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Gut microbiota modulates lung fibrosis severity following acute lung injury

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Abstract

Independent reports note the significance of gut microbiota on lung disease severity; however, studies using murine models to define the role of the gut microbiome in pulmonary fibrosis progression are missing. We used the bleomycin murine model to quantify lung fibrosis in C57BL/6J mice housed in germ-free, animal biosafety level 1 (ABSL-1), or animal biosafety level 2 (ABSL-2) environments. Mice housed in gnotobiotic facilities are protected from bleomycin-induced pulmonary fibrosis, while ABSL-1 and ABSL-2 mice develop mild fibrosis and severe lung fibrosis, respectively. Metagenomic analysis of the gut microbiota revealed greater microbial diversity in ABSL-1 compared to ABSL-2 mice, with an increased presence of *Lactobacilli* and *Bifidobacterium* in ABSL-1 mice. Flow cytometric analysis of single-cell lung suspensions revealed enhanced IL-6/STAT3 /IL-17A signaling in CD4⁺ T cells of ABSL-2 mice, compared to ABSL-1 or germ-free mice. Fecal microbiota transplantation (FMT) of low microbial diverse stool (ABSL-2) into germ-free mice before bleomycin administration recapitulated the severe fibrosis phenotype, whereas FMT of ABSL-1 stool induced minimal fibrosis. These findings strongly support a causal role of the gut microbiota in augmenting pulmonary fibrosis severity after acute lung injury.

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Introduction

Accumulating evidence suggests that the gut microbiota have a profound impact on lung pathophysiology, such as the impact of early gut microbiome on childhood asthma¹. Fibrotic lung disease, a major cause of morbidity and mortality worldwide, occurs when fibroblasts are stimulated to overproduce collagen and other extracellular matrix components in response to inflammatory signals². Current treatments rely on slowing disease progression, yet the mortality rate for some fibrotic lung diseases remains unacceptably high³⁻⁵. Studies in patient cohorts with idiopathic pulmonary fibrosis (IPF) have described altered lung microbiota, and correlate high lung bacterial burden with enhanced disease^{6,7}. Additionally, metagenomic sequencing of fecal samples from autoimmune fibrotic lung diseases, such as systemic sclerosis and silicosis, revealed dysbiosis and lack of gut community diversity^{8,9}. Despite these striking clinical correlations, mechanistic studies using murine models to define the role of the gut microbiota in pulmonary fibrosis development and progression are missing.

A growing body of literature supports a gut-lung axis, where processes occurring in the gut associate with outcomes of lung pathologies^{10,11}. The gut microbiota can influence the lung inflammatory environment in several ways. Production of inflammatory cytokines such as IL-6, which play a role in fibrosis development, can be induced by gut microbes¹²⁻¹⁴. Enhanced IL-6 signaling has been shown to contribute to interstitial lung disease (ILD) pathogenesis, through the IL-6/STAT3/IL-17A signaling pathway¹⁵⁻¹⁷. Conversely, microbial metabolites, such as the short chain fatty acid (SCFA) butyrate, produced by *Lactobacilli* and *Bifidobacterium*, can have anti-inflammatory properties and bias T cells toward a Th1 or T regulatory rather than Th17 phenotype^{18,19}. These contrasting roles for the gut microbiota in immune activation suggest that

the specific microbial composition of gut communities is important for enhancing or suppressing pathogenic immune responses contributing to lung fibrosis.

This study used direct modulation of the gut microbiota via rearing environments, fecal microbiota transplantation (FMT) in a bleomycin-induced murine model to demonstrate that a functional gut-lung axis modulates lung fibrosis. While germ-free (GF) mice developed significantly less lung fibrosis than ABSL-1 or ABSL-2 housed mice, FMT of the ABSL-2 stool into germ-free mice before bleomycin administration recapitulated the severe fibrosis phenotype. These results specify that degrees of lung fibrosis severity can be modulated by the absence and/or diversity of the gut microbiota.

Results

Rearing environment impacts severity of pulmonary fibrosis

To assess the contribution of the gut microbiota to lung fibrosis severity, we housed mice in three distinct environments: GF (no microorganisms), ABSL-1 (no experiments involving infectious agents; mice possess only commensal organisms), and ABSL-2 (experiments involving infectious agents of moderate potential hazard to personnel are present in environment). None of the mice in our investigations were ever infected; instead, 6-week-old male and female mice in each environment were administered either saline or bleomycin intranasally and monitored for 14 days. When given intranasal bleomycin, all GF mice exhibited 100% survival (Fig. 1a). ABSL-2 mice administered bleomycin exhibited the greatest mortality, and ABSL-1 survival was intermediate of GF and ABSL-2 mice (Fig. 1a). Significant distinctions in survival were noted among the ABSL-2 and GF mice ($p=0.026$, log-rank Mantel-Cox test). When treated with saline intranasally, mice in all housing cohorts had similar survival

