Microbiota by Antibiotic or Probiotic Aerosol Therapy: A Strategy to Promote Immunosurveillance against Lung Metastases

Graphical Abstract

Authors

Valentino Le Noci, Simone Guglielmetti, Stefania Arioli, ..., Andrea Balsari, Elda Tagliabue, Lucia Sfondrini

Correspondence

andrea.balsari@unimi.it

In Brief

Le Noci et al. reveal that modulation of pulmonary microbiota by antibiotic or probiotic aerosolization decreases tumor growth in the lung. Antibiotic treatment induces a reduction of immunosuppressive cells in the lung, while probiotic administration promotes maturation of resident antigen-presenting cells.

Highlights

- Aerosolized antibiotic reduces lung microbiota and a tolerogenic microenvironment

- Aerosol delivery of an antibiotic or probiotic decreases tumor seeding in the lung

- Antibiotic or probiotic aerosol improves chemotherapy against experimental metastases

CellPress

Modulation of Pulmonary Microbiota by Antibiotic or Probiotic Aerosol Therapy: A Strategy to Promote Immunosurveillance against Lung Metastases

Valentino Le Noci,[1,5] Simone Guglielmetti,[2,5] Stefania Arioli,[2] Chiara Camisaschi,[3] Francesca Bianchi,[1,4] Michele Sommariva,[4] Chiara Storti,[4] Tiziana Triulzi,[1] Chiara Castelli,[3] Andrea Balsari,[1,4,7,*] Elda Tagliabue,[1,6] and Lucia Sfondrini[4,6]

[1]Molecular Targeting Unit, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan 20133, Italy
[2]Dipartimento di Scienze degli Alimenti, Nutrizione e Ambiente (DeFENS), Università degli Studi di Milano, Milan 20133, Italy
[3]Immunotherapy Unit, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan 20133, Italy
[4]Dipartimento di Scienze Biomediche per la Salute, Università degli Studi di Milano, Milan 20133, Italy
[5]These authors contributed equally
[6]Senior author
[7]Lead Contact
*Correspondence: andrea.balsari@unimi.it
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SUMMARY

Pulmonary immunological tolerance to inhaled particulates might create a permissive milieu for lung metastasis. Lung microbiota contribute to pulmonary tolerance; here, we explored whether its manipulation via antibiotic or probiotic aerosolization favors immune response against melanoma metastasis. In lungs of vancomycin/neomycin-aerosolized mice, a decrease in bacterial load was associated with reduced regulatory T cells and enhanced T cell and NK cell activation that paralleled a significant reduction of melanoma B16 lung metastases. Reduction of metastases also occurred in lungs transplanted with bacterial isolates from antibiotic-treated lungs. Aerosolized *Lactobacillus rhamnosus* strongly promoted immunity against B16 lung metastases as well. Furthermore, probiotics or antibiotics improved chemotherapy activity against advanced B16 metastases. Thus, we identify a role for lung microbiota in metastasis and show that its targeting via aerosolization is a therapy that can prevent metastases and enhance responses to chemotherapy.

INTRODUCTION

The lung microenvironment is characterized by a high immune tolerance, which is essential for preventing excessive inflammation in response to inhaled particulates. This status is maintained by lung antigen-presenting cells (APCs), primarily alveolar macrophages (AMs) and dendritic cell (DC) subpopulations (Hussell and Bell, 2014), which promote immunosuppression, inducing regulatory T cells (Tregs) (Soroosh et al., 2013) and the release of prostaglandin E2 (PGE2), transforming factor β (TGF-β), and interleukin (IL)-10 (Hussell and Bell, 2014). This physiological immunosuppressive status could explain the high

susceptibility of the lungs to metastasis implantation from various extrapulmonary neoplasms, including melanoma, breast cancer, and colon cancer.

Lung microbiota are thought not only to provide resistance to the colonization of respiratory pathogens but also to play a role in the immune tolerance of the lung microenvironment, a hypothesis supported by much evidence. In mice, increased bacterial load in the lungs and the appearance of specific bacterial taxa during the first two weeks after birth mediate the development of Tregs through the microbe-induced polarization of lung dendritic cells (Gollwitzer et al., 2014). Moreover, the strong immune activation observed in the airways of germ-free mice sensitized and challenged intranasally with ovalbumin (OVA) is reduced after reconstitution with commensal bacteria (Herbst et al., 2011). In humans, an attenuated immune response to lipopolysaccharide (LPS) stimulation was reported for AMs in the presence of a specific lung microbiota (Segal et al., 2016), and the presence of *Staphylococcus aureus*, a lung commensal bacterium reported to polarize alveolar CD11b+ monocytes to the M2-suppressive phenotype, is essential for promoting resistance to acute inflammation induced by influenza virus (Wang et al., 2013). Moreover, members of the Bacteroidetes phylum have been described to decrease lung inflammation (Larsen et al., 2015), while the lung bacteria *Prevotella* spp. and *Veillonella* spp. are associated with increased Th17 cell-mediated lung inflammation (Segal et al., 2016). Furthermore, a study in HIV patients with pneumonia demonstrated that distinct lower-airway microbiota are associated with specific local host immune responses (Shenoy et al., 2017).

Nasal sprays and aerosolization are efficient and non-invasive methods to deliver molecules such as antibiotics (Zarogoulidis et al., 2013), antibodies (Le Noci et al., 2016), cytokines (Storti et al., 2015), Toll-like receptor agonists (Le Noci et al., 2015; Sfondrini et al., 2013), and bacterial cells (Marchisio et al., 2015) and therefore represent a strategy to locally modify the lung microbiota, limiting the exposure of other organs. In mice, bacterial 16S rRNA profiling revealed that the lung microbiome was efficiently modified through nasal exposure, but not oral
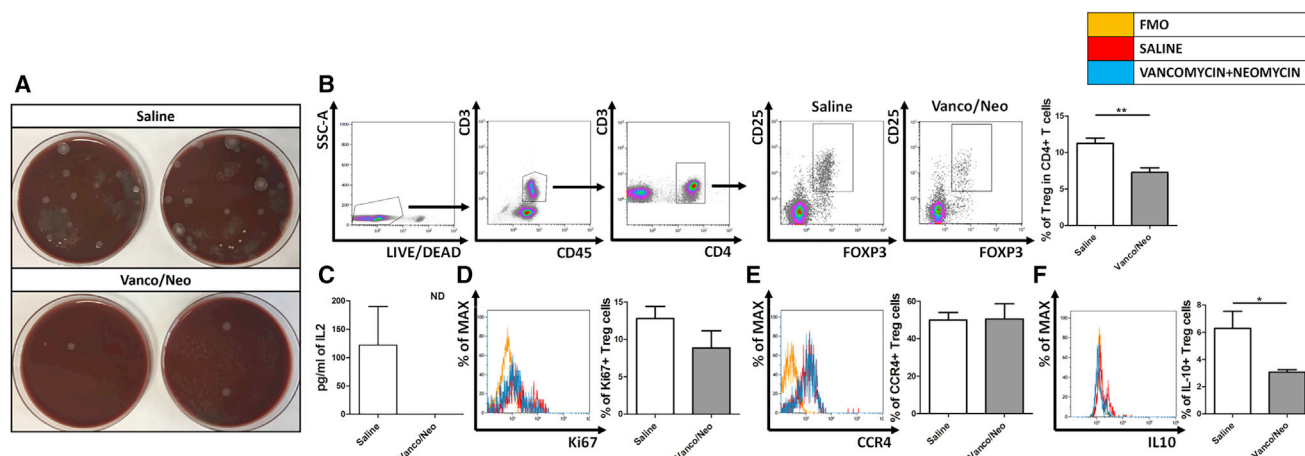
Figure 1. Effects of Antibiotic Aerosolization on the Lung Bacterial Microbiota and Tregs
Mice were treated for 5 days with aerosolized vancomycin and neomycin or with saline (4–5 mice/group).
(A) Bacterial load in 2 representative BAL samples collected aseptically 2 hr after the last treatment and plated on chocolate agar.
(B) Gating strategy and representative dot plots of Tregs (CD45+CD3+CD4+CD25+FoxP3+ live cells after doublet cell exclusion) in lung immune infiltrates obtained from the digested lungs of saline- or antibiotic (vancomycin- and neomycin [Vanco/Neo])-treated mice. Bars (mean ± SEM) represent the percentage of Tregs in the CD4+ T cell population (5 mice/group).
(C) IL-2 level (in picograms per milliliter) in BAL samples of 5 mice/group (ND, non-detectable).
(D–F) Representative overlay histogram plots and bars (mean ± SEM) showing the percentage of Ki-67-expressing Tregs (D), CCR4-expressing Tregs (E), and IL-10-producing Tregs (F) (FMO, fluorescence minus one control) (5 mice/group).
*p ≤ 0.05, **p ≤ 0.01.

