Gut Microbiota Can Impact Chronic Murine Lung Allograft Rejection

To the Editor:

The role of the microbiome in regulation of immune homeostasis is well established, and its alteration underlies the immunopathogenesis of a variety of diseases (1, 2). Clinical and animal studies have recently found that derangement in the microbiome may impact transplant outcomes by contributing to allograft dysfunction after liver, small bowel, and skin transplantation (3–5). Clinical studies have also shown that lung transplantation alters the composition of the lung microbiome (6, 7). However, whether the gut microbiome can modulate lung transplant outcomes remains unknown. Lung allografts are limited by the development of chronic lung allograft rejection (8), and transplant-associated ischemia–reperfusion injury is a major risk factor for chronic lung allograft rejection (9–11). A recent animal study showed that administration of broad-spectrum antibiotics to reduce gut microbiota can ameliorate lung ischemia–reperfusion injury (12). In a previous report, we found that $CD4^+$ T lymphocytes were required for the expansion of an IL-17A response in the lung allograft that is necessary for the development of obliterative bronchiolitis, the histological correlate of chronic lung allograft rejection (13). Gut microbiota can regulate T-helper cell type 17 (Th17) cell homeostasis (14), raising the possibility that alterations in the gut microbiota can impact lung allograft rejection. Hence, we hypothesized that alterations in microbiota may also impact lung transplant outcomes, particularly chronic lung allograft rejection, which is characterized by airway remodeling and fibrosis.

To address this question, we used a minor mismatch model of orthotopic lung transplantation with C57BL/10 mice as donors and C57BL/6N mice as recipients in a pathogen-free facility, as we previously described (13). To alter the microbiota at the time of transplantation, both donor and recipient mice were treated by daily oral gavage (200 μl) with a cocktail of broad-spectrum antibiotics we previously reported (5), including gentamycin (0.35 mg/ml), kanamycin (5.25 mg/ml), colistin (8,500 U), metronidazole (2.15 mg/ml), and vancomycin (0.5 mg/ml) diluted in autoclaved water, starting 10 days before transplantation and ending the day before transplantation. In the control group, mice were treated with 200 μl of autoclaved water orally. This treatment timing was intended to reduce microbial diversity at the priming phase of the alloresponse, when T cells first encounter donor antigens, so that we could investigate whether dysbiosis at the priming stage affected Th17 differentiation after lung transplantation. We also hypothesized that the microbiota from not only the recipient but also the donor would modulate the alloresponse after lung transplantation. Therefore, we treated both donor and recipient mice with antibiotics. In the context of skin grafts, we previously showed that pretreatment of both donor and recipient mice with antibiotics prolonged graft survival, but pretreatment of only the donor or only the recipient did not alter graft outcomes (5). Moreover, continuing antibiotics after transplantation did not further prolong skin graft survival (M.-L.A., unpublished results).

On Day 21 after transplantation, antibiotic pretreatment was associated with a decrease in the severity of rejection, as determined using established criteria (Figure 1). Five of the six mice pretreated with antibiotics developed only mild acute rejection, whereas the majority of control allografts developed moderate or severe acute rejection. In addition, allografts from antibiotic-pretreated mice developed less lung fibrosis compared with control mice (Figure 1; Table E1 in the data supplement). Remarkably, allografts from mice pretreated with the antibiotic regimen did not exhibit obliterative bronchiolitis injury, in contrast to the control mice. These data suggest that antibiotic pretreatment can ameliorate cellular rejection and obliterative airway fibrosis.

To further determine whether antibiotic pretreatment in our animal model led to changes in the gut microbiome, we analyzed the composition of the fecal microbiota. We found that C57BL/6N and C57BL/10 fecal pellets had similar relative abundances of phyla under physiological conditions (Figure 2A). On Days 0 and 21 after transplantation, however, the antibiotic pretreatment regimen significantly increased Bacteroidetes and decreased Firmicutes (Figure 2B). We also identified 11 species (10 *Clostridial* species and *Bacteroides dorei*) whose variance was significantly better predicted by treatment and treatment interaction with time compared with time alone (Table E2). Importantly, the abundance of *B. dorei* was negatively correlated with allograft rejection and fibrosis, whereas several *Clostridial* species showed a positive correlation (Figure 2C).