plots (Fig. S1a), did not experience significantly different weight loss (Fig. S1b) and had similar lung collagen content (Fig. S1c), suggesting no acute lung injury. There were significant morbidity distinctions, in terms of weight loss, to mice administered bleomycin. Except for mice housed in gnotobiotic facilities, ABSL-1 and ABSL-2 mice administered saline demonstrated significantly less weight loss than those receiving bleomycin ($p < 0.0001$ and $p < 0.01$, respectively; one-way ANOVA with Tukey's multiple comparison's test) (Fig 1b). Also, GF mice receiving bleomycin exhibited significantly less weight loss compared to ABSL-1 mice ($p < 0.001$, one-way ANOVA with Tukey's multiple comparison's test), as well as ABSL-2 mice ($p < 0.01$, one-way ANOVA with Tukey's multiple comparison's test) (Fig. 1b). Qualitative and quantitative histologic assessments of the lung following bleomycin administration in the three different rearing environments were conducted. To analyze fibrosis parameters, we conducted pulmonary trichrome staining and measured the collagen content by the Sircol assay. Representative hematoxylin and eosin (H&E) and trichrome sections of bleomycin-treated mice under the three rearing conditions (Fig. 1c). Fibrosis severity was less severe by Ashcroft scoring in GF mice compared to ABSL-1 mice, as well as compared to ABSL-2 mice (Fig. 1d). Notably, quantification of collagen content revealed that ABSL-2 mice had significantly higher lung collagen content than GF ($p < 0.0001$, one-way ANOVA with Tukey's multiple comparison's test) or ABSL-1 mice ($p < 0.0001$, one-way ANOVA with Tukey's multiple comparison's test). The ABSL-1 mice had intermediate lung fibrosis of GF and ABSL-2 mice ($p < 0.01$, one-way ANOVA with Tukey's multiple comparison's test) (Fig. 1e). Remarkably, the GF mice administered bleomycin had pulmonary collagen contents similar to mice administered intranasal saline (Figure 1e). These findings indicate that the environment in which the murine host lives contributes to lung fibrosis severity.

Lack of gut microbial diversity associates with severity of lung fibrosis

To investigate the hypothesis that the gut microbiota is an important contributor to the differences in fibrosis severity between ABSL-1 and ABSL-2 housed mice, we performed metagenomic analysis on fecal pellets from mice in each housing cohort. We did not detect microorganisms in the stool of GF mice by sequencing and culture as expected. Shannon alpha diversity, a measure of species richness and evenness, was significantly higher in ABSL-1 mice compared with ABSL-2 mice using a Wilcoxon rank sum test ($W = 166$, $p = 0.010$) (Fig. 2a). Shannon alpha diversity did not differ between treatments (bleomycin vs. saline) using a Wilcoxon rank sum test ($W=367$, $p=0.278$), suggesting that bleomycin treatment does not alter the diversity of the gut microbiota. We compared the relative abundance of phyla using a generalized linear model (GLM) with a negative binomial distribution. ABSL-1 mice had significantly higher relative abundances of Proteobacteria (GLM, $\chi^2=20.221$, $p<0.001$) and Verrucomicrobia (GLM, $\chi^2=4.487$, $p=0.034$) (Fig. 2b). Increased proteobacteria abundance has been linked with both inflammatory gut and airway environments, as in Crohn's disease²⁰, asthma²¹ and after lung transplantation²². Beta diversity differences between ABSL-1 and ABSL-2 microbiota compositions also differed significantly when analysis was conducted using both the Bray-Curtis dissimilarity metric (Fig. 2c) and Jaccard index (Fig. S2) that account for taxon abundance variation and presence/absence of taxa, respectively (PERMANOVA, Bray-Curtis: $F_{2,47}=4.676$, $R^2=0.089$, $p=0.006$; Jaccard: $F_{2,47}=4.735$, $R^2=0.090$, $p=0.003$). Treatment (bleomycin vs. saline) was not significantly associated with taxonomic composition using either metric, indicating that bleomycin treatment does not alter the gut microbiota taxonomic composition (Bray-Curtis: $F_{2,47}=0.696$, $R^2=0.013$, $p=0.546$; Jaccard: $F_{2,47}=0.992$, $R^2=0.019$, $p=0.390$) (Fig. 2c).

