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Airway microbiota signals anabolic and catabolic remodeling in the transplanted lung

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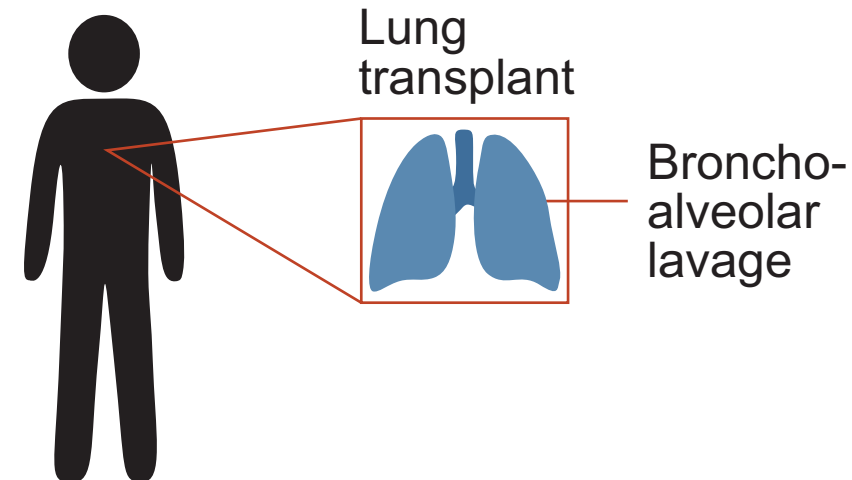
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Microbiota Impact on Airway Remodeling

ACCEPTED MANUSCRIPT



Microbiota:

Bacterial communities



Prevotella, Streptococcus
Veillonella, Neisseria



Staphylococcus, Pseudomonas
Haemophilus, Corynebacterium

Host:

Cell differential



M2 macrophage



Myofibroblast



M1 macrophage



Neutrophil



Fibroblast

Gene expression



Platelet-derived growth factor



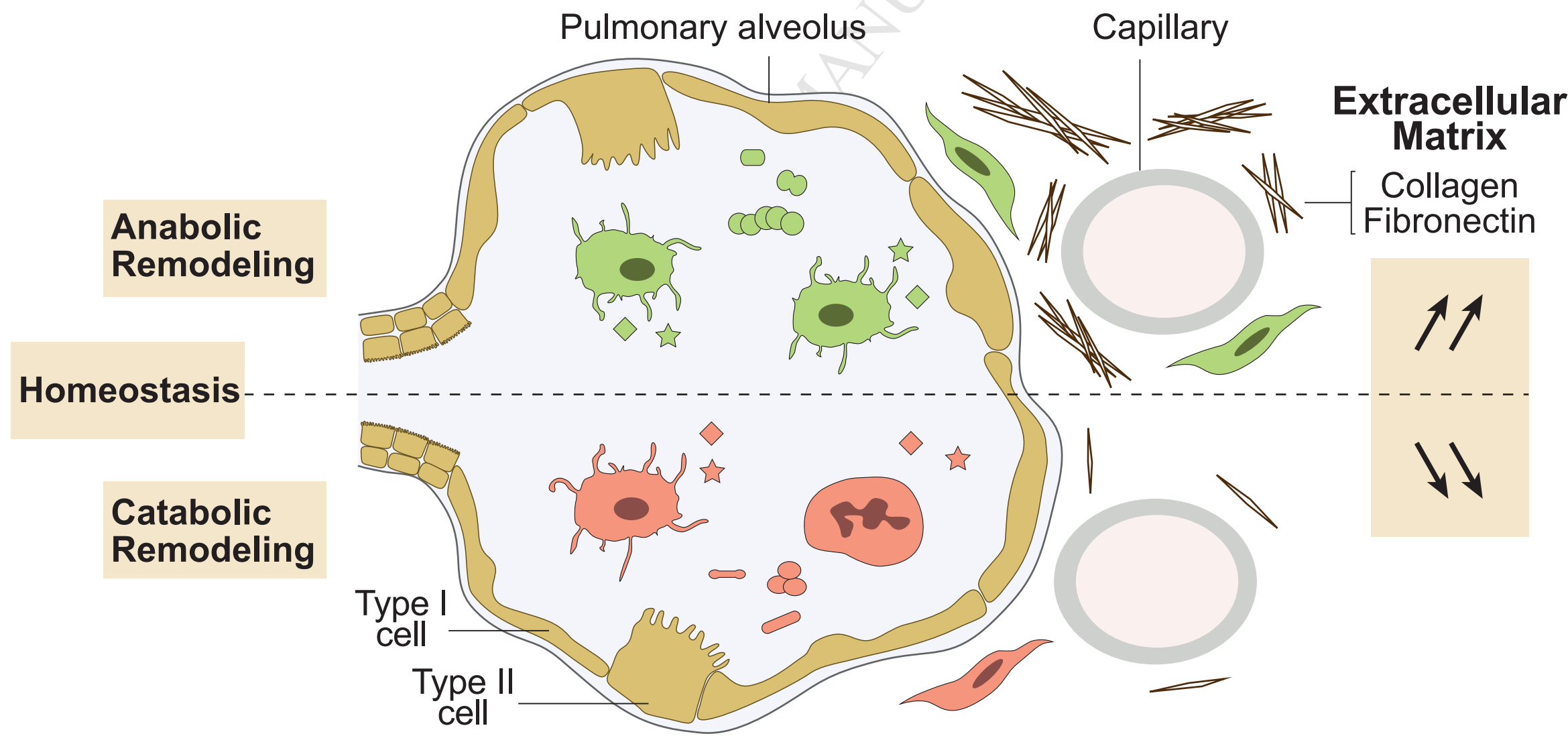
Thrombospondin



Matrix metalloproteinases



Osteopontin



Airway microbiota signals anabolic and catabolic remodeling in the transplanted lung

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Capsule Summary: The rate of matrix deposition varies with the microbial composition in the transplanted lung, potentially impacting upon graft survival.

Key messages: 1) Four host remodeling gene expression profiles that align with different bacterial communities prevail in the transplanted lung. 2) Typical bacterial pathogens that occasionally bloom during the first months post-transplant appear to promote the degradation of

the extracellular matrix, while bacteria belonging to a healthy steady state microbiota permit fibroblast to myofibroblast differentiation and matrix deposition.

Key words: microbiota; macrophages; fibroblasts; matrix; airway remodeling.

Abbreviations: BAL: Bronchoalveolar lavage; CLAD: Chronic Lung Allograft Dysfunction; COPD: Chronic obstructive pulmonary disease; GO: Gene Ontology; IPF: Idiopathic pulmonary fibrosis; PCA: Principal component analysis; THP-DM: THP1-derived macrophages.

ABSTRACT

Background Homeostatic turnover of the extracellular matrix conditions the structure and function of the healthy lung. In lung transplantation, long-term management remains limited by Chronic Lung Allograft Dysfunction (CLAD), an umbrella term used for a heterogeneous entity ultimately associated with pathological airway and/or parenchyma remodeling.

Objective To assess whether the local cross-talk between the pulmonary microbiota and host cells is a key determinant in the control of lower airway remodeling post-transplantation.

Methods Microbiota DNA and host total RNA were isolated from 189 bronchoalveolar lavages obtained from 116 patients post-lung transplantation. Expression of a set of 11 genes encoding either matrix components, or factors involved in matrix synthesis or degradation (anabolic and catabolic remodeling, respectively), was quantified by real-time quantitative PCR. Microbiota composition was characterized using 16S ribosomal RNA gene sequencing and culture.

Results We identified four host gene expression profiles, amongst which catabolic remodeling, associated with high expression of metalloproteinase-7, -9, and -12, diverged from anabolic

remodeling linked to maximal thrombospondin and platelet-derived growth factor D expression. While catabolic remodeling aligned with a microbiota dominated by pro-inflammatory bacteria (e.g. *Staphylococcus*, *Pseudomonas* and *Corynebacterium*), anabolic remodeling was linked to typical members of the healthy steady state (e.g. *Prevotella*, *Streptococcus* and *Veillonella*). Mechanistic assays provided direct evidence that these bacteria can impact upon host macrophage-fibroblast activation and matrix deposition.

Conclusion Host-microbes interplay potentially determines remodeling activities in the transplanted lung highlighting new therapeutic opportunities to ultimately improve long-term lung transplant outcome.