Prior studies have established a causal link between $CD4^+$ T effector lymphocytes and chronic lung allograft rejection in the murine model of lung transplantation (13). We have also found that depletion of $CD4^+$ T lymphocytes abrogates the IL-17 response in the minor–major histocompatibility complex mismatch model (13). Hence, we further analyzed T-cell subsets and IL-17 expression to determine whether the protection in lung allograft rejection associated with antibiotic pretreatment was due to a reduction in the IL-17 response. We did not find any changes in either the cellular composition or the absolute number of lymphocytes in the allografts (Figure E1). Specifically, there was no difference in T-cell subpopulations ($CD4^+CD25^+Foxp3^+$ regulatory T cells [Tregs] and IL-17–producing T cells) between the treatment and control groups (Figure E1).

Our data suggest that changes in the microbiota associated with reduced lung allograft pathology do not occur via impaired Th17 responses. Instead, the mechanism may be similar to that by which antibiotic pretreatment results in prolonged skin graft survival, namely, a decreased basal state in dendritic cell activation associated with diminished proliferation of graft-reactive T cells (5). Prior studies have demonstrated that metabolism of short-chain fatty

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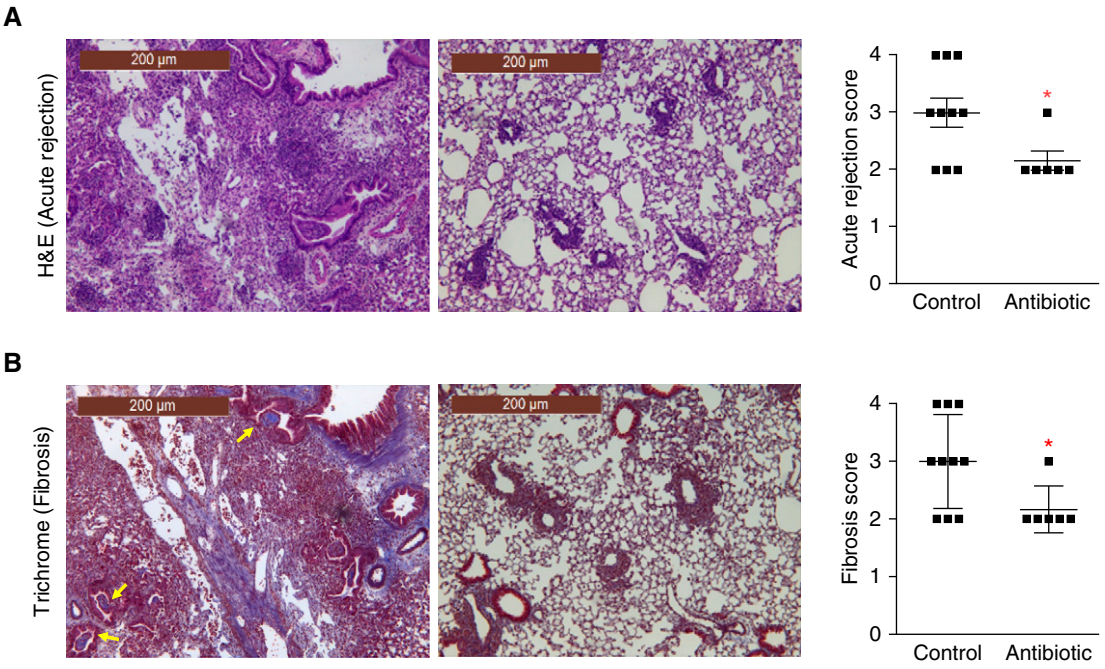