Using linear discriminant analysis (LDA) to examine species-level differences in the gut microbiota, 10 taxa were overrepresented in ABSL-1 mice and 5 taxa were *overrepresented in ABSL-2 mice* (Fig. 2d). The overrepresented taxa in ABSL-2 mice, included *Lachnospiraceae bacterium A2*, *Lachnospiraceae bacterium 28-4*, *Firmicutes bacterium ASF500*, and *Romboutsia ilealis*. Relative abundance of Firmicutes in the lung microbiota of bleomycin-treated mice with fibrosis has been reported⁷. Species overrepresented in ABSL-1 mice include *Staphylococcus nepalensis*, *Dubosiella newyorkensis*, *Acetatifactor muris*, *Lactobacillus animalis*, *Lactobacillus murinus*, and *Acutalibacter muris*. Of note, *Lactobacillus* species, which are overrepresented in ABSL-1 mice, have been shown to reduce IL-17A-mediated disease severity in other experimental animal models²³⁻²⁵, and is associated with significant reductions in respiratory symptoms²⁶.

Beta diversity differences in metagenomic functional gene profiles between rearing environment and treatment conditions were also examined using Jaccard distances (Fig. 3a and 3b) and Bray-Curtis dissimilarities (Fig. S3a and S3b). Functional profiles across gene family and MetaCyc reaction pathway abundances were significantly associated with environment and treatment specifically when examining Jaccard distances (Fig. 3c). MetaCyc reaction pathway abundances differed significantly between the gut microbiota composition of ABSL-1 and ABSL-2 mice (Jaccard: $F_{2,47}=3.055$, $R^2=0.058$, $p<0.001$) (Fig. 3c). Similarly, rearing environment was significantly associated with gene family profiles (PERMANOVA; Jaccard: $F_{2,47}=7.030$, $R^2=0.125$, $p<0.001$) (Fig. 3b). A difference between rearing environments only when using Jaccard distances, and not Bray-Curtis dissimilarities, indicates that low abundance pathways are driving the differences between rearing environments. Shannon diversity was higher in ABSL-1 mice for both MetaCyc reaction pathways ($W=544$, $p<0.001$) and gene families ($W=149$, $p=0.003$), but did not differ between saline and bleomycin treatments. GLMs did not identify any differentially

abundant pathways between ABSL-1 or ABSL-2 mice. However, LDA identified 24 pathways that were overrepresented in ABSL-1 mice and one pathway that was overrepresented in ABSL-2 mice (Fig. 3d). Microbial community compositional and functional differences between mice in separate rearing groups with differing amount of fibrosis support the hypothesis that the gut microbiota may directly contribute to the degree of lung fibrosis.

Gut microbial diversity and taxa associate with proinflammatory lung responses

To determine the immunologic drivers of lung fibrosis under distinct rearing environments, we performed flow cytometric analysis of single cell lung suspensions obtained from GF, ABSL-1, and ABSL-2 mice 14 days after inoculation with either saline or bleomycin. We noted no difference in CD4+IL-6+ T cells in the saline-treated control group between the three facilities (Fig. S4a); however, with bleomycin treatment, there was a significant increase in CD4+IL-6+ T cells in ABSL-2 mice compared to GF or ABSL-1 mice (Fig. 4a; representative histogram in Fig. S5a). The same trend occurred for the IL-6 co-receptor, gp130, where bleomycin-treated mice in the ABSL-2 cohort had the highest percentage of CD4+ gp130+ T cells (Fig. 4b representative histograms in Fig. S5b). We previously reported that PD-1+ Th17 cells contribute to the development of lung fibrosis. We also noted nonsignificant levels of Programmed Death-1+(PD-1) Th17 cells in GF and ABSL-1 mice, compared to ABSL-2 mice (Figure 4c). Representative histograms and gating strategy are shown (Fig. 4d, e, S5c). Bleomycin treatment increases pSTAT3 expression in CD4+ T cells in the ABSL-2 cohort mice (Fig. 4f). There was no difference across any treatment or housing cohort in the expression of IL-23R (Fig. S4b), suggesting that increased pSTAT3 is due to enhanced IL-6 rather than IL-23 signaling. The distinctions in the percentages of CD4+IL-17A+ T cells in GF and ABSL-2 mice support microbiota-induced alterations to the IL-6/ STAT3/IL-17A pathway in pulmonary fibrosis.

As there were differences between immune variables in the mice with different microbiota, we looked for associations between individual taxa abundances and immunological variables using Spearman correlation tests. Several taxa, including members of the Firmicutes and Proteobacteria, were found to be negatively correlated with multiple immunological values (Table 1), and *Romboutsia ilealis* was positively correlated with all immunological values (Table 1). The species negatively correlated with immune activation include *Lactobacillus* species, which have been shown to produce butyrate that dampens Th17 polarization^{18,19}.