INTRODUCTION

Despite a marked improvement over the last two decades, lung transplantation remains associated with a lower survival rate (54% at 5 years post-transplantation in 2013),¹ compared to the heart, kidney, liver and pancreas. In fact, long-term management of the lung allograft faces a major, as yet incompletely characterized condition: Chronic Lung Allograft Dysfunction (CLAD), an umbrella term used to describe different phenotypes of chronic lung allograft rejection, for which a general consensus on the precise clinical definition is still lacking. The heterogeneity of CLAD phenotypes reflects the complexity of the underlying mechanisms, whereby inflammation combined with aberrant airway and/or parenchyma remodeling progressively impairs lung function, ultimately leading to re-transplantation or death.² Consequently, there is an urgent need for increasing our understanding of CLAD pathophysiology and identifying biomarkers specific for the different CLAD phenotypes.

A rich and balanced airway microbiota has been linked with the maintenance of local tissue homeostasis. Perturbation in the composition of this microbial community, known as dysbiosis, has been reported in a variety of respiratory conditions, including lung transplantation.³⁻⁶ We recently showed that predominance of pro-inflammatory (e.g. *Staphylococcus* and *Pseudomonas*) or low stimulatory (e.g. *Prevotella* and *Streptococcus*) bacteria aligned with inflammatory or tissue remodeling gene expression profiles, respectively, in bronchoalveolar lavage (BAL) cells following transplantation, suggesting that the pulmonary microbiota may impact upon long-term graft survival.⁷

In the present study, we sought to dissect host-microbe interactions in the context of the complex remodeling processes taking place following lung transplantation. We show that the transplanted lung presents distinct remodeling profiles, characterized by the expression of genes differentially involved in matrix synthesis or degradation. In addition, we provide evidence that the constituents of microbial communities dominated by these bacterial taxa fine-tune gene expression profiles in macrophages and fibroblasts - two key cell types in the regulation of remodeling processes.

MATERIALS AND METHODS

Patient sample collection and ethics statement

BAL samples were collected during surveillance bronchoscopies carried out during the first 14 months post-transplant, from October 2012 to July 2014 in 6 Swiss and French transplantation centres, within the framework of the European project SysCLAD (System prediction of Chronic Lung Allograft Dysfunction).⁸ The national and local ethics committees approved the study, and all subjects, whose details are provided in Table 1, gave written informed consent.

Detail on BAL fluid collection and processing is provided in this article's Online Repository. BAL fluid was submitted to cell differential determination, culture-dependent bacterial and fungal detection, and PCR-based detection of viral infection, according to routine clinical procedures. Negative controls obtained upon washing a ready-to-use endoscope with sterile saline were prepared following the same procedure.

RNA-seq and data analysis

Total BAL cellular RNA converted into cDNA libraries using the Illumina TruSeq RNA Sample Preparation Kit (Illumina, San Diego, CA) was submitted to high throughput sequencing using the Illumina HiSeq 2500 System. Gene expression quantification was based on reads per kilobase of exon model per million mapped reads.

Extraction of remodeling genes from Gene Ontology database

A panel of 627 remodeling-related genes were extracted from Gene Ontology (GO) database (release May 2016) using the browser AmiGo⁹ (version 2.3), based on GO Terms listed in Figure 1, B.

Real-time quantitative PCR for characterizing gene expression profiles

BAL cellular RNA was extracted, cDNA synthesized and amplification performed using custom oligonucleotide primers and probes (Microsynth, Balgach, Switzerland; see detail in the Methods section and Table E1 in this article's Online Repository).

Bacterial 16S rRNA gene amplification, sequencing, and metagenome prediction

The 16S content of BAL fluid DNA was characterized either by quantitative PCR using previously reported primers specific to pan bacteria or major phyla (Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria and Fusobacteria), or Illumina MiSeq sequencing using primers targeting the V1V2 region, as previously described.¹⁰ Sequences were processed and the resulting table of operational taxonomic units (OTUs) was used for metagenome prediction, using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) software¹¹ (see detail in the Methods section in this article's Online Repository).

Bacterial cultures

Culture conditions for *Staphylococcus aureus* (ATCC 25904), *Pseudomonas aeruginosa* (ATCC BAA-47), *Streptococcus pneumoniae* (NCTC 7466) and *Prevotella melaninogenica* (ATCC 25845) are provided in this article's Online Repository. For stimulation experiments, suspensions were diluted to reach a concentration of 4×10^{-3} (*P. aeruginosa* and *S. pneumoniae*) or 2×10^{-3} (*S. aureus* and *P. melaninogenica*) optical density at 600 nm corresponding to 10^6 colony-forming units (CFU) per milliliter.

In vitro macrophage-fibroblast co-culture model of matrix deposition

Conditions for maintenance of human THP-1 monocytic cell line (ATCC TIB-202) and MRC5 fibroblasts (ATCC CCL-171), and Macrophage Colony-Stimulating Factor 1-driven generation of THP-1-derived macrophages (THP-DM) are provided in this article's Online Repository. Stimulation using bacterial suspensions described above, at a density of 5 CFU per eukaryotic cell at day 0, was performed for 30 hours (gene expression analysis) or 6 days (quantification of matrix deposition) in minimum essential medium (Thermo Fisher Scientific, Rochester, NY), supplemented with 70- and 400-kDa Ficoll (37.5 and 25 mg/mL, respectively; GE Healthcare,

Little Chalfont, UK) and transforming growth factor- β 1 (TGF- β 1, 5 ng/ml; eBioscience, San Diego, CA) for optimal matrix deposition, along with a mixture of prednisolone (500 nM; Sigma, St. Louis, MO) / FK506 (tacrolimus, 25 nM; Enzo Life Sciences, Lausen, Switzerland) / mycophenolic acid (10 μ M; Tocris Bioscience, Ellisville, MO). Penicillin and streptomycin (1000 U/mL and 100 μ g/mL, respectively; Thermo Fisher Scientific) were used to prevent bacterial growth, and cell viability was assessed using a dedicated colorimetric assay (Cell Counting Kit-8, Dojindo Molecular Technologies, Rockville, MD). Immunostaining for collagen type 1 and fibronectin, and automated image acquisition are detailed in this article's Online Repository.

Principal component and statistical analysis

To analyze the distribution of BAL cell gene expression profiles, the target gene/reference gene copy number ratio was log-transformed and principal component analysis (PCA) was performed using prcomp (scaled) routine in R.¹² Multiple-group comparisons were performed using Kruskal-Wallis test with Dunn's post-hoc analysis and post-transplant differences in the relative abundance of the remodeling gene expression profiles were compared using Chi-square test (R package PMCMR). In metagenomic prediction analysis, statistical significance was evaluated using DESeq2 package in R (p-value <0.05; log2 fold-change >2). Graphs were generated using Prism 6.0 software (GraphPad, La Jolla, CA).

RESULTS

Experimental toolkit for determining remodeling gene expression profiles in BAL cells

In a previous study, we showed that a majority of BAL samples obtained from lung transplant recipients up to 14 months post-transplant can be distinguished based upon the expression levels of a set of genes involved in inflammation and remodeling.⁷ However, a substantial group

representing 43% of total samples showed an intermediate profile and remained poorly characterized. Moreover, we noted an overlap across the whole sample set in the expression of the selected remodeling markers, platelet-derived growth factor D (PDGFD) and the tissue inhibitor of metalloproteinase 1 (TIMP1)/ matrix metalloproteinase 12 (MMP12) ratio. These data highlighted the need to develop an analytical toolkit able to further dissect the complexity of remodeling gene expression profiles post-transplantation.

To this end, we employed two complementary approaches (experimental scheme detailed in Fig 1, A). A subset of 9 BAL samples, that represented an inflammatory, remodeling or intermediate gene expression profile in our first-round real-time PCR, was analyzed by RNA sequencing. Data were filtered based on adjusted *p*-value (0.05 threshold) and ranked by fold-change. In parallel, we selected 6 Gene Ontology (GO) terms linked to remodeling (see Fig 1, B for details) and interrogated the GO database using the application AmiGO version 2.3,⁹ generating a list of 627 candidate genes. Table 2 presents the restricted list of 11 remodeling genes obtained upon combining the two approaches, and further assessing potential candidates based on a literature survey on the remodeling-fibrosis axis, in the context of various respiratory conditions. Real-time PCR validation analysis indicated that expression of the selected genes in our initial set of 9 BAL samples was associated with a wide-spread distribution of corresponding eigenvectors, as obtained by PCA, consistent with implications in diverse remodeling-related activities (Fig 1, C). Indeed, the corresponding encoded proteins have been linked to multiple aspects of remodeling, including matrix composition, accumulation and degradation.¹³⁻²⁰