exposure, to the antibiotic vancomycin, a glycopeptide used for inhalation in patients (ClinicalTrials.gov: NCT01509339) (Barfod et al., 2015), and nasal administration of lactobacilli was found to stimulate respiratory immunity and to increase resistance against viral infections (Harata et al., 2010; Youn et al., 2012). In humans, inhaled antibiotics are used to treat critical lung infections (Geller, 2009), while the administration of *Streptococcus salivarius* by nasal spray has been reported to prevent acute otitis in children (Marchisio et al., 2015).

To our knowledge, no studies aiming to alter the pulmonary microbiota, to subvert the immune-suppressive lung microenvironment and to establish local immunity against cancer, have been performed. In this study, we evaluated whether the nebulization of antibiotics or probiotics to modify the lung microbiota results in immunosurveillance against lung cancer metastases.

RESULTS

Vancomycin and Neomycin Aerosolization Modifies Lung Tissue Immunity and Prevents Tumor Implantation
To evaluate whether a reduction of the lung microbiota mitigates the immunosuppressive status in the lung microenvironment, mice were treated with broad-spectrum antibiotics by airway delivery. Specifically, mice were aerosolized with both vancomycin, which is directed to Gram-positive bacteria, and neomycin, which acts against several Gram-negative bacteria and partially against Gram-positive bacteria, or with saline for 5 days. The commensal bacterial load decreased in the bronchoalveolar lavage (BAL) of antibiotic-treated mice (mean colony-forming unit [CFU]/mL: 313.3 ± 173.7 in antibiotic-treated mice versus

1,390 ± 152.4 in saline-treated mice, p = 0.0056) (Figure 1A). Fluorescence-activated cell sorting (FACS) analysis of digested lung suspensions revealed that the decrease in bacterial load induced by antibiotic aerosolization did not significantly change the percentage of T cells, natural killer (NK) cells, DCs, and AMs infiltrating the lungs (Figure S1). Conversely, it resulted in a reduction of the percentage of the tolerogenic population of CD4+CD25+FoxP3+ Tregs (Figure 1B). This reduction was associated with a drop in IL-2 level in BAL, a factor essential for expansion and survival of Tregs, suggesting that the decline of Tregs might be related to a reduced bioavailability of IL-2 in the lung microenvironment (Figure 1C). A diminished fraction of proliferating Tregs, defined by the expression of the Ki-67 proliferation marker, was observed in antibiotic-treated lungs (Figure 1D), while no difference in CCR4 expression (Figure 1E), a chemokine receptor involved in migration of Treg, was detected. A decrease of IL-10-producing Tregs, a cytokine implicated in their functional activity, was also observed *ex vivo* in lung suspensions from antibiotic-treated mice (Figure 1F).

The effects of antibiotic treatment on the implantation of tumor cells in the lungs were evaluated in mice pre-treated with aerosolized antibiotics or saline for 2 weeks, intravenously (i.v.) injected with B16 melanoma cells, and treated again with antibiotics for another 3 weeks. At the end of the treatment, the number of macroscopic melanotic metastases was significantly reduced in antibiotic- versus saline-treated mice (p < 0.0001) (Figure 2A). No overt signs of toxicity, such as weight loss, hunching, ruffled fur, or difficulty breathing, were observed in mice treated with antibiotics (Figure S2A). Histopathological examination of the lung tissues showed the absence of injury in the lung parenchyma (Figure S2C).
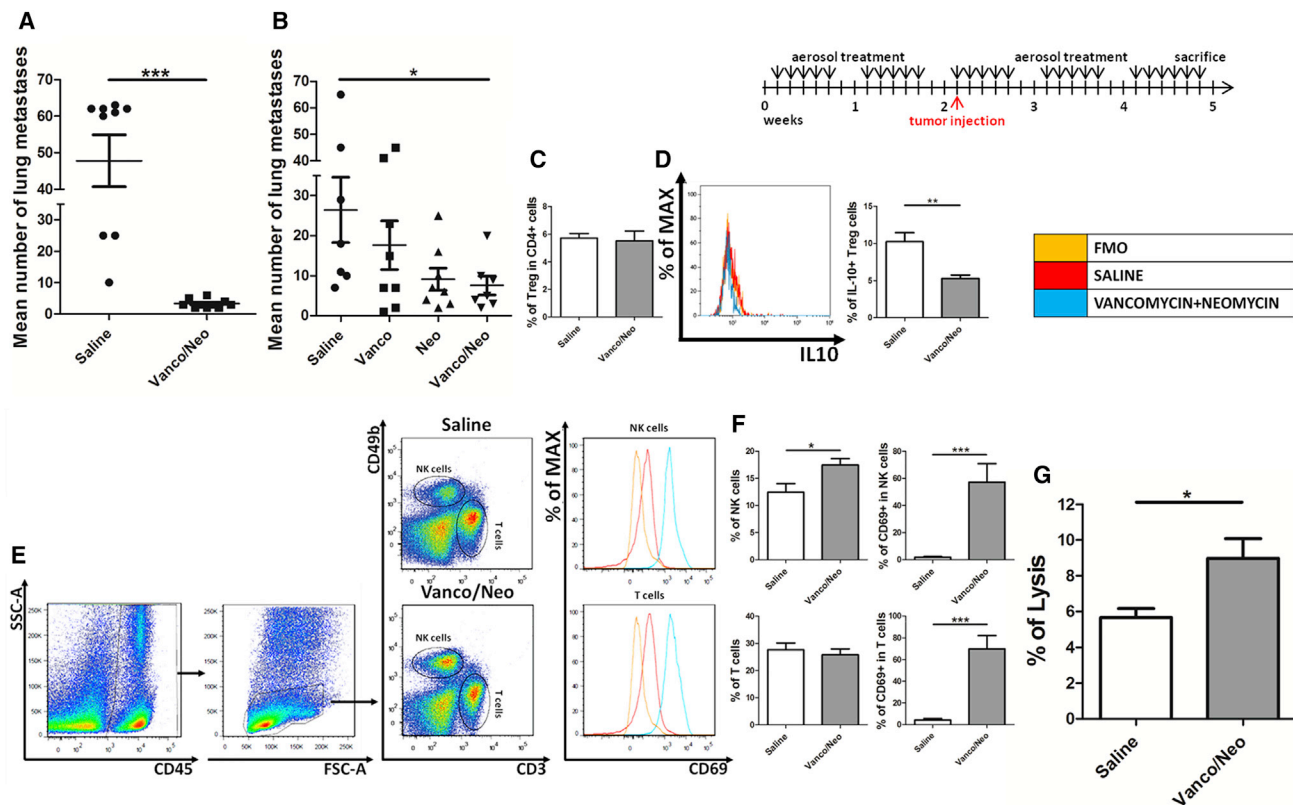
Figure 2. Effects of Antibiotic Aerosolization on the Growth of Experimental B16 Lung Metastases and on the Activation of Lung Immune Effector Cells