Reconstitution of low diversity gut microbiota enhances pulmonary fibrosis

To test the hypothesis that the gut microbiota causes fibrosis severity, we performed fecal microbiota transplantation (FMT) experiments. GF mice were gavaged every other day with ABSL-1 or ABSL-2 stool for one week prior to bleomycin inoculation and during the 14 days following the induction of lung injury. We observed a trend in increased weight loss at day 14 compared to untreated GF mice in mice treated with ABSL-2 stool, but not ABSL-1 stool (Fig. 5a). This corresponded with a significant increase in lung collagen content in the GF mice that underwent FMT of ABSL-2 stool. No increase in lung fibrosis was detected among GF mice following FMT of ABSL-1 stool (Fig. 5b). This data suggests that lung injury alone is not sufficient to induce fibrosis but that bacteria capable of inducing profibrotic cytokine expression also need to be present in the gut.

Discussion

Using the murine model of pulmonary fibrosis under distinct rearing environments, we establish the impact of variation in gut microbial diversity on lung fibrosis severity. In particular, low gut

microbial diversity was present among ABSL-2 mice that possessed severe lung fibrosis, whereas higher microbial diversity was present among ABSL-1 mice with less lung fibrosis following bleomycin administration (Fig. 2). Despite intranasal bleomycin treatment, GF mice did not develop lung fibrosis. FMT of ABSL-2 stool into GF mice before bleomycin treatment enhanced fibrosis severity, directly linking gut microbial composition with disease outcomes. (Fig. 5).

We observed protection from bleomycin-induced acute lung injury in GF mice (Fig. 1), supporting the hypothesis that acute lung injury alone is not sufficient to induce lung pathology, but rather “two-hits” are necessary to induce pulmonary fibrosis: 1) acute lung injury (ALI) and 2) presence of microorganisms capable of inducing profibrotic cytokine expression, such as IL-17A. ABSL-2 mice do not spontaneously develop lung fibrosis, even if housed for seven months (data not shown). Remarkably, GF mice demonstrate minimal collagen deposition following intranasal bleomycin instillation. The significance of the gut microbiota to lung fibrosis progression was further supported by FMT of ABSL-2 stool into GF mice to recapitulate fibrotic lung disease severity. FMT of ABSL-1 stool into GF mice did not induce significant lung fibrosis. A recent clinical trial noted reductions in the incidence of upper respiratory tract infections among patients randomized to probiotics containing *Lactobacilli* and *Bifidobacteria* compared to placebo²⁶, further supporting the importance of the gut-lung axis in lung disease.

Investigation of the pulmonary inflammatory milieu among the different cohorts of mice provides an interesting linkage between profibrotic cytokine expression and reduced microbial diversity. Independent investigators support the capacity of the gut microbiota to induce systemic inflammation, specifically increased IL-6, IFN- γ and IL-17A, through mechanisms such as 1) formation of IsoLG-protein adducts in dendritic cells, which drive IFN- γ and IL-17A production by T cells²⁷; 2) augmentation of gut permeability allows translocation of bacterial endotoxin

(lipopolysaccharide, LPS) from the gut microbiota to blood circulation, leading to microbiota-dependent interleukin-17A (IL-17A) secretion from lamina propria derived Th17 cells²⁸; or 3) Reduced expression of short chain fatty acids, such as butyrate, by the gut microbiota enhances Th17 cell expansion²⁹. It is also noteworthy that microbial strains shown to improve gut permeability, such as *Bifidobacterium*, were most prevalent in the ABSL-1 stool (Fig. 2). Importantly, many of the organisms enriched in the ABSL-1 cohort, including *Dubosiella newyorkensis*, *Staphylococcus nepalensis*, and two *Lactobacillus* species were also correlated with decreased immune activation (Table 1). The only species that had a positive correlation with immune activation, *Romboutsia ilealis*, was enriched in the ABSL-2 mice. *Romboutsia* species are associated with the metabolic changes associated with obesity³⁰, which serves as an independent risk factor for pulmonary progression in the ILD sarcoidosis³¹. Further investigation of the impact of specific microbial species such as *Romboutsia* on metabolic disorders associated with lung fibrosis is warranted. Metagenomic analysis also revealed relatively higher abundance of *Lactobacillus* species in ABSL-1 housed mice (Fig. 2), which demonstrated fewer Th17 cells (Fig. 4). Various *Lactobacilli* species can reduce IL-17A expression via TLR2/4 in epithelial cells²⁵ or through production of metabolites such as butyrate²³. SCFA, like butyrate, reduce inflammatory conditions following insult through their capacity to decrease IL-17A synthesis, as well as promote IL-10 generation through T regulatory cell development¹⁹. Modulation of the gut microbiota of mice by oral gavage of *Lactobacillus reuteri* protected mice from multiple sclerosis-like disease symptoms by decreasing IL-17A levels²⁴. Future investigation of the impact of microbial diversity on lung fibrosis is warranted, including assessing the ability of probiotic treatment with *Lactobacillus spp.* to reduce disease severity in patients with lung fibrosis.