Four host remodeling gene expression profiles prevail in the transplanted lung

To gain further insight into remodeling gene expression profiles post-transplantation, we analyzed by real-time PCR a total of 189 BAL samples obtained from 116 patients between 0.5 and 24 months post-transplant (see Table 1 for details), using our new analytical toolkit. PCA (Fig 2, A) and hierarchical clustering (Fig 2, B) allowed us to identify four profiles that we defined as low, intermediate, anabolic and catabolic remodeling, representing 21, 47, 20 and 12% of total samples, respectively. Per-gene analysis confirmed that the low and intermediate groups were indeed associated with low and intermediate expression levels, respectively, of virtually all genes tested (Fig 2, C). Anabolic remodeling was characterized by maximal expression of thrombospondin 1 (THBS1) and PDGFD, two factors previously linked to TGF- β -mediated repair in different experimental and clinical settings (Fig 2, C).^{19, 21} In contrast, expression of several metalloproteinases (MMP7, MMP9 and MMP12), typically associated with inflammation and matrix degradation, distinguished the catabolic remodeling group. This latter profile was also linked to a high expression of additional remodeling markers, including the matrix constituents collagen type VI alpha 2 (COL6A2) and fibronectin 1 (FN1), the growth-promoting insulin-like growth factor 1 (IGF1), and the matricellular proteins osteopontin (SPP1), chitinase 3-like 1 (CHI3L1) and IGF binding protein 2 (IGFBP2), all involved in the regulation of matrix turnover (Fig 2, C). Spearman's correlation analysis confirmed these associations, with a coefficient (ρ) ranging from -0.27 (PDGFD vs. SPP1) to 0.78 (MMP9 vs. CHI3L1) indicating negative and positive correlation, respectively (see Figure E1 in this article's Online Repository). We next observed that the different remodeling gene expression profiles identified were linked to distinct kinetics following transplantation (Fig 2, D; $p < 0.05$ by Chi-square test). Specifically, low remodeling was almost limited to the first 12 months post-transplant, while the intermediate

profile predominated in each of the four time windows considered (Fig 2, *D*). We further observed that catabolic remodeling peaked between 3 and 6 months, clearly preceding the maximal relative frequency of anabolic remodeling, from 12 months onwards (Fig 2, *D*). This latter divergence in the kinetics of catabolic versus anabolic remodeling was confirmed when we considered 87 combinations of paired samples obtained from 34 patients, who were lavaged multiple times (median 3, range 2-6; Fig. 2, *E* and Table E2 in this article's Online Repository) . A schematic representation of these longitudinal transitions between two different remodeling profiles further highlighted the marked difference between the catabolic and anabolic profiles, testified by one single direct transition (Fig. 2, *F*). In contrast, the intermediate remodeling profile was often connected to either the low, anabolic or catabolic remodeling profile, consistent with a central position (Fig. 2, *F*). Furthermore, we observed a substantial degree of stability in remodeling gene expression over time. Indeed, 34 paired samples (39%) were linked to a stable profile, while 52 others (61%) showed a profile transition. Moreover, when further focusing on 14 patients who provided from 4 to 6 BAL samples (median 4.5, interquartile range 4-5) over a range of 5.5 to 21 months (see details in Table E2 in this article's Online Repository), we found a median number of 2 profiles (interquartile range 2.0-2.3). Here, the relative frequency of paired samples with a stable profile decreased from the intermediate, anabolic, catabolic to low remodeling background (50%, 23%, 15% and 12%, respectively). Overall, these observations support the existence in the transplanted lung of distinct remodeling activities, differentially interconnected, and regulated in a time-dependent manner.

Relation between airway microbiota composition and host remodeling gene profiling

We previously reported that the airway microbiota composition varies in concert with BAL cell gene profiling post-transplant.⁷ In particular, we showed that dysbiosis, defined by a strong predominance of one single phylum exceeding 70% of relative abundance, was associated with a high expression of two contrasting sets of genes, linked to either inflammation or remodeling, depending on the predominant phylum, while more balanced bacterial communities aligned with a neutral gene expression profile. We therefore investigated whether the airway microbiota composition aligned with the four host remodeling gene expression profiles identified in the present study, with a special interest in the contrasting catabolic and anabolic profiles. For this purpose, we relied on a combination of molecular analysis and culture, which proved complementary in distinguishing the composition of the airway microbiota associated with a catabolic remodeling gene expression profile, versus that linked to a low, intermediate or anabolic profile. Specifically, PCR amplification of the 16S rRNA gene using phylum-specific primers indicated that dysbiosis observed in 37% of total BAL samples was increased when driven by Firmicutes or Actinobacteria, and decreased when Bacteroidetes predominated, on a catabolic remodeling background (Fig 3, A). Moreover, sequence analysis at genus-level underscored the association of catabolic remodeling with *Staphylococcus*, *Corynebacterium*, *Stenotrophomonas* and *Haemophilus* (Fig 3, B). In contrast, a microbial community comprising *Prevotella*, *Streptococcus*, *Veillonella* and *Neisseria* was poorly represented in this catabolic remodeling context, while strongly predominating in association with low, intermediate or anabolic remodeling (Fig 3, B). These specificities in the airway microbiota composition linked to host catabolic remodeling gene expression profiling were also accompanied by a reduced diversity compared with the three other remodeling profiles (Fig 3, C).

Assessment of positive cultures confirmed *Staphylococcus spp.* and *Corynebacterium spp.*, and additionally included *Pseudomonas spp.*, as part of the bacteria primarily linked to a catabolic remodeling profile (Fig 3, D). In contrast, BAL samples tested positive for oropharyngeal flora, which typically includes bacteria of the genera *Prevotella*, *Streptococcus*, *Veillonella* and *Neisseria*,³ predominated in association with an anabolic remodeling gene expression profile (Fig 3, D).

To explore further the links between the airway microbiota composition and host gene expression, and given the early onset of the catabolic remodeling profile, versus the late onset of the anabolic remodeling profile, we next determined the relative abundance of the different microbiota states within four time windows post-transplantation (Fig 3, E). Here, we found that microbiota dysbiosis was more likely to occur during the first six months post-transplant when driven by Firmicutes, while becoming more frequent over time when driven by Bacteroidetes, thus aligning with the kinetics of catabolic and anabolic remodeling, respectively. Furthermore, we applied PICRUSt analysis to gain insight on the predictive metagenome functions of the bacterial communities linked to the four remodeling profiles.¹² PICRUSt links OTUs, as identified by 16S RNA gene sequencing, to KEGG genes, their corresponding KEGG orthologs (KOs) and, ultimately, functions. Global analysis of the predicted metagenome indicated a segregation of the microbial communities found on a catabolic remodeling background, which especially differed from the communities associated with an anabolic remodeling profile (Fig 3, F). To investigate potential differences in respect to host remodeling more specifically, we next considered a subset of 159 KOs related to bacteria-matrix interaction and/or protein degradation. We found that 24 genes were significantly differentially represented (threshold set to log₂ fold-change >2) within the communities associated with a catabolic, compared to an anabolic,

remodeling profile (Fig 3, G). Of note, 23 of those genetic markers, which included, however not exclusively, virulence factors linked to infections of various etiology, including *Staphylococcus aureus* and *Pseudomonas aeruginosa*, were enriched (median log₂ fold-change 6.1, interquartile range 2.3-10.3; adjusted p-value ≤ 0.01) in the communities associated with a catabolic remodeling profile (Fig 3, G).

Host-microbe associations and the underlying clinical situation

The relatively limited follow-up time in our study did not allow us to look for associations, between the observed features in host remodeling gene expression or microbiota composition, and the onset of chronic rejection. Moreover, we found no link with the diagnosis of acute cellular rejection (A grade), as established upon histological analysis of transbronchial biopsies (see Table E3 in the Online Repository). In addition, when considering samples with a histological diagnosis of airway inflammation (lymphocytic bronchiolitis, B grade), we found only a slight increase between the percentage of samples with a catabolic remodeling signature, versus those of samples with either a low, intermediate or anabolic profile (21.4 vs 11.4, 10.9 and 15.4%, respectively; see Table E3 in the Online Repository). However, confirming our previous findings,⁷ Firmicutes, Proteobacteria and Actinobacteria-driven dysbiosis, rather than the absence of dysbiosis or Bacteroidetes-driven dysbiosis, was found to be linked to histologically determined airway inflammation (27.8, 15.9 and 4.8%, respectively; see Table E3 in the Online Repository).

To assess potential links between host remodeling, microbiota states and inflammation directly in the BAL, we next looked at the cell differential and found a marked enrichment in neutrophils, a hallmark of acute inflammation, accompanied by a corresponding relative decrease in

macrophage and lymphocyte counts, on a catabolic remodeling background (Fig 4, A). Consistently, we also observed a stronger expression of the inflammation marker genes cyclooxygenase-2 (COX2) and tumor necrosis factor-alpha (TNF) (Fig 4, B) in this setting. These observations suggested that the microbial communities linked to catabolic remodeling exhibit increased inflammatory properties in comparison to the principal members of the community dominated by *Prevotella*,^{7, 22} as previously reported.