Mice were aerosolized with vancomycin and neomycin or saline for 2 weeks and then i.v. injected with B16 melanoma cells and aerosolized again for another 3 weeks (the scheme of the experiment is shown).

(A and B) Number of macroscopic metastases (A) in the lungs of mice treated with combined antibiotics or saline (9–10 mice/group) and (B) in mice treated to compare the effects of the vancomycin and neomycin combination with those of each antibiotic (7–8 mice/group).

(C) Bars (mean ± SEM) representing the percentage of Tregs in the CD4+ T cell population from lung immune infiltrates of 4 mice/group aerosolized with saline or combined vancomycin and neomycin.

(D) Representative overlay histogram plots and bars (mean ± SEM) showing the percentage of IL-10-producing Tregs.

(E) Gating strategy and representative dot plots and overlay histogram plots of NK cells (CD45+CD49b+CD3− cells) and T cells (CD45+CD3+CD49b− cells) and of CD69 expression in NK and T cell gates from mice aerosolized with saline or combined vancomycin and neomycin (4 mice/group) (FMO, fluorescence minus one control).

(F) Bars (mean ± SEM) representing the percentage of NK cells, T cells, and CD69-expressing NK and T cells from 4–5 mice/group.

(G) Percentage of the specific lysis of NK-sensitive YAC target cells cultured for 4 hr with non-adherent cells obtained from the lung suspensions of 4 mice/group.

*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

To examine the effect of each antibiotic, the experiment was repeated by treating the mice with vancomycin or neomycin alone or their combination. A reduction in the number of B16 lung metastases was observed in both the vancomycin- and the neomycin-treated groups, even if a significant decrease compared to the saline-treated mice was achieved only in the combination group (p = 0.395 in vancomycin-treated mice, p = 0.053 in neomycin-treated mice, and p = 0.0464 in vancomycin- and neomycin-treated mice versus saline-treated mice) (Figure 2B).

FACS analysis of lung cell suspensions obtained from mice i.v. injected with tumor cells and aerosolized with vancomycin and neomycin or saline revealed no modulation of Tregs (Figure 2C), but a significant reduction of IL-10-producing Tregs was found in antibiotic-treated mice (Figure 2D). No difference in the percent-

age and a strong upregulation of CD69 were observed in T cells (Figures 2E and 2F), whereas both increased recruitment and a strong upregulation of CD69 were detectable in NK cells (Figures 2E and 2F). The increased recruitment and activation of NK cells were associated with increased cytotoxic activity evaluated *in vitro* against NK-sensitive YAC target cells (Figure 2G).

These results indicate that local antibiotic treatment reduces the implantation of experimental lung metastases and that this effect is associated with a modulation of the immune response.

Pulmonary Transplantation of Bacteria Isolated from Vancomycin- and Neomycin-Treated Lungs Reduces Tumor Implantation

16S rRNA gene profiling revealed that 5-day administration of vancomycin and neomycin via aerosol significantly increased
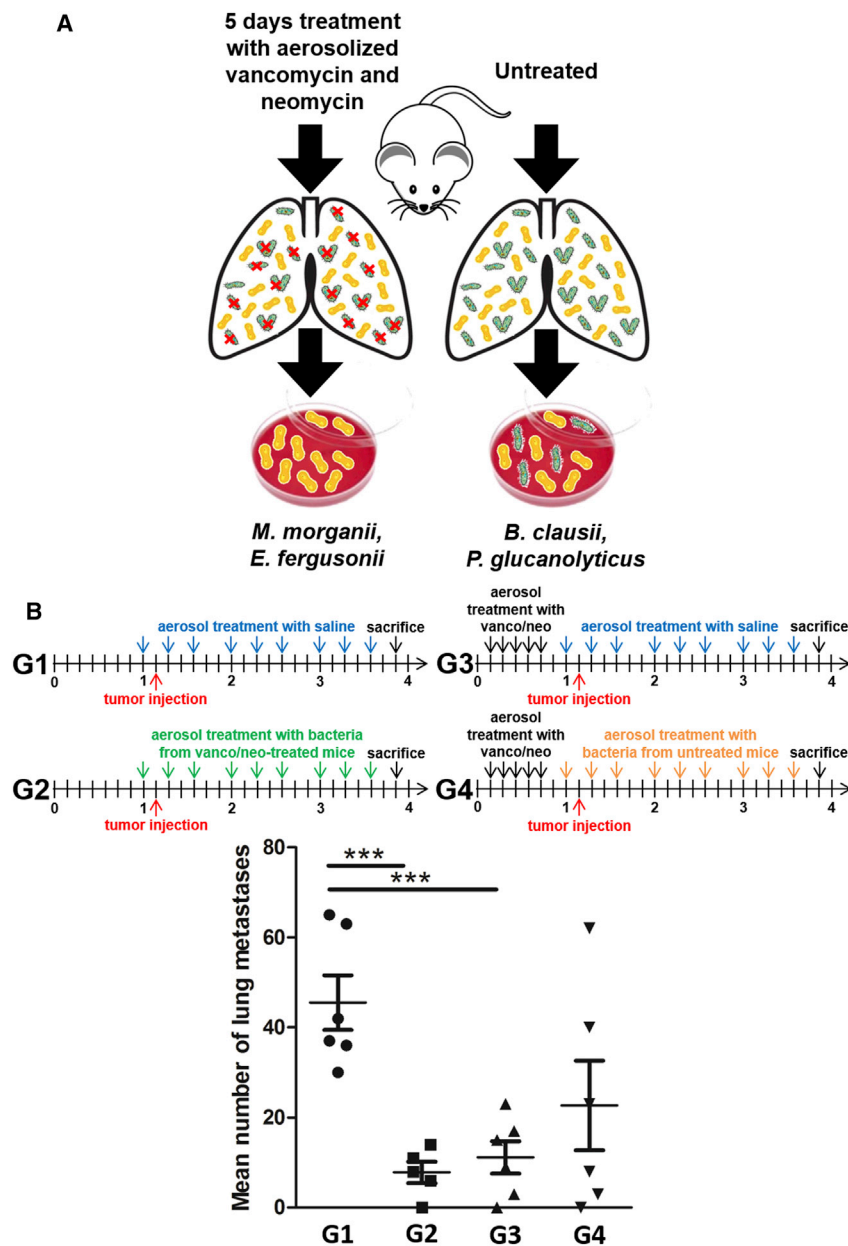
Figure 3. Effects of Commensal Bacteria Transplantation from Antibiotic-Treated or Untreated Lungs on the Growth of Experimental B16 Lung Metastases