In addition to identifying members of the gut microbiota that may contribute to or protect from severe lung fibrosis, our investigation suggests that increased diversity in gut community composition is beneficial with respect to pulmonary disease severity (Figs. 1, 5). This observation follows with the ecological principle that more diverse ecosystems are more resistant to perturbations than less diverse systems. Diversity has been shown to be beneficial for both community stability and functionality over time, whether members of the ecosystem are animals, plants, or microorganisms³²⁻³⁵. The human gut microbiota represents a thriving ecosystem with taxonomic and functional diversity within and between individuals³⁶. Fecal microbiota transplantation (FMT) has been explored clinically to increase gut microbial diversity to combat complications such as those occurring after allogeneic hematopoietic stem cell transplantation³⁷ and *Clostridium difficile* infection³⁸. Additional murine studies using FMT have demonstrated increased murine survival after acute liver injury and during acute respiratory distress syndrome by decreasing IL-17A levels^{39,40}. Our investigations add to this growing body of FMT literature, suggesting that functional and taxonomic diversity in the gut microbiota of patients with lung fibrosis may improve their clinical outcomes, and that decreased gut microbial diversity may predispose individuals to more severe manifestations of fibrotic lung disease (Fig 6). Molecular examination of ABSL-1 stool demonstrated that there were some proinflammatory bacteria present, such as proteobacteria which has been linked with both inflammatory gut and airway environments asthma²¹ and after lung transplantation²²; however, companion microorganisms, such as *lactobacilli*, limit their capacity to induce lung disease severity.

There are limitations to this investigation. While our sequencing studies focused on the bacterial members of the gut community, the potential role of fungal and viral contributors to the gut microbiota's impact on pulmonary fibrosis cannot be discounted. In addition, other components

of stool, including host-derived cells and metabolites, are transferred to the naïve host during FMT⁴¹. Also, this study did not probe lung microbial communities among mice in the different rearing environments; altered lung microbiota have been noted to be important in ILD patients^{7,42}. Further investigation of the influence of the gut microbiota on the lung microbial community and vice versa is warranted.

Overall, our study implicates the low-diversity gut microbiota as a contributor of pulmonary fibrosis severity through their capacity to activate the IL-6/STAT3/IL-17A pathway. These findings support that acute lung injury in a host with low microbial diversity leads to more severe lung disease, compared to hosts with increased microbial diversity. Validation of the potential benefit of enhancing gut microbial diversity in ILD patients is warranted.

Methods

Murine model of lung fibrosis

We reared mice in either germ-free (gnotobiotic conditions with no microbial interactions, animal biosafety level 1 (ABSL-1) experimental conditions in which no infections were conducted, or animal biosafety level 2 (ABSL-2) environments in which infections with pathogenic microorganisms occur. None of the mice in any of the facilities underwent inoculation with any microorganisms; only bleomycin was administered intranasally to induce lung fibrosis. For the murine model of bleomycin-induced pulmonary fibrosis, 6- to 8-week-old C57BL/6J mice from Jackson Labs weighing approximately 17-22g were used. Mice were anesthetized by intraperitoneal injection of 80 µl of 20mg/ml Ketamine/1.8 mg/ml Xylazine solution, then 75µl containing 0.04 Units of bleomycin (Novaplus Lake Forest IL) in saline (0.9% sodium chloride) (Hospira Inc, Lake forest IL) as control were administered intranasally to mice in each housing

cohorts as previously described⁴³. Lungs were harvested for histology, flow cytometry, or single-cell isolation as previously described⁴⁴. All murine procedures were performed according to the protocol approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center (protocol #M1700043; ABSL-1 and ABSL-2 facilities, and FMT experiments) or University of Louisville (protocol #20786; Germ-free facilities). Germ-free isolators at the University of Louisville are sampled (swabs of the environment plus fresh fecal pellets from at least 3-4 random cages) and cultured aerobically on Chocolate Agar and TSA w/ 5% Sheep Blood Agar at 37°C and anaerobically on Anaerobic PEA Agar, LKV/BBE Agar (Laked Kanamycin Vancomycin/Bacteroides Bile Esculin) and Brucella Agar at 37°C each time the isolator is opened (roughly once/week but opened as few times as possible). No microbial growth was detected in any isolator used in this study.

Metagenomic analysis of gut microbiota

Fecal pellets were collected from mice in each housing cohort and genomic DNA (gDNA) was extracted with the Qiagen DNAeasy extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The gDNA concentration and quality were confirmed using the Bioanalyzer 2100 system (Agilent, Santa Clara, CA).