Given that the bacterial genera associated with catabolic remodeling comprise common respiratory pathogens, we next aimed to evaluate the links between host remodeling-microbe associations and the diagnosis of infection. For this purpose, we focused our attention on a subset of 29 BAL samples obtained from patients who presented with clinical evidence of infection, and had either a positive result in BAL culture by routine microbiological examination, and/or microbiota dysbiosis detected by 16S rRNA gene amplification. BAL samples with catabolic and anabolic remodeling gene expression profiles aligned with maximal and minimal rates of clinical infection, respectively (Fig 4, C). Moreover, when considering all of those samples associated with a clinical suspicion of infection, we found a striking difference in the bacteria identified by culture and/or type of detected dysbiosis, depending on the remodeling context (Fig 4, D). Hence, combined with the information we obtained on the kinetics of gene expression profiles (Fig 2, D and E) and microbiota states (Fig 3, E and F), this dataset indicated that the early acquisition of a catabolic remodeling profile and dysbiosis driven by highly stimulatory bacteria, is linked to inflammation and a more frequent association with clinical infection. In contrast, the clinical implications of the coincident anabolic remodeling profile and dysbiosis driven by low stimulatory bacteria, which are likely to be expressed later following lung transplantation, remain to be defined.

The constituents of airway microbial communities set the balance between anabolic and catabolic remodeling

The identification of anabolic and catabolic gene expression profiles linked to different airway microbiota compositions raised the possibility that the extracellular matrix turnover in the transplanted lung is differentially influenced by the constituents of local bacterial communities. To address this, we used an *in vitro* model system that exploits the microbial sensing and remodeling capacities of THP1-derived macrophages (THP-DM) and MRC5 fibroblasts, to compare the direct effects of bacterial mixes on remodeling gene expression profiles and matrix deposition. Culture conditions were optimized for efficient matrix deposition, and included immunosuppressive drugs, that mimicked the transplanted lung environment, and antibiotics, which effectively prevented bacterial over-growth (see Materials and Methods for details). This ensured consistent cell viability levels across the different experimental conditions, in spite of a median 19% decrease in the presence of *Staphylococcus aureus* and *Pseudomonas aeruginosa* vs *Prevotella melaninogenica* and *Streptococcus pneumoniae* (Fig E2). In this setting, bacteria identified in lung transplant recipients on a catabolic remodeling background (a mix of *Staphylococcus aureus* and *Pseudomonas aeruginosa*) induced a globally distinct remodeling gene expression profile in macrophage-fibroblast co-cultures, as compared to the representatives of the community linked to anabolic remodeling (*Prevotella melaninogenica* and *Streptococcus pneumoniae*; Fig 5, A). Specifically, *Staphylococcus* and *Pseudomonas* induced maximal expression of MMP7, -9 and -12, while *Prevotella* and *Streptococcus* were linked to higher THBS1 expression, two features aligning with catabolic and anabolic gene expression profiling in BAL cells of lung transplant recipients, respectively (Fig 5, B). *Staphylococcus* and *Pseudomonas* were more potent in repressing expression of the marker of fibroblast to

myofibroblast differentiation, alpha-smooth muscle actin (ACTA2), along with that of the matrix components collagen type I alpha 1 (COL1A1) and fibronectin 1 (FN1) (Fig 5, B). These divergent gene expression profiles were ultimately reflected by a marked decrease in matrix deposition linked to *Staphylococcus* and *Pseudomonas* versus *Prevotella* and *Streptococcus* exposure, as determined by type 1 collagen and fibronectin immunostaining (Fig 5, C and D). Consequently, this dataset underscored the critical role of bacterial stimulation in the maintenance of homeostatic matrix turnover, suggesting that the detrimental impact of airway microbiota dysbiosis following transplantation may consist of exaggerated anabolic or catabolic remodeling processes.

DISCUSSION

The view of the lower airways representing an ecosystem implies that host cells and the constituents of the microbiota vary in a coordinated manner, particularly in the case of a breakdown in local homeostasis. Accordingly, different microbial communities have been observed in association with lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), and idiopathic pulmonary fibrosis (IPF), versus the healthy state, with further changes prior, during or following exacerbations.^{4-6, 22} The ecosystem associated with the transplanted lung is shaped by the early and continuous implementation of immunosuppression and the preventive or therapeutic use of antibiotics. We have previously reported that this lung habitat is linked to distinct microbial communities which can influence macrophage gene expression.⁷

The present study aimed to discriminate between anabolic and catabolic remodeling following lung transplantation, given the contrasting implications for graft fate these

mechanisms may have. In addition, we investigated the composition of lower airway microbial communities on the different remodeling backgrounds, and addressed the impact of bacterial stimulation on remodeling using an *in vitro* system of matrix deposition.

The 11 remodeling-related target genes we selected have all previously been linked to respiratory conditions, though their pathological role remains elusive.^{14, 15, 17, 20, 24-26}

Functionally, the corresponding encoded factors can be divided in four main categories: i. matrix structural constituents (COL6A2, FN1), which in myeloid cells are further known to modulate cell-matrix adhesion properties;^{17, 27} ii. matricellular proteins (THBS1, SPP1, CHI3L1, IGFBP2), which comprise a variety of non-structural, multifunctional factors, that transiently bind to the matrix under specific conditions, notably in association with lung injury, wound healing and fibrogenesis;²⁸ iii. matrix metallopeptidases (MMP7, -9, -12), that fulfil multiple functions, but overall associated with an enhanced matrix degradation or turnover; iv. growth factors (PDGFD, IGF1), that regulate the proliferation, migration and survival of key remodeling cell players.

Beside their implication in homeostatic matrix turnover, all of these factors are involved in the control of lung injury, where they function to ensure a time-dependent sequence of inflammatory and remodeling processes, which ultimately lead to tissue repair. Likewise, they have been linked to different respiratory conditions, where their production varies with disease progression. Hence, both the physiological and pathophysiological functions of these factors appear to be orchestrated in a time-dependent manner. In this respect, our study, supported by longitudinal data, shows marked differences in the kinetics of the four remodeling profiles identified following transplantation. Of particular interest, the frequency of the catabolic remodeling profile was maximal between 3 and 6 months, while

the anabolic remodeling profile peaked between 12 and 24 months post-transplantation. Several factors associated with the catabolic remodeling profile have been linked to inflammatory and/or acute conditions, such as acute lung injury (SPP1),²⁹ severe asthma (CHI3L1)³⁰ or COPD exacerbation (CHI3L1).²⁴ Moreover, the up-regulated production of MMPs by inflammatory, epithelial and stromal cells, and the associated release in the lung and circulation of matrix degradation products, have been observed in a large panel of respiratory conditions, including CLAD versus stable lung transplant recipients.³¹⁻³⁴

In contrast, THBS1 and PDGFD, two factors linked to the anabolic remodeling profile, have been reported to be part of a positive feedback loop with the prototypical pro-fibrogenic factor TGF- β 1, during tissue repair,^{19, 35} promoting fibroblast proliferation and myofibroblast differentiation.^{35, 36} Collectively, these observations suggest that the factors linked to the catabolic and anabolic remodeling profiles identified are active in distinct remodeling processes and/or stages.

The lower airway microbiota composition also varies over time post-transplant. We observed that a maximal frequency of communities, strongly dominated by highly stimulatory bacteria (from 3 to 6 months), coincided both with the peak of bacterial infection and that of the catabolic remodeling profile. In marked contrast, the maximal frequency of communities dominated by low stimulatory bacteria (from 12 months onwards), aligned with the kinetics of the anabolic remodeling profile, suggesting that the interplay between host cells, microbes and the matrix represents a key determinant of the lower airway micro-environment. In this respect, data suggests that increases in BAL and/or circulating matrix degradation products coincide with shifts in airway microbiota composition during exacerbations in COPD and IPF.^{5, 6, 33, 37}

Furthermore, matricellular proteins play important roles in innate immune mechanisms linked to

both physiological bacterial colonization and infection, such as the regulation of bioactive antibacterial peptide release.^{38, 39}

Our *in vitro* dataset indicates that *Staphylococcus aureus* and *Pseudomonas aeruginosa*, typically associated with pro-inflammatory microbial communities, trigger a stronger catabolic remodeling profile, in comparison to *Prevotella melaninogenica* and *Streptococcus pneumoniae*. This is consistent with recent observations linking the two former bacterial species to increased BAL levels of matrix glycosaminoglycans, as compared to those associated with *Streptococcus pneumoniae*, in acute exacerbation and stable COPD, respectively.⁴⁰ There are different ways bacteria could modulate matrix production in our *in vitro* system. Figure 5A-D indicates that this occurred through stimulation of fibroblasts and macrophages, by shifting the gene expression balance between matrix components and metalloproteinases. In addition, bacteria could have impacted upon remodeling through direct degradation of the matrix. However, given the presence of antibiotics in the culture medium, which efficiently prevented bacterial metabolic activities, this second mode of action is likely to be insignificant in this setting. Consequently, we propose that the observed effects are linked to the higher stimulatory potential of the microbe-associated molecular patterns (MAMPs), such as peptidoglycan and lipopolysaccharide, which contribute to the structural properties of bacterial cells in *Staphylococcus* and *Pseudomonas*, versus those in *Prevotella* and *Streptococcus*. Regardless of the mechanisms involved, these observations suggest that the net effect of host-microbe interactions on matrix regulation closely depends on the microbiota constituents. We propose that in the healthy state, the lower airway micro-environmental conditions are linked to low-grade matrix turnover and a balanced microbiota, which contribute to local homeostasis. However, in respiratory diseases associated with acute inflammation or fibrogenesis, local conditions linked to a catabolic and

anabolic remodeling profile, respectively, promote the growth of specific bacteria, which in turn differentially impact on matrix regulation.