(A) Bacterial strains were isolated from the BAL of mice aerosolized for 5 days with vancomycin and neomycin and from the BAL of untreated mice and were taxonomically identified by 16S rRNA gene sequencing.

(B) Number of macroscopic lung metastases in mice aerosolized, starting 1 day before they were i.v. injected with B16 cells, with saline (G1), or a combination of *in vitro*-cultivated bacterial strains isolated from the BAL of vancomycin- and neomycin-treated mice (G2) and in mice aerosolized with saline (G3) or a combination of *in vitro*-cultivated bacterial strains isolated from the BAL of untreated mice (G4) after reducing their commensal flora through a 5-day aerosolization with vancomycin and neomycin (5–6 mice/group).
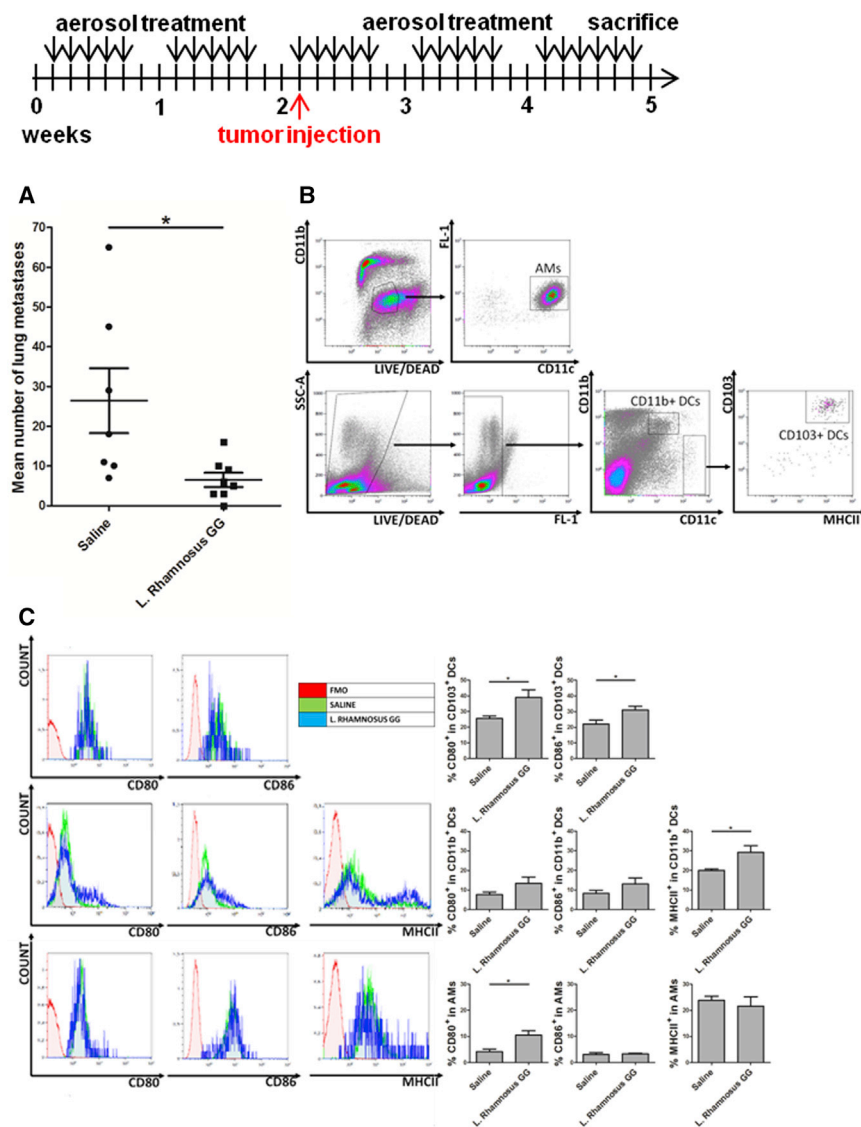
***p $\leq$ 0.001.

(α) diversity in terms of bacterial richness, estimated by the number of observed operational taxonomic units (OTUs) and the Chao1 index. On the contrary, no difference was observed with indexes that estimate biodiversity while considering bacterial evenness (i.e., Shannon, Simpson, and inverse Simpson). At the taxonomic level, antibiotic treatment modified BAL microbiota, drastically reducing the population of the genus *Streptococcus*, which are common Gram-positive Firmicutes commensal of the respiratory tract; concomitantly, antibiotic administration led to the expansion in BAL microbiota of the generally less represented genera of the Gram-negative phylum Proteobacteria. In addition, we found expansion of members of the Gram-positive phylum Actinobacteria (Figure S3).

To assess the effect of bacteria expanded upon antibiotic treatment on metastasis growth, we treated or did not treat mice with vancomycin and neomycin as done previously and isolated bacterial strains from the BAL. According to the colony morphology, from the BAL of antibiotic-treated mice, we isolated two bacterial strains that were taxonomically assigned to the Gram-negative Proteobacteria taxa (*Morganella morganii* and *Escherichia fergusonii*) (Figure 3A). In contrast, the three strains isolated from the BAL of the untreated mice were taxonomically identified as Gram-positive Firmicutes species (two morphologically and genetically different isolates of *Paenibacillus glucanolyticus* and *Bacillus clausii*) (Figure 3A).

To determine the effects of commensal bacteria isolated from antibiotic-treated or untreated lungs on metastasis growth, a transfer experiment was performed. Specifically, a group of mice was aerosolized for 3 weeks with a bacterial cells suspension of the two strains isolated from the BAL of mice treated with vancomycin and neomycin aerosol (group G2), while another group was aerosolized with saline (group G1). Conversely, to assess whether the strains isolated from the untreated lungs were able to reverse the antitumor effect of antibiotic treatment, the third and fourth groups of mice were treated by 5-day aerosolization with vancomycin and neomycin to reduce their commensal flora and then aerosolized with a combination of the three bacterial strains isolated from untreated mice (group G4), to re-colonize their lungs, or with saline (group G3). All groups were i.v. injected with B16 melanoma cells 24 hr after the first bacterial or saline

aerosol treatment   aerosol treatment   sacrifice

weeks   0   1   2   3   4   5

tumor injection

A

B

C

Mice were aerosolized with *L. rhamnosus* or saline for 2 weeks and then i.v. injected with B16 melanoma cells and aerosolized again for another 3 weeks (the scheme of the experiment is shown).