Paired end sequences were trimmed, quality-filtered, and host contaminants were removed in KneadData (v.0.7.6) using default values and the C57BL mouse reference database. Taxonomic composition was profiled using MetaPhlAn (v.3.0.4)⁴⁵ and functional composition was profiled using HUMAnN3 (v3.0.0)⁴⁶. Samples with less than 10 million sequence reads remaining after quality filtering and removing host reads were removed prior to downstream analysis. Permutational multivariate analysis of variance (PERMANOVA) was used to assess the effects of

both housing (ABSL-1 vs. ABSL-2) and treatment (saline vs. bleomycin) on Bray-Curtis dissimilarity and Jaccard distance matrices constructed from species-level relative abundance tables. PERMANOVAs were performed using the *vegan* package in R^{47,48}. Wilcoxon Rank Sum tests in R were used to examine differences in Shannon diversity between the ABSL-1 and ABSL-2 environments. We used linear discriminant analysis in LEfSe⁴⁹ to identify differentially abundant taxa and pathways across both housing and treatment groups. Generalized linear models (GLMs) using a negative binomial distribution using the *glmmTMB* package in R⁵⁰ were additionally used to identify differentially abundant taxa and functional pathways. Spearman correlations were used to examine associations between individual taxa abundances and physiological variables. False discovery rate (FDR) corrected p-values were calculated using the *fdrtool* package in R⁵¹ and are reported for all GLMs and Spearman correlations. Code for all analyses can be found at github.com/emallott/PulmonaryFibrosisMicrobiota.

Flow cytometry

All flow cytometry experiments were acquired with a LSR-II flow cytometer (BD Biosciences), and information on all antibodies used in this study is listed in Supplemental Table 1. Live cells were gated based on forward and side scatter properties, and surface staining of cells was performed as previously described⁵². Th17 cells were identified using flow cytometry as previously described⁵³. Cells were gated on singlets, live CD3⁺ and CD4⁺ cells. Data analysis was performed using FlowJo software (Tree Star, Ashland, OR). A minimum of 50,000 events were acquired per sample.

Fecal Microbiota Transplantation Experiments

Fecal pellets were collected from mice housed in the ABSL-1 and ABSL-2 facilities. These pellets were suspended in sterile PBS to create fecal slurries at 100mg feces/ mL PBS. Germ-free mice were given 100 uL of the fecal slurry by oral gavage every other day, beginning one week before intranasal inoculation with bleomycin. The germ-free mice gavaged with stool were also fed non-sterile food that originated from either the ABSL-1 or ABSL-2 facility, to match the stool received. Mice were humanely euthanized, and tissues collected 14 days after bleomycin treatment.

Statistics

Multiple-group comparisons were performed using a one-way analysis of variance (ANOVA) with Tukey's post hoc test. Statistical analysis for all figures was carried out using Prism version 7.02 (GraphPad Software). For a result to be considered statistically significant, a *P* value of less than 0.05 was used.

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Author Contributions

O.S.C. and L.E.H. designed and performed experiments, analyzed data, and wrote the manuscript. E.K.M. performed the metagenomic analysis. A.C., J.C.V., H.W., and B.S. performed experiments. S.R. Bordenstein and G.R.B. contributed to the intellectual content and editing of the manuscript and study design. S.D., S.R. Bodduluri, and H.B. aided with the germ-free experiments. W.P.D. conceived the study, designed experiments, analyzed data, and wrote the manuscript.

Table 1. Spearman correlation associations between individual taxa abundances and immunological variables. Spearman correlation coefficients and FDR-corrected p-values are reported. NR = no significant relationship after FDR correction.

	CD4+ PD-1+ IL-17A+ T cells	CD4+ pSTAT3+ T cells	CD4+ gp130+ T cells
<i>Dubosiella newyorkensis</i> (Firmicutes)	$\rho = -0.548$, $p_{\text{fdr}}=0.011$	NR	NR
<i>Lactobacillus animalis</i> (Firmicutes)	NR	$\rho = -0.619$, $p_{\text{fdr}}=0.012$	$\rho = -0.532$, $p_{\text{fdr}}=0.029$
<i>Lactobacillus murinus</i> (Firmicutes)	NR	$\rho = -0.619$, $p_{\text{fdr}}=0.001$	$\rho = -0.541$, $p_{\text{fdr}}=0.029$
<i>Parasutterella</i> <i>excrementihominis</i> (Proteobacteria)	NR	NR	$\rho = -0.585$, $p_{\text{fdr}}=0.029$
Proteobacteria bacterium CAG.139 (Proteobacteria)	NR	NR	$\rho = -0.564$, $p_{\text{fdr}}=0.029$
<i>Romboutsia ilealis</i> (Firmicutes)	$\rho = 0.412$, $p_{\text{fdr}}=0.043$	$\rho = 0.599$, $p_{\text{fdr}}=0.012$	$\rho = 0.542$, $p_{\text{fdr}}=0.029$
<i>Staphylococcus nepalensis</i> (Firmicutes)	$\rho = -0.407$, $p_{\text{fdr}}=0.044$	$\rho = -0.608$, $p_{\text{fdr}}=0.012$	$\rho = -0.528$, $p_{\text{fdr}}=0.029$
<i>Turicimonas muris</i> (Proteobacteria)	NR	NR	$\rho = -0.511$, $p_{\text{fdr}}=0.033$