There were limitations to this study. First, although our *in vitro* dataset revealed a link between remodeling gene expression levels and activities, we acknowledge that remodeling in the transplanted lung cannot be inferred solely from the identification of BAL cell gene expression profiles, given the importance of post-transcriptional regulation in this process. Nonetheless, the different remodeling gene expression profiles we identified correspond to distinct micro-environmental conditions, as corroborated by specific features in the lung microbiota composition and the underlying clinical state. Second, while the anabolic and catabolic remodeling profiles distinguished two well-delineated sample groups, as further confirmed by their association with contrasting bacterial communities, we found no such associations for the low and intermediate remodeling profiles. A possible underlying reason is that these two latter profiles reflect local conditions that are convergent enough to accommodate similar microbial communities. Alternatively, the analytical tools we used to characterize gene expression profiling and the composition of the airway microbiota might not have reached the power required to further dissect existing associations. Third, given that remodeling gene expression was measured from unsorted BAL cells, we could not ascertain the cellular source of gene transcripts, which would inform on the underlying pathophysiological processes. Fourth, the post-transplant follow-up period was not long enough to enable us to assess any distinction between stable transplant recipients and patients with CLAD. Given the progressive nature and prevalence of this major obstacle to long-term graft survival, further studies with a minimal 3-year longitudinal follow-up are required to elucidate a link between pulmonary bacterial dysbiosis, host gene expression profiling, and eventually pathophysiological tissue remodeling.

Fifth, a direct assessment of the impact of antibiotics and immunosuppressive drugs was precluded by the broad use, large variability, and partly overlapping effects of the applied regimens, within the context of lung transplantation. Sixth, a full picture of the airway ecosystem should include an in-depth characterization of the viral and fungal microbiota constituents. Notwithstanding these shortcomings, our collective findings shed light on the role of host-microbe interactions in the control of matrix turnover post-lung transplantation. Further increasing our understanding of the mechanisms implicated may help decipher the pathophysiology of CLAD, and by inference that of other respiratory conditions associated with lower airway remodeling.

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ACCEPTED MANUSCRIPT

Table 1. Patient characteristics.

Patients/Samples	Total (n)	116/189
	Male	58 (50.0%)
	Age at transplant (yr)	52 (35-61)
	Sampling time point (days post-transplant)	339 (92-376)
Type of transplant	Bilateral lung	101 (87.0%)
	Single lung	15 (12.9%)
Pre-transplant diagnosis	Chronic obstructive pulmonary disease	38 (32.8%)
	Cystic fibrosis	31 (26.7%)
	Interstitial lung disease	24 (20.7%)
	Graft failure (re-transplantation)	5 (4.3%)
	Other	18 (15.5%)
Transbronchial biopsies* [†]		167 (88.4%)
	A0	128 (76.6%)
	A1	21 (12.6%)
	A2	5 (3.0%)
	B0	105 (62.9%)
	B1	19 (11.4%)
	B2	1 (0.6%)
Immunosuppression*	Tacrolimus	176 (93.1%)
	Cyclosporin	13 (6.9 %)
Antibiotics*	TMP/SMX	143 (75.7%)
	Azithromycin	25 (13.2%)
	Other (inhaled, oral or intravenous routes)	54 (28.6%)
BAL positive bacterial culture (excl. oropharyngeal flora)*	<i>Staphylococcus aureus</i> (n= 10), <i>Pseudomonas aeruginosa</i> (n=10), <i>Staphylococcus epidermidis</i> (n=5), <i>Corynebacterium</i> sp. (n=4), <i>Enterococcus</i> sp. (n=3), <i>Streptococcus</i> sp. (n=1), <i>Klebsiella pneumoniae</i> (n=1), <i>Haemophilus influenzae</i> (n=1), <i>Escherichia coli</i> (n=1), <i>Enterobacter</i> sp. (n=1)	32 (16.9%)
BAL positive fungal culture*	<i>Aspergillus</i> sp. (n=4), <i>Penicillium</i> sp (n=3), <i>Candida</i> sp. (n=4)	13 (6.9%)
BAL positive viral PCR* [‡]	CMV (n=11), EBV (n=2), metapneumovirus (n=1), parainfluenza (n=1)	14 (13.2%)

Abbreviations: CMV=cytomegalovirus, EBV=Epstein-Barr virus, TMP/SMX=trimethoprim/sulfamethoxazole

Data presented as n (% of group) or median (interquartile range); *at sampling; [†]grading of pulmonary allograft rejection according to guidelines of the International Society for Heart and Lung Transplantation;⁴¹ [‡]conducted in a subset (n=106) of samples. Virological investigations ranged from CMV only to more extensive testing, as per case requirement.

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Table 2. Remodeling gene set used for the characterization of BAL cell profiling*.

Gene name, alias [†]	Gene symbol [†]	Molecular functions
Chitinase 3 like 1	CHI3L1	Regulator of cell-matrix interactions
Collagen type VI alpha 2 chain	COL6A2	Matrix constituent
Fibronectin 1	FN1	Matrix constituent
Insulin like growth factor 1	IGF1	Fibroblast proliferation and activation
Insulin like growth factor binding protein 2	IGFBP2	Fibroblast proliferation and activation
Matrix metalloproteinase 7	MMP7	Mediator of matrix turnover; basement membrane proteolysis
Matrix metalloproteinase 9	MMP9	Mediator of matrix turnover; collagen and fibronectin proteolysis
Matrix metalloproteinase 12	MMP12	Mediator of matrix turnover; elastin proteolysis
Platelet derived growth factor D	PDGFD	Fibroblast proliferation and survival
Secreted phosphoprotein 1, osteopontin	SPP1	Regulator of cell-matrix interactions
Thrombospondin 1	THBS1	Regulator of cell-matrix interactions; major activator of TGFβ1

*Primer and probe sequences are available in Table E1 in this article's Online Repository.

[†]According to HUGO Gene Nomenclature Committee at the European Bioinformatics Institute.

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FIGURE LEGENDS

FIG 1. Identification of a set of remodeling genes. **A**, Schematic outline of experimental approach. **B**, Gene Ontology criteria used in candidate gene selection process. **C**, Principal component (PC) analysis, and associated eigenvectors, based upon quantitative PCR determination of expression of the restricted list of 11 remodeling genes (see Table 2 for details) in the initial subset of 9 BAL samples (dots).

FIG 2. Identification of four remodeling gene expression profiles in post-transplant BAL samples. Principal Component Analysis (**A**) and hierarchical clustering (**B**) based upon qPCR determination of gene expression (**C**). Medians and interquartile ranges are indicated. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Transversal (**D**) and intraindividual (**E**) post-transplant variations in remodeling gene expression profile. In **E**, data shows the frequency of sample pairs that displayed acquisition of a new remodeling profile. **F**, Schematic diagram of intraindividual remodeling profile transitions with indicated occurrences.

FIG 3. Associations between the pulmonary microbiota and host remodeling gene expression. Microbiota status (**A**), the 10 most abundant microbial genera (**B**), Shannon diversity index with medians and interquartile ranges, * $p < 0.05$ (**C**) and culture outcome (**D**) as per remodeling profile. **E**, Kinetics of microbiota status post-transplant. **F**, Bray-Curtis Principal Coordinate Analysis of inferred metagenomic content. **G**, Differential abundance analysis focused on KEGG orthologs related to bacteria-matrix interaction or proteolysis, on a catabolic versus anabolic remodeling gene expression background.