Figure 1. Pretreatment with antibiotics inhibits rejection, fibrosis, and the development of obliterative bronchiolitis. B10 left lungs were transplanted orthotopically into B6 recipients. Ten days before transplantation, the recipients were treated by daily oral gavage with an antibiotic cocktail of gentamycin, kanamycin, colistin, metronidazole, and vancomycin. Lungs were harvested and analyzed on Day 21 after lung transplant. (*A*) Hematoxylin and eosin–stained lung allografts and rejection acute (A) score (top panels). (*B*) Masson's trichrome–stained lung allografts and fibrosis (F) score. Arrows show obliterative bronchiolitis injury. *$P < 0.05$ using unpaired *t* test. Scale bar: 200 μm. H&E = hematoxylin and eosin.

acids leads to the induction of Tregs. We did not find any changes in the levels of Foxp3$^+$ Tregs, but our previous results suggest that Foxp3$^-$IL-10$^+$ Tregs play an important role in the suppression of antigen-specific T effector cells after human lung transplantation (15). It may thus be of interest to explore whether antibiotic-induced dysbiosis ameliorates lung allograft rejection through the induction of Foxp3$^-$IL-10$^+$ Tregs.

In this study, we were not able to analyze the lung microbiome because survival BAL in mice is challenging, but we recognize that the antibiotic regimen likely changed the lung microbiota and that its role in murine lung allograft rejection needs to be further determined. In conclusion, we demonstrate that alterations in microbial diversity are associated with decreased signs of lung allograft rejection, but future studies are needed to determine whether they are sufficient to mediate this effect. ■

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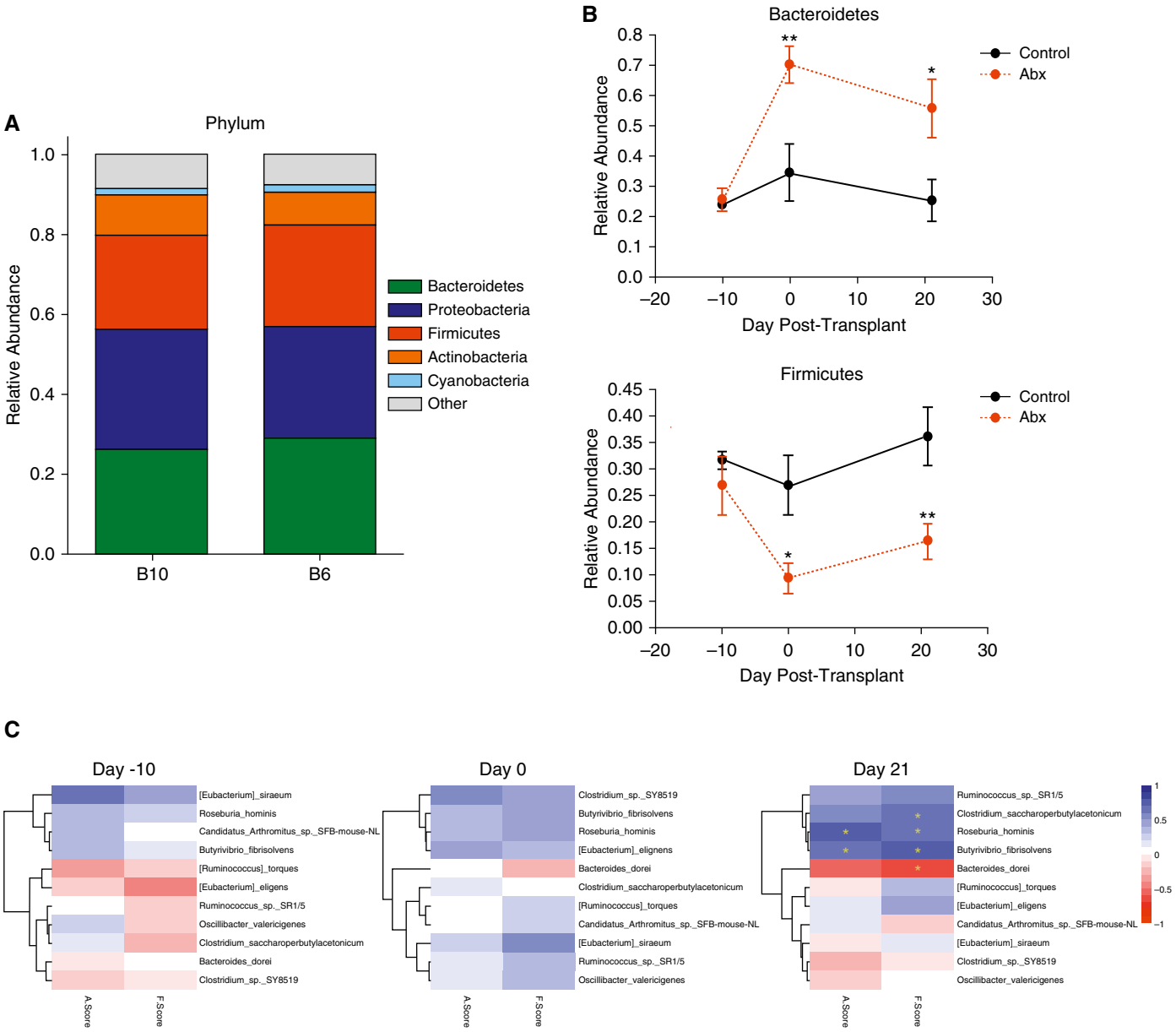