Figure 1

Figure 1. Germ-free mice are protected from lung fibrosis morbidity and mortality.

(a) Survival curve of mice housed in gnotobiotic, ABSL-1, or ABSL-2 facilities and intranasally inoculated with bleomycin.

(b) Body weight of mice in the housing and treatment cohorts was continuously measured and recorded daily from day 1 to day 14.

(c) Representative images for H&E- and trichrome-stained lungs of GF, ABSL-1 and ABSL-2 mice, 14 days after bleomycin injury with Scale bar, 100 µm. (d) Ashcroft Scoring of trichrome-stained lungs by rearing environments following bleomycin administration to mice (n = 6-12 per cohort).

(e) Soluble collagen content of RLL of mice exposed to bleomycin or saline treatment and housed in gnotobiotic, ABSL-1, or ABSL-2 facilities by Sircol assay; Data are reported as mean ± SD with each dot representing an individual mouse, n= 6-25 mice. Statistical significance was assessed using a one-way ANOVA with Tukey's multiple comparison's test. *P<0.05 **P < 0.01, ***P< 0.001 ****P< 0.0001; GF: Germ-free, ABSL-1: Animal Biosafety Level 1, ABSL-2: Animal Biosafety Level 2, ns = no significance; RLL: right lower lobe.

Figure 2. Increased gut microbial diversity is associated with better outcomes in murine model of fibrosis. (a) Shannon diversity index scores for ABSL-1 and ABSL-2 mice (b) Phyla-level composition of the gut microbiome of ABSL-1 and ABSL-2 mice (c) Nonlinear multidimensional scaling (MDS) plot showing differences in microbial taxonomic composition based on Bray-Curtis dissimilarities (d) Results of linear discriminant analysis showing taxa that are overrepresented in ABSL-1 or ABSL-2 mice

Figure 3

Figure 3. Functional gene profiles are significantly associated with rearing environment and treatment. (a) MetaCyc reaction pathway abundance and (b) gene family abundance profiles using nonlinear multidimensional scaling (MDS) plots based on Jaccard distance matrices (c) Heatmap showing normalized abundances of MetaCyc reaction pathway abundances between rearing environment and treatments (d) Results of linear discriminant analysis showing MetaCyc reaction pathways that are overrepresented in ABSL1 or ABSL2 mice

Figure 4

Figure 4. Germ-free mice do not mount a proinflammatory response upon bleomycin treatment.

Flow cytometric analysis of T cells from single cell lung suspensions at day 14. Single cell lung suspensions were obtained from mice housed in gnotobiotic, ABSL-1, or ABSL-2 facilities (GF, ABSL-1 and ABSL-2). Flow cytometry analysis was conducted for CD4+ (a) IL-6 (b) gp130 (c) PD-1+ IL-17A+ (d) gating strategy (e) representative histograms and (f) pSTAT3^{Y705} expression on T cells. Comparisons between cohorts were performed using one-way ANOVA with Tukey's post-hoc. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: no significance. Bars are mean $\pm$ SD; each symbol represents an individual mouse, n = 2-19 mice.

Figure 5

Figure 5. Reconstitution of an ABSL-2 microbiome in GF mice restores lung fibrosis morbidity. Germ-free mice were gavaged with ABSL-1 or ABSL-2 fecal slurries and given bleomycin to induce lung fibrosis (a) Weight loss at day 14 and (b) RLL collagen content by Sircol assay were determined across the gavage treatment groups. Each symbol represents an individual mouse. Statistical significance was assessed using a one-way ANOVA with Tukey's post-hoc. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = no significance

Figure 6

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Figure 6. Model of gut contribution to fibrotic lung disease.

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Supplemental Information for *Gut microbiota modulates lung disease severity following acute injury*

Supplementary Figure 1

Supplemental Figure 1. Housing conditions does not impact morbidity and mortality during saline treatment. (a) Survival curve of mice housed in 3 separate facilities and intranasally inoculated with saline. (b) Weight loss of mice in the saline treatment cohorts (c) Sircol assay of mice housed in 3 separate facilities and treated with saline (control group); Data are reported as mean $\pm$ SD with each dot representing an individual mouse. Statistical significance was assessed using a one-way ANOVA with Sidak's multiple comparison's test. * $P < 0.05$ ** $P < 0.01$, **** $P < 0.0001$; ns = no significance; RLL: right lower lobe. Data in (b) and (c) are shown as part of Figure 1.