FIG 4. Associations between host remodeling, inflammation and infection. Relationship between host remodeling and either BAL cell differential (**A**), expression of inflammatory genes

cyclooxygenase-2 (COX2) and tumor necrosis factor-alpha (TNF) (**B**), prevalence of suspected clinical infection (**C**) and bacteria isolated by culture and/or driving dysbiosis. In panels A and B, medians and interquartile ranges are indicated. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

FIG 5. Impact of bacterial stimulation on remodeling gene expression in macrophage-fibroblast co-cultures. **A**, Gene expression-based Principal Component (PC) analysis. Dots represent the mean coordinates obtained from duplicates in three independent experiments. **B**, qPCR-based gene expression analysis. Immunostaining (**C**) and quantification (**D**) of deposited matrix proteins. Scale bar represents 100 μm . In panels B to D, data were from six independent experiments. Values represented as mean with standard error of mean are expressed as fold-change over baseline. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

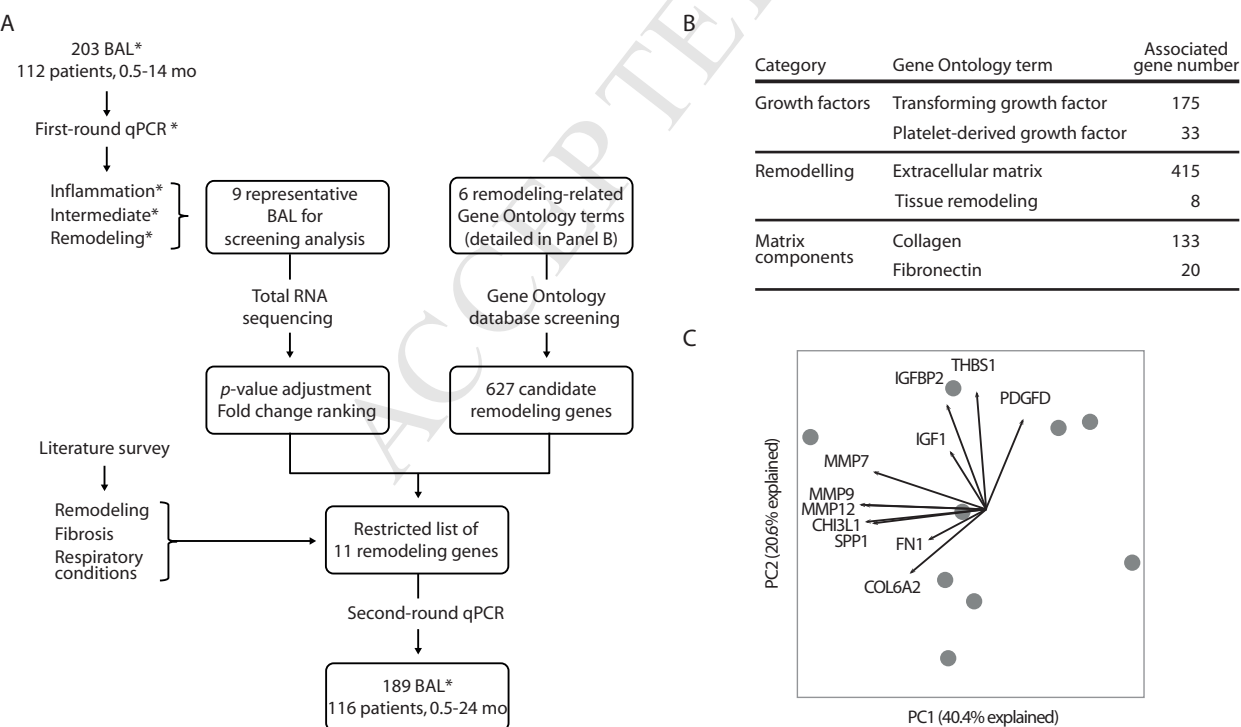


Figure 1

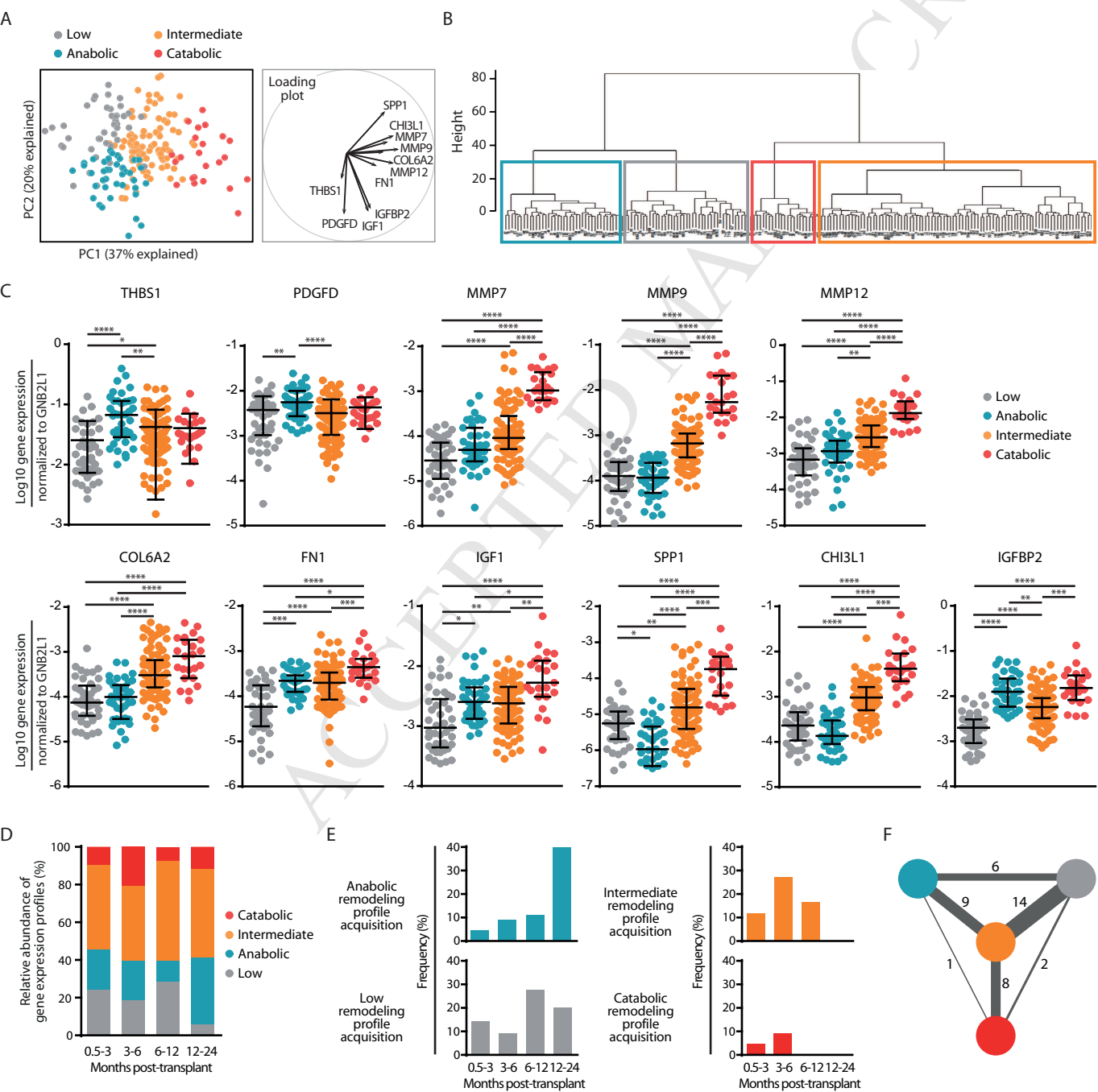


Figure 2

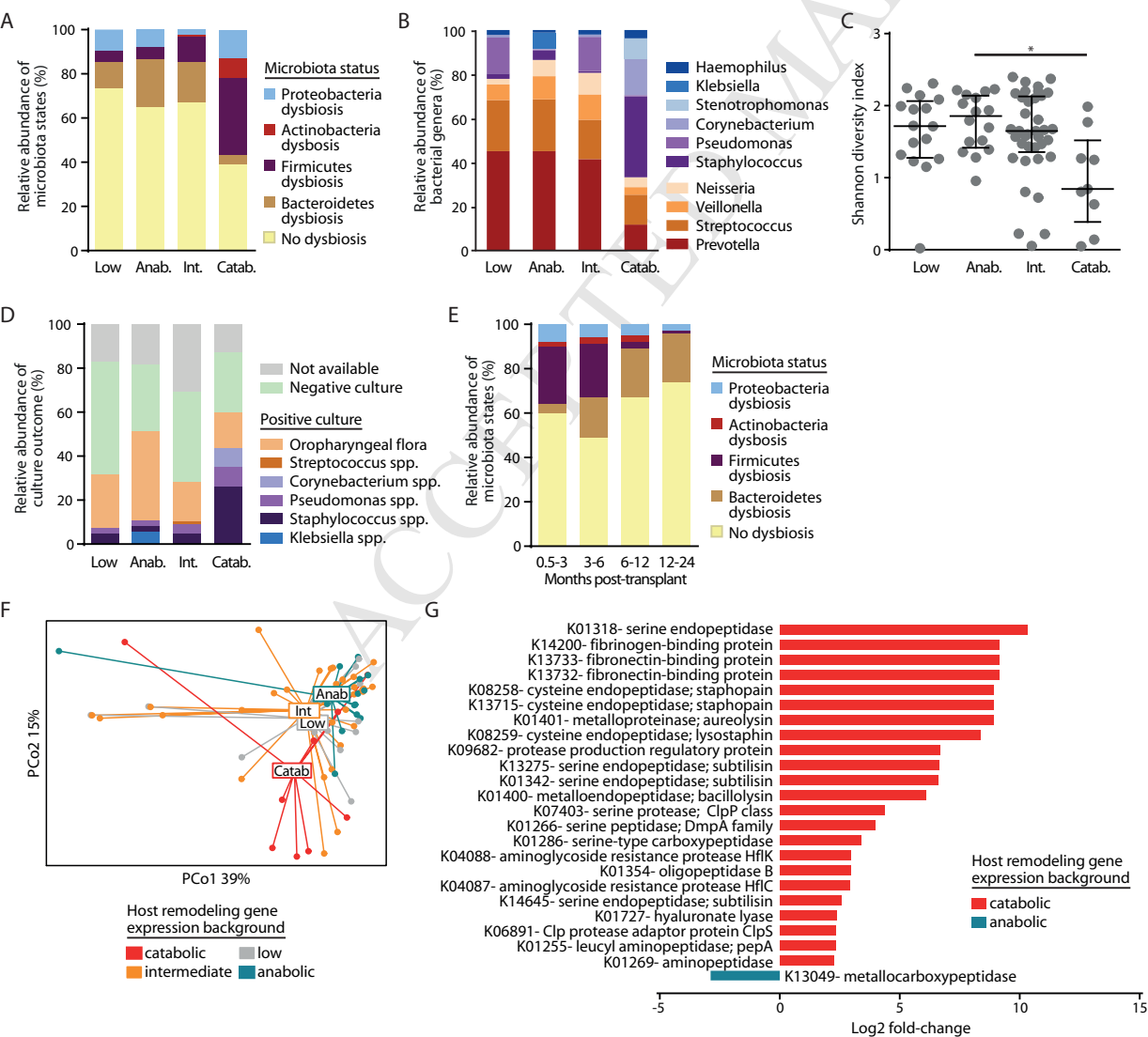


Figure 3

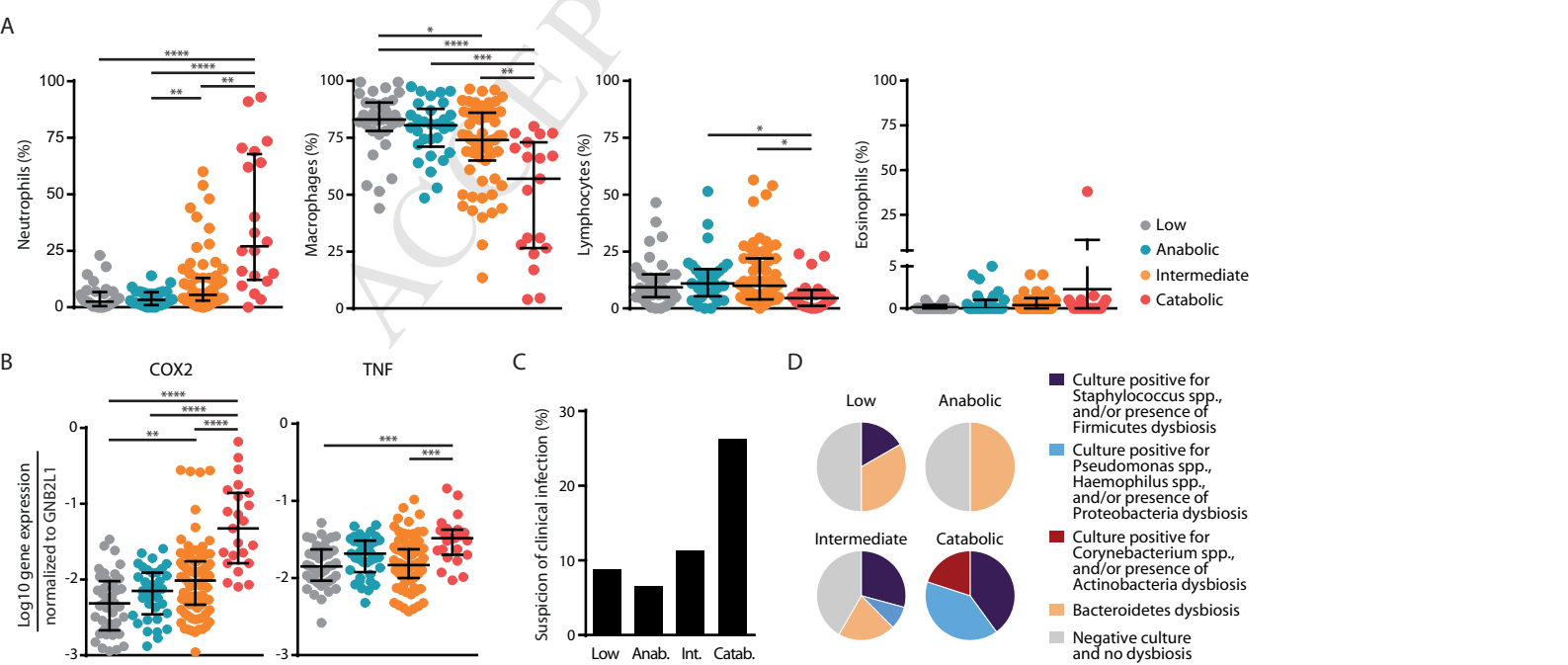


Figure 4

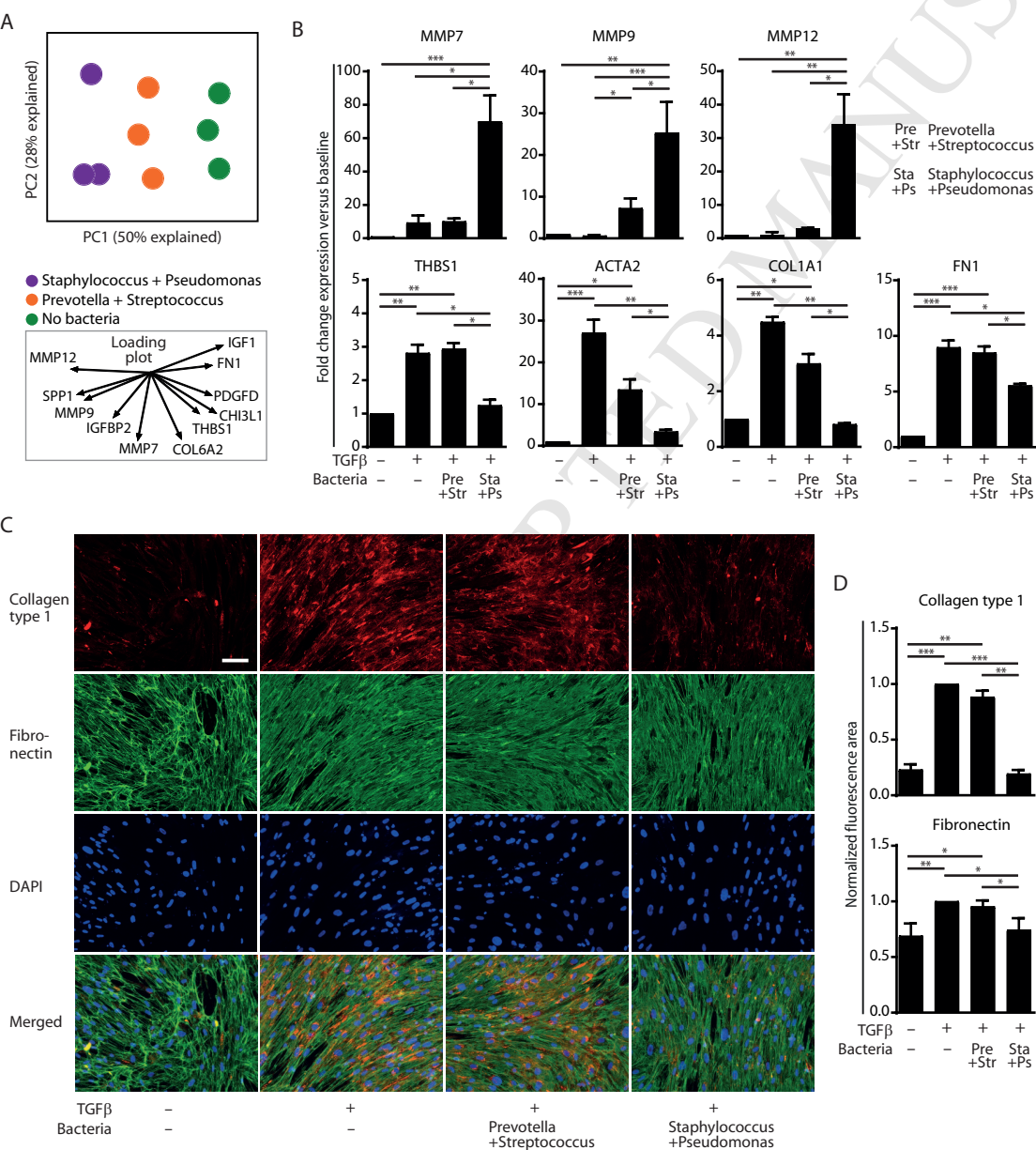


Figure 5

ONLINE REPOSITORY

Airway microbiota signals anabolic and catabolic remodeling in the transplanted lung

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- **List of SysCLAD Consortium members**
- **Supplemental methods**
- **Supplemental references**
- **Table E1.** Oligonucleotide primers and probes for analysis of BAL cell profiling
- **Table E2.** Timing of BAL sample collection in patients with multiple lavages
- **Table E3.** Sample distribution based upon transbronchial biopsy evaluation
- **Figure E1.** Spearman correlation coefficient matrix for the selected 11 remodeling genes
- **Figure E2.** Assessment of cell viability in fibroblast-macrophage co-cultures

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Supplemental methods

Patient sample collection

Patients underwent transoral or transnasal bronchoscopy. For BALF collection, the bronchoscope was wedged either in the middle lobe or lingula of the allograft and 100-150 ml of normal saline were instilled. Different fractions of collected samples were submitted to cell differential determination, culture-dependent bacterial and fungal detection, and PCR-based detection of viral infection, according to routine clinical procedures. Two fractions of 3 ml were stored at 4°C and centrifuged within 3 h at either 2,000 or 14,000 $\times g$ for 10 min, for future isolation of BALF cellular RNA and total DNA, respectively. Pellets were snap frozen, either after cell lysis in RLT buffer (Qiagen, Hilden, Germany) to preserve RNA integrity, or directly, and were stored at minus 80°C until further processing. Samples obtained upon washing the endoscope with sterile saline prior an examination were prepared following the same procedure and were used as negative controls.