(A) Number of macroscopic lung metastases in the mice at the end of the experiment (7–8 mice/ group).

(B) Gating strategy to analyze alveolar macrophages (AMs) (CD11c+FL-1+ cells among live CD11blow cells; the FL-1 channel was reserved for the assessment of autofluorescence), CD103+ DCs (CD103+MHC class II+ in CD11c+CD11blow cells gated among live FL-1− cells), and CD11b+ DCs (CD11b+CD11c+ cells gated among live FL-1− cells).

(C) Overlay histogram plots of CD80- and CD86-expressing CD103+ DCs; of CD80, CD86, and MHC class II-expressing CD11b+ DCs; and of CD80, CD86, and MHC class II-expressing AMs in the lung immune infiltrates of B16 tumor-bearing mice aerosolized with saline or *L. rhamnosus*. Bars (mean ± SEM) represent data from 3–4 mice/ group (FMO, fluorescence minus one control). $*p \leq 0.05$.

Aerosolized *Lactobacillus rhamnosus* Probiotic Promotes an Immune Response that Prevents Tumor Implantation in the Lungs
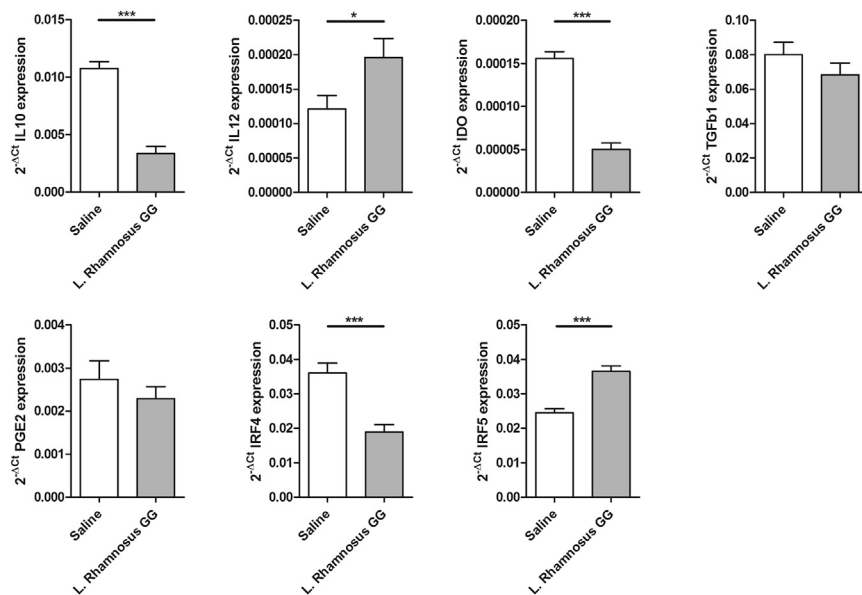
Our data indicate that aerosolization of bacteria isolated from lung microbiota of antibiotic-treated mice reduces lung metastasis implantation. Thus, because these bacteria include opportunistic pathogen species that typically arise after antibiotic treatment, we evaluated whether the immunosuppressive effects of the resident microflora can be overcome by the aerosolization of safe bacteria with immunostimulatory properties such as *Lactobacillus rhamnosus* GG, which reportedly enhances cell-mediated immunity by intranasal administration in viral infection models (Harata et al., 2010; Youn et al., 2012). Aerosolized *L. rhamnosus* GG reached the lung in a viable form (Figure S4) and significantly reduced the number of lung metastases compared to saline treatment when aerosolized for 2 weeks before B16 melanoma cells were i.v. injected and for the 3 weeks before sacrifice (Figure 4A). No weight loss or other signs of toxicity and no injury to the lung parenchyma were observed (Figures S2B and S2C).

Numerous reports have shown that *Lactobacillus* spp. may mediate anticancer effects through DC maturation (Mohamadzadeh et al., 2005). In agreement, as shown in Figures 4B and 4C, analysis of lung suspensions from mice i.v. injected with B16 tumor cells and aerosolized with *L. rhamnosus* revealed that this treatment enhanced the maturation of AMs and of CD103+ DCs and CD11b+ DCs, the two major DC populations

aerosolization, and the number of lung metastases was counted 3 weeks later. A significant reduction in the number of metastases was observed in the group aerosolized with bacteria isolated from antibiotic-treated lungs (group G2) versus that from the saline-treated group (group G1) (p = 0.0005) (Figure 3B). Moreover, as shown in the figure, antibiotic aerosol treatment, even when limited to the 5 days before tumor injection, significantly reduced the implantation of lung metastases (group G3), but re-colonization of antibiotic-treated mice by aerosolization of commensal bacteria isolated from the lungs of untreated mice (group G4) attenuated the protective effect of antibiotic treatment compared to saline-treated mice (Figure 3B).

These results exclude a direct effect of the antibiotics on tumor implantation and growth and indicate that resident bacteria control tumor implantation in the lung by influencing the immune landscape of the lung tissue.

Figure 5. Effects of Probiotic Aerosolization on the Tumor Immune Microenvironment
Mean relative expression ± SEM of IL-10, IL-12, IDO, TGF-β1, PGE2, IRF4, and IRF5 mRNA levels, evaluated by real-time PCR in the adherent cell fraction of digested lungs from mice aerosolized with *L. rhamnosus* GG or saline for 2 weeks before and 3 weeks after they were i.v. injected with B16 melanoma cells (4–8 mice/group). Results are presented as $2^{-\Delta Ct}$. *$p \le 0.05$, ***$p \le 0.001$.

residing in the lung that are involved in migration to the lymph nodes for tumor-derived antigen presentation (Sung et al., 2006); however, no increase was detected in the percentage of these three populations (data not shown). Specifically, the expression of CD80 and CD86 maturation markers on CD103+ DCs was significantly upregulated, CD11b+ DCs displayed higher positivity for major histocompatibility complex class II (MHC class II) molecules, and there was a trend suggesting the upmodulation of CD80 and CD86 (Figures 4B and 4C). Moreover, *L. rhamnosus* significantly upregulated CD80 on AMs (Figures 4B and 4C). The increased maturation of CD103+ DCs and AMs following the aerosolization of *L. rhamnosus* was also observed in the lungs of tumor-free FVB mice (data not shown), indicating that the maturation of resident APCs by *L. rhamnosus* increased in the absence of tumor cells and that this effect was not restricted to the specific reactivity of APCs in C57BL/6 mice. Moreover, the maturation of resident APCs was associated with reduced expression of M2 genes, as revealed by real-time PCR analysis performed on mRNA extracted from the adherent cell fraction of digested lungs that contains macrophages and myeloid cells. M2-related genes, such as Il10, Ido, and Irf4, significantly declined in adherent cells from the lungs of *L. rhamnosus*-aerosolized mice versus untreated mice (Figure 5). Moreover, there was a trend suggesting a reduction in TGF-β and PGE2 expression. In contrast, M1 markers, such as IL-12 and IRF5, were significantly upregulated (Figure 5).