Supplementary Figure 2

Supplemental Figure 2. Rearing environment alters gut microbiota composition. Nonlinear multidimensional scaling (MDS) plot showing differences in microbial taxonomic composition based on Jaccard index

Supplemental Figure 3. Functional profiles are not different between floor or treatment when using Bray-Curtis dissimilarity matrices (a) Gene family abundance and (b) MetaCyc reaction pathway abundance profiles based on Bray-Curtis dissimilarity matrices.

Supplementary Figure 4

Supplemental Figure 4. Gut microbial diversity and taxa associate with inflammatory pulmonary lung responses. Single cell lung suspensions were obtained from mice housed in three different facilities (GF, ABSL-1 and ABSL-2). Flow cytometry analysis was conducted for (a) IL-6 and (b) IL-23R expression on CD4+T cells. Statistical significance was assessed using a one-way ANOVA with Tukey's multiple comparison's test. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, ns = no significance $n=2-19$.

Supplementary Figure 5a

a Germ Free (CD4⁺ IL-6⁺ T Cells (%))

b ABSL-1 (CD4⁺ IL-6⁺ T Cells (%))

c ABSL-2 (CD4⁺ IL-6⁺ T Cells (%))

Supplementary Fig. 5a: Representative histogram depicting IL-6 expression on CD4⁺ T cells in GF, ABSL-1 and ABSL-2 saline and bleomycin treated mice

a) Representative histogram depicting IL-6 expression on CD4⁺ T cells in GF mice b) Representative histogram depicting IL-6 expression on CD4⁺ T cells in ABSL-1 mice c) Representative histogram depicting IL-6 expression on CD4⁺ T cells in ABSL-2 mice. GF: Germ-free, ABSL-1: Animal Biosafety Level 1, ABSL-2: Animal Biosafety Level 2

Supplementary Figure 5b

Supplementary Fig. 5b: Representative histogram depicting GP130 expression on CD4⁺ T cells in GF, ABSL-1 and ABSL-2 saline and bleomycin treated mice

a) Representative histogram depicting GP130 expression on CD4⁺ T cells in GF mice b)
 Representative histogram depicting GP130 expression on CD4⁺ T cells in ABSL-1 mice c)
 Representative histogram depicting GP130 expression on CD4⁺ T cells in ABSL-2 mice

GF: Germ-free, ABSL-1: Animal Biosafety Level 1, ABSL-2: Animal Biosafety Level 2

Supplementary Figure 5c

Germ Free (CD4⁺ PD-1⁺ IL-17A⁺ T Cells (%))

Supplementary Fig. 5c: Representative histogram depicting PD-1+ IL-17A+ expression on CD4⁺ T cells in GF, ABSL-1 and ABSL-2 saline and bleomycin treated mice. a) Representative histogram depicting PD-1+ IL-17A+ expression on CD4⁺ T cells in GF mice b) Representative histogram depicting PD-1+ IL-17A+ expression on CD4⁺ T cells in ABSL-1 mice c) Representative histogram depicting PD-1+ IL-17A+ expression on CD4⁺ T cells in ABSL-2 mice. GF: Germ-free, ABSL-1: Animal Biosafety Level 1, ABSL-2: Animal Biosafety Level 2

Supplemental Table 1. Antibodies used in this study

Antibody	Vendor Catalog No	Host organism; antibody type	Clone	Conc (µg)/volume (µL) per test*
CD3 Alexa Fluor 700	Biolegend 100216	Rat Monoclonal	17A2	0.25 µg/µL
CD4 APC-Cy7	Biolegend 100414	Mouse Monoclonal	GK1.5	0.1 µg/µL
IL-6 APC	Biolegend 504808	Rat Monoclonal	MP5-20F3	0.04 µg/µL
GP-130 PE	Biolegend 149404	Rat Monoclonal	4H1B35	0.02 µg/µL
PD-1 PE-Cy7	Biolegend 135216	Rat Monoclonal	29F.1A12	0.1 µg/µL
IL-17A PE	Biolegend 506904	Rat Monoclonal	TCII-18H10.1	0.1 µg/µL
STAT3 (pY703) Pacific Blue	BD 560312	Mouse Monoclonal	4/P-STAT3	10 µg/µL
IL-23R APC	Biolegend 150906	Rat Monoclonal	12B2B64	0.02 µg/µL

*Test = 1 million cells in 50µl of staining buffer