BALF cellular RNA extraction and real-time quantitative PCR for gene expression analysis

BALF cell lysates were transferred into a QIAshredder column (Qiagen) for homogenization and total RNA was extracted using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA concentration was determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and reverse transcription was performed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Characterization of BALF cell remodeling profiles was based on multiplex real-time PCR analysis of expression of a set of 11 genes (Table 2). We used guanine nucleotide-binding protein, beta polypeptide 2-like 1 (GNB2L1) gene as a reference gene, given its high expression stability in BALF cells in both health and disease (E1). Custom oligonucleotide primers and probes (Table E1) were designed using NCBI primer blast software and were purchased from Microsynth (Balgach, Switzerland). Amplification was carried out using iQ Multiplex Powermix and a CFX96 Real-Time detection system. Absolute quantification was performed using the CFX Manager software 2.1 (all from Bio-Rad) based upon values obtained with a set of purified amplicons used as standards.

Bacterial metagenome prediction

The operational taxonomic units (OTUs) table obtained from 16S rRNA gene sequencing data was normalized relative to the 16S rRNA gene copy number, and the metagenomic content was predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) software. The predicted metagenome was exported as KEGG orthologs (KOs) for principal coordinate analysis (PCoA) on Bray-Curtis distance, using cmdscale function in R. To investigate the potential functional capacity of the bacterial metagenome in respect to host remodeling, a subset of 159 KOs related to matrix interactions (search terms chondroitin*, collagen*, fibrinogen, fibronectin, hyaluron*) or protein/peptide degradation (search terms

protease, proteinase, peptidase, *lysin, *pain, *staphin) were selected. All of these KOs were previously reported to be linked to bacterial genomes. *Bacterial cultures*

Staphylococcus aureus (ATCC 25904) and *Pseudomonas aeruginosa* (ATCC BAA-47) were cultured on tryptic soy agar or in tryptic soy broth at 37°C with 130 and 200 rpm agitation, respectively (all media were obtained from Becton–Dickinson, Franklin Lakes, NJ, USA unless otherwise specified). *Streptococcus pneumoniae* (NCTC 7466) was grown on Mueller Hinton agar with 5% sheep blood or in tryptic soy broth at 37°C, in absence of agitation in a 5% CO₂ incubator. *Prevotella melaninogenica* (ATCC 25845) was cultured on Columbia agar with 5% sheep blood or in chopped meat broth (Remel Laboratories, Lenexa, KS, USA) at 37°C under anaerobic conditions. For stimulation experiments, bacteria were grown for 16 h, washed twice in phosphate buffered saline (PBS; BioConcept, Allschwil, Switzerland) at 3,000 x g for 10 min at 4°C, and resuspended in X-VIVO 15 medium (Lonza, Basel, Switzerland). Suspensions were finally diluted to reach a concentration of 4×10^{-3} (*P. aeruginosa* and *S. pneumoniae*) or 2×10^{-3} (*S. aureus* and *P. melaninogenica*) optical density at 600 nm corresponding to 10^6 colony-forming units per milliliter.