In contrast to that observed in antibiotic-treated lungs, no significant reduction in the percentage of Treg was observed in lung suspensions obtained from *L. rhamnosus*-treated mice (mean ± SEM of the percentage of CD25+FoxP3+ cells in the CD45+CD3+CD4+ fraction: 9.8 ± 0.8 in probiotic-treated mice versus 11.2 ± 0.7 in saline-treated mice). No differences were observed in the recruitment of NK cells or T cells in the lungs of tumor-bearing mice between the two groups of mice (data not shown). However, the increased expression of maturation markers on APCs in B16 tumor-bearing lung suspensions from

mice aerosolized with *L. rhamnosus* was associated with the enhanced expression of CD69 on both T and NK populations and with increased levels of the activatory NK receptor NKG2D (Figure 6). Depletion experiments confirmed the role of NK cells in the antitumor effects of *L. rhamnosus* in the low immunogenic NK-sensitive B16 tumor model (Figure S5). However, we cannot exclude that *L. rhamnosus* might also promote T cell-mediated cytotoxic activity against immunogenic tumors.

These results suggest that aerosolized *L. rhamnosus* promotes the maturation of resident APCs in the lungs and reduces the M2 microenvironment, resulting in the increased activation of effector cells.

Aerosolization with Probiotics or Antibiotics after Tumor Implantation Increases Therapeutic Effects of Dacarbazine

In addition to lactobacilli, *Bifidobacterium* is the probiotic genus most frequently investigated for its immunomodulatory properties (Vlasova et al., 2016). In particular, the species *B. bifidum*, which exclusively colonizes the healthy human colon (Turroni et al., 2014), has been demonstrated to stimulate DC to acquire Th1 stimulatory capacity (Guglielmetti et al., 2014). Thus, after establishing its ability to significantly mature resident APCs in the lungs when administered by aerosol (data not shown), the strain *B. bifidum* MIMBb23sg was applied, and its effects were compared to those of *L. rhamnosus* in subsequent experiments.

To investigate whether aerosolization with probiotics boosts the immune response against a growing tumor after its implantation in the lung, we evaluated the efficacy of aerosolized lactobacilli in a therapeutic protocol by initiating aerosolization 7 days after tumor injection, when metastatic foci are detectable in the lungs (Le Noci et al., 2016). This treatment was combined with dacarbazine (DTIC), a conventional chemotherapeutic agent used in metastatic melanoma patients that exerts immunostimulatory effects (Hervieu et al., 2013).

The mice were i.v. injected with B16 melanoma cells and treated from day 7 to day 21 with DTIC alone or in combination with aerosolized *L. rhamnosus* or *B. bifidum*. Two other groups of mice received *L. rhamnosus* or *B. bifidum* alone, and one group was left untreated. The antitumor effect of DTIC was significantly increased when combined with *L. rhamnosus* or *B. bifidum* (Figure 7A) with a comparable improvement, but no
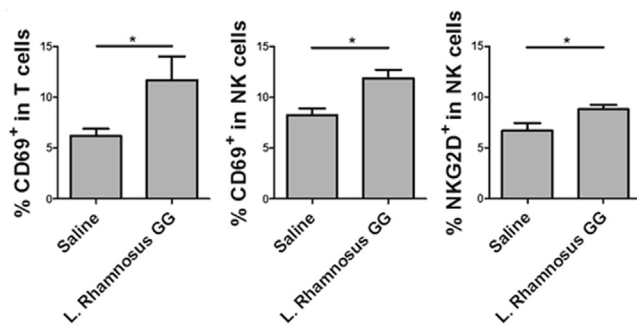
Figure 6. Effects of Probiotic Aerosolization on the Activation of Lung Antitumor Effector Cells

Mice were aerosolized with *L. rhamnosus* or saline for 2 weeks and then i.v. injected with B16 melanoma cells and aerosolized again for another 3 weeks. Bars represent the percentages (±SEM) of CD69-expressing T cells and of CD69- or NKG2D-expressing NK cells, pooled from 3–4 mice/group. *p ≤ 0.05. (The gating strategy was performed as described in the legend of Figure 2.)

significant effect was observed following treatment with each probiotic alone. FACS analysis of cell suspensions obtained from mice i.v. injected with tumor cells and then aerosolized with *L. rhamnosus* revealed no differences in the recruitment of NK cells or T cells (data not shown); nonetheless, a significant increased CD69 expression was observed in both NK and T cells in DTIC and probiotic- versus DTIC-treated mice (Figure 7B). This increased activation of effector cells was associated with a higher ability to lyse B16 tumor cells (Figure 7C).

Because the preceding experiments revealed that a reduction in the commensal lung microbiota promoted immune activation, we next evaluated whether aerosolization with antibiotics improved the antitumor efficacy of DTIC in the therapeutic protocol. A significant increase in the antitumor effect of DTIC was observed in mice i.v. injected with B16 melanoma cells and treated as described previously from day 7 to day 21 with the chemotherapeutic drug and aerosolized vancomycin and neomycin (Figure 7D). Increased cytotoxic activity was observed against B16 target cells in lung suspensions from mice treated with DTIC and vancomycin and neomycin compared to those from mice treated with DTIC alone (Figure 7E).

These results suggest that the local administration of immunostimulatory probiotics or the reduction of immunosuppressive commensal species by antibiotics improves the antitumor effect of DTIC.

DISCUSSION

In this proof-of-concept study, we demonstrate that the commensal lung microbiota are manipulated by antibiotic or probiotic aerosolization and that changes induced by these treatments are associated with a reduction in the immune suppression present in the lung microenvironment, thus favoring an immune response against cancer cells.

Antibiotic aerosolization is a compelling strategy used to achieve high antibiotic concentrations in the airway to maximize bacterial killing with minimal systemic side effects (Zarogoulidis

et al., 2013). Antibiotic aerosolization has been revealed to be safe and to have a significant impact on clinical outcomes in the treatment of chronic respiratory tract infections in patients with cystic fibrosis (Geller, 2009). Moreover, pulmonary administration of antibiotics has the advantage of minimally affecting gut microbiota, avoiding the intestinal dysbiosis reported to play a critical role in systemic immunity (Belkaid and Naik, 2013) and eventually to increase the frequency (Boursi et al., 2015; Friedman et al., 2006; Velicer et al., 2004) and the growth (Cheng et al., 2014; Rossini et al., 2006) of tumors.