Figure 2. Antibiotic treatment shifts the composition of gut microbiota, which is associated with transplant outcomes. (*A*) Comparison of the top five phyla abundances from B6 and B10 mice at baseline conditions. Stacked bars are averages of $n = 4$ mice in each group. (*B*) Abundances of Bacteroidetes and Firmicutes phyla over the course of the experiment. Points are represented at mean and SEM. $P < 0.05$ was considered significant, Tukey's *post hoc t* test. (*C*) Correlations between significantly different species and A/F scores. $P < 0.05$ was considered significant, Spearman's correlation. * and ** show the points that achieved statistical significance with $P < 0.05$. Abx = antibiotics.

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Retraction: Phenazine Content in the Cystic Fibrosis Respiratory Tract Negatively Correlates with Lung Function and Microbial Complexity

In attempting to determine whether the correlation we previously reported between FEV_1 and phenazine concentrations in cystic fibrosis (CF) sputum (Hunter and colleagues, 2012 [1]) extends to sputum samples from a new patient cohort, we discovered a technical error in the measurement of CF sputum phenazines in our previous study.

At the time, we were using an older high-performance liquid chromatography (HPLC) system for phenazine analysis that lacked tandem mass spectrometry capability. Even though we performed controls by adding phenazines to CF sputum, and used a standard protocol for phenazine analysis that reliably identifies phenazines in bacteriological growth medium and is used by many laboratories, when we performed our new CF sputum analyses with a liquid chromatography–mass spectrometry system, we became aware that under our standard HPLC protocol, a non-phenazine metabolite has nearly identical separation dynamics as phenazines. In reanalyzing our old CF sputum data, we realized that we had unwittingly assigned a specific chromatographic peak to phenazines although that peak most likely represented a different compound (heme). Phenazines were present in some of our sputum samples; however, we had overestimated their abundance. This was an unfortunate but innocent mistake; we maintain that there was no outside interference or research misconduct. (We have prepared a detailed preprint report explaining how we discovered the technical error, the nature of the error, and the new, correct assignment in a manuscript available on bioRxiv [http://biorxiv.org/cgi/content/short/475525v1]; please note this report has not been peer-reviewed or approved by the *AJRCMB*).

We have no reason to question any other data reported in the original manuscript (e.g., microbial community analysis, *Pseudomonas aeruginosa* isolate enumeration and phenazine abundance in bacteriological growth medium, FEV_1 values, or other clinical data). However, we no longer have confidence in the main claim of the paper—that phenazine content in CF sputum negatively correlates with lung function decline and microbial community structure. Rather, heme appears to be responsible for this correlation. Therefore, in conjunction with the Journal editors, we are retracting the article.

The authors of Hunter *et al.,* 2012. ■

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