In vitro macrophage-fibroblast maintenance and stimulation experiments

The MRC5 (ATCC CCL-171) human fibroblast cell line was maintained in Minimum Essential Medium with Earle's salts (EMEM), supplemented with non essential amino acids, sodium pyruvate, 10% (v/v) heat inactivated fetal bovine serum (FBS) and 100 U/ml penicillin and 100 µg/ml streptomycin (all from Thermo Fisher Scientific). The THP-1 (ATCC TIB-202) human monocytic cell line was maintained in RPMI 1640 medium supplemented with 10% (v/v) heat inactivated FBS, 20 µM 2-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Thermo Fisher Scientific). Incubation was at 37° in a humidified 5% CO₂ atmosphere. For generating THP-1-derived macrophages (THP-DM), 24-well plates were precoated with FBS for 60 min. THP-1 cells were centrifuged at 300 x g for 10 min, resuspended in differentiation medium, which corresponded to maintenance medium supplemented with human recombinant Macrophage-Colony Stimulating Factor (M-CSF; Peprotech, Rocky Hill, NJ, USA), FBS (20% final concentration) and 10 mM HEPES, and were seeded into precoated 24-well plates at a density of 10^5 cells per well. Incubation was for 7 days, without medium change. To assess gene expression levels in macrophage-fibroblast co-cultures in the presence or absence of bacteria, fibroblasts and THP-DM were seeded at a density of 40'000 and 20'000 cells/cm², respectively, in serum-free EMEM containing transforming growth factor-β1 (TGF-β1, 5 ng/ml; eBioscience, San Diego, California, USA), a mixture of prednisolone (500 nM; Sigma, St. Louis, Missouri, USA) / FK506 (tacrolimus, 25 nM; Enzo Life Sciences, Lausen, Switzerland) / mycophenolic acid (10 µM; Tocris Bioscience, Ellisville, Missouri, USA), and penicillin and streptomycin (1000 U/mL and 100 µg/mL, respectively; Thermo Fisher Scientific). Bacterial suspensions were added 16 h post-seeding, at a total density of 3×10^5 colony-forming units (CFU)/cm², which corresponded to 5 CFU per eukaryotic cell at day 0, and the effects of stimulation were assessed

30 h later, when supernatants were removed and cells were lysed in RLT buffer (Qiagen) to preserve RNA integrity. Cell viability was assessed 30 h following the addition of bacterial mixtures into macrophage-fibroblast co-cultures, using a WST-1-based colorimetric assay (Cell Counting Kit-8, Dojindo Molecular Technologies, Rockville, MD), according to the manufacturer's instructions.

In vitro assessment of matrix deposition

To optimize for maximal matrix deposition, macrophage-fibroblast co-culture medium described above was supplemented with 70- and 400-kDa Ficoll (37.5 and 25 mg/mL, respectively; GE Healthcare, Little Chalfont, UK)