Here, we observed that the decrease in the lung bacterial flora resulting from antibiotic aerosolization reduced tumor implantation in the lung and that this treatment increased the activation of NK and T effector cells. In contrast to results obtained in non-tumor-bearing mice, which showed a reduction of the Treg percentage and of IL-10-producing Tregs, antibiotic treatment in tumor-bearing lungs did not reduce the percentage of Tregs but significantly diminished the percentage of IL-10-producing cells. This result might be related to the presence of tumor cells in the lung that are known to modulate Treg expansion and recruitment through cytokine and chemokine production. Tregs, regardless of their antigenic specificity, establish a state of dominant and stable tolerance through a bystander effect (Tang and Bluestone, 2008) that limits effector T cell activation against tumor cells. Because a significant reduction of lung metastases was achieved only using combined wide-spectrum antibodies, it is plausible that the decrease of Tregs induced by aerosolization is due to the reduction or elimination of combinations of signaling pathways activated by different microbe-associated ligands in the lung microenvironment. In agreement with our results, endogenous microbiota have been reported to drive suppressive cells in pancreas, and bacterial ablation by antibiotic treatment has been demonstrated to promote an antitumor immune response in pancreatic cancer by reducing the immunosuppressive microenvironment (Pushalkar et al., 2018).

Numerous bacteria, many not previously recognized using culture-dependent analysis, have been demonstrated to colonize both mouse and human lungs by genomic approaches, the most abundant of which are the Gram-positive firmicutes and the Gram-negative bacteroidetes and proteobacteria phyla, with the prominent genera being *Streptococcus*, *Pseudomonas*, *Veillonella*, and *Prevotella* (Dickson et al., 2016). Few studies have analyzed the immunological properties of bacterial components in the lung, and whether specific taxa or biotypes of bacteria with dedicated functions that promote immunosuppression or immunostimulation exist in the lung remains virtually unknown and difficult to define. Our results demonstrated a significant reduction of metastases in lungs of mice aerosolized with bacteria that arose in lungs of antibiotic-treated mice, including the Gram-negative bacterial species *Morganella morganii* and *Escherichia fergusonii*, ubiquitous opportunistic pathogens taxonomically belonging to Proteobacteria phylum, that might exert an immunostimulatory effect through their typical production of virulence factors (Liu et al., 2016; Rimoldi and Moeller, 2013).

Because *Morganella morganii* and *Escherichia fergusonii* were not repeatedly expanded in both experiments, we can speculate that the shift from Firmicutes to Proteobacteria, or
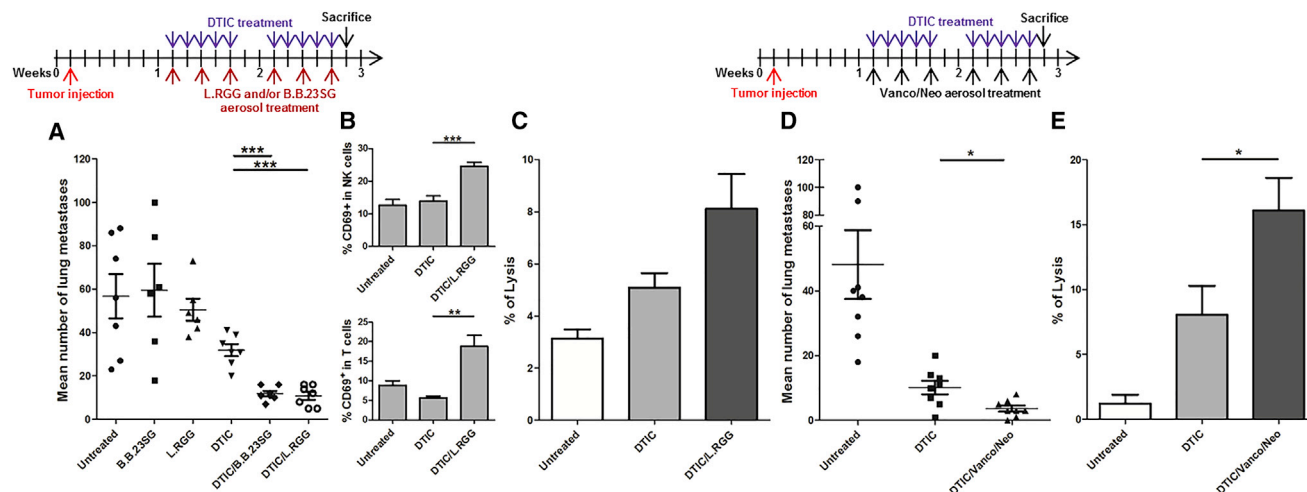
Figure 7. Effects of Dacarbazine and Aerosolized Probiotics or Antibiotics on the Growth of Experimental B16 Lung Metastases

Mice were i.v. injected with B16 melanoma cells and then left untreated or treated starting 7 days after tumor injection with dacarbazine (DTIC) administered i.p. alone or with aerosolized probiotics or antibiotics (the scheme of each experiment is shown).

(A) Number of macroscopic lung metastases in B16 melanoma-injected mice untreated or treated with *L. rhamnosus* GG, *B. bifidum* MIMBb23sg, DTIC alone, or DTIC combined with aerosolized *L. rhamnosus* GG or *B. bifidum* MIMBb23sg (6–7 mice/group).

(B) Bars (mean ± SEM) representing the percentage of CD69-expressing NK and T cells from 4–5 mice/group treated with DTIC alone or combined with aerosolized *L. rhamnosus* GG.

(C) Percentage (mean ± SEM) of the specific lysis of B16 target cells cultured for 4 hr with non-adherent cells obtained from lung suspensions of DTIC or DTIC and *L. rhamnosus*-treated mice (3 mice/group).

(D) Number of macroscopic lung metastases in mice injected as described earlier and untreated or treated with DTIC alone or combined with aerosolized vancomycin and neomycin (8 mice/group).

(E) Percentage of the specific lysis of B16 target cells cultured for 4 hr with non-adherent cells obtained from lung suspensions of DTIC or DTIC-, vancomycin-, and neomycin-treated mice (6 mice/group).

$*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

other Gram-positive microbes commonly induced by antibiotic treatment, rather than specific species, is relevant to the reduced immunosuppression. These findings point to a role of lung bacteria in antitumor immune response and suggest that a balance among different bacteria is critical for antitumor immune responses. Accordingly, local administration of bacteria such as *L. rhamnosus*, a Gram-positive lactic acid bacterium considered a stable commensal of the oral microbiota and an allochthons intestinal bacterium in humans (Badet and Thebaud, 2008; Walter, 2008), was found to overcome immunosuppressive signaling derived from commensal microbes. This bacterium is regarded as safe and extensively performed for food products and supplements, and it has already been shown to stimulate respiratory immunity in mice when intranasally administered (Harata et al., 2010; Youn et al., 2012). The reduction of lung metastasis implantation induced by aerosolized *L. rhamnosus* was associated with increased maturation of resident APCs, reduced expression of M2 genes, and activation of T and NK cells in the lung, whereas the percentage of Tregs was only lightly modified. Increased activation of APCs, resulting in the promotion of type 1 immune responses, has also been reported in humans following *in vitro* incubation of *L. rhamnosus* with mononuclear cells derived from healthy blood donors (Fong et al., 2015). Thus, two strategies to modify the lung microbiota—that is, aerosolization of antibiotics or probiotics—promote similar antitumor activity with different immune mechanisms. The activation of

antitumor effector cells by antibiotics seems related to a pauperization of microbial signals necessary to the recruitment of Tregs in the lung, while probiotics appear to overcome commensal bacteria-induced tolerance, promoting the maturation of resident APCs.

A boost of immune response induced by the two strategies similarly improved the response to treatment with DTIC, a genotoxic drug shown to exert immune-mediated effects by promoting NK cell cytotoxicity (Hervieu et al., 2013). Thus, targeting the microbiota in the lung by aerosolization could represent a therapeutic intervention to maintain an immune microenvironment that favors the immunostimulatory effects exerted by several chemotherapeutic drugs (Zitvogel et al., 2008).

Altogether, our study reveals an unravel role of pulmonary microbiota in controlling tumor growth in the lung and points to the promise of aerosolization with probiotics or antibiotics as a clinical therapeutic procedure to prevent metastases in the lungs of high-risk patients and to enhance the response of patients to chemotherapy.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents

B16 mouse melanoma cells and YAC-1 mouse lymphoma cells (ATCC) were routinely maintained at 37°C in a 5% CO_2 atmosphere in DMEM (Gibco-Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS,

Gibco-Thermo Fisher Scientific) and 2 mM glutamine (Lonza). Authentication of the cell line was verified by short tandem repeat DNA profiling, and cultures were regularly tested for mycoplasma using the mycoAlert Plus Kit (Lonza). Vancomycin was provided by PharmaTex, and neomycin trisulfate salt hydrate was purchased from Sigma-Aldrich. DTIC was provided by Medac. ^{51}Cr (1 mCi) was purchased from PerkinElmer. *Lactobacillus rhamnosus* GG and *Bifidobacterium bifidum* MIMBb23sg were grown anaerobically at 37°C in de Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories) and cysteine-MRS (cMRS) supplemented with 0.05% L-cysteine hydrochloride, respectively. After growth, the cells were collected by centrifugation, washed, counted by flow cytometry, diluted in saline solution (9 g/L NaCl) with 10% (w/v) glycerol to a final concentration of 1×10^9 cell/mL, and stored at −80°C until use.

Isolation of Bacteria and Lung Suspensions

BAL was performed in euthanized mice as described (Sfondrini et al., 2013). The recovered BAL fluid was then centrifuged and plated on agar chocolate medium to isolate commensal bacterial colonies. For details on the protocols used to (1) evaluate the presence of *L. rhamnosus* GG in the BAL of *L. rhamnosus*-aerosolized mice and (2) isolate and taxonomically identify bacterial strains from BAL of untreated or antibiotic-treated mice, see Supplemental Experimental Procedures.

Isolation of lung immune cells from the lungs of healthy or tumor-bearing mice treated with antibiotics, probiotics, or saline was performed as described (Le Noci et al., 2016). Cell suspensions were directly stained for flow cytometry or plated to separate adherent and non-adherent cell fractions as described (Sommariva et al., 2017).

Mice and Experimental Protocols

Female C57BL/6 mice, aged 6–8 weeks (Charles River), were maintained in laminar flow rooms at constant temperature and humidity, with food and water given *ad libitum*. The experiments were approved by the Ethics Committee for Animal Experimentation of Fondazione IRCCS Istituto Nazionale dei Tumori, Milan. To evaluate the effects of aerosolized antibiotic or probiotic on tumor implantation, mice were treated with vancomycin and/or neomycin (5 days/week at 12-hr intervals) or with *L. rhamnosus* GG (3 times/week), starting 2 weeks before they were i.v. injected with 5×10^5 B16 melanoma cells and continuing throughout the experiment.

For depletion of effector cells, mice were i.v. injected with CD3 F(ab')2 fragments (145-2C11 f(ab')2) (BioXcell) at a dose of 50 μg/day for 5 days/week starting 1 day before tumor injection to deplete T cells or were intraperitoneally (i.p.) injected with 500 μg of α-NK1.1 antibody (PK136) (BioXcell) 1 day before tumor injection, followed by injection of 200 μg every 5 days throughout the experiment, to deplete NK cells. The efficacy of cell depletion was verified by staining peripheral blood leukocytes for specific subsets after depletion. In therapeutic experiments, mice were i.v. injected with 5×10^5 B16 melanoma cells and treated starting 7 days after tumor cell injection with antibiotics (vancomycin and neomycin) or probiotics (*L. rhamnosus* GG or *B. bifidum* MIMBb23sg) alone or in combination with DTIC, administered i.p. at 70 mg/kg (5 days/week). For details of aerosol administration, see Supplemental Information. In all experiments, mice were weighed and inspected for any sign of sufferance twice weekly and euthanized at day 21 after tumor injection to count macroscopic lung metastases. To exclude any effects of aerosolized antibiotics or probiotics on the architecture and structure of the lung parenchyma, lung samples were analyzed as described (Le Noci et al., 2016). All *in vivo* experiments were repeated at least twice.

Pulmonary Microbiota Transplantation

The experiment was performed using four groups of mice: two groups were aerosolized 3 times/week for 3 weeks with saline (group 1) or with bacteria grown *in vitro* obtained from the BAL of mice treated for 5 days with aerosolized vancomycin and neomycin (10 mL of saline with 10% glycerol containing 10^9 units/mL of *Morganella morganii* and *Escherichia fergusonii* at 33% and materials) (group 2). Two other groups were aerosolized with vancomycin and neomycin for 5 days and then aerosolized with saline (group 3) or with bacteria grown *in vitro* from the BAL of untreated mice

(10 mL of saline with 10% glycerol containing 10^9 units/mL of *Bacillus clausii* and two *Paenibacillus glucanolyticus* isolates at 14%, 17%, and 68%, respectively) (group 4). All mice were i.v. injected with B16 cells 1 day after the first treatment, and the number of melanotic foci was counted 3 weeks later.

In Vitro Cytotoxicity Assays

The ability of effector immune cells from the lung immune infiltrates of mice to promote antitumor activity was evaluated by measuring cytotoxic activity of non-adherent cells obtained from the lung suspensions on ^{51}Cr-B16 target cells or NK-sensitive ^{51}Cr-YAC-1 target cells as described (Sommariva et al., 2017). The radioactivity of the supernatant (80 μL) was measured as described (Di Modica et al., 2016).

Statistical Analysis

Differences among groups were compared using a two-tailed unpaired Student's t test and were considered significant at $p \leq 0.05$. All analyses were performed using GraphPad Prism v.5.0 for Windows.

Intra-subject bacterial richness and evenness (α diversity) were analyzed using five algorithms: observed OTUs, Chao1, Shannon, Simpson, and inverse Simpson. Significance was estimated according to Mann-Whitney U test (**$p < 0.01$, *$p < 0.05$). Inter-sample (β) diversity analysis was carried out with the UniFrac algorithms. LEfSe analysis represent differences between the two groups of mice at the taxonomic levels of phylum, class, order, family, and genus (represented by the concentric circles moving from the center of the cladogram). Analyses were performed using R v.3.4.2.

DATA AND SOFTWARE AVAILABILITY

The accession numbers for the partial sequences of the 16S rRNA gene of BAL bacterial isolates and the raw sequencing data of the microbiomic analysis reported in this paper are ENA: PRJEB28069 and Dataverse: https://doi.org/10.5072/FK2/WV6XKI.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.08.090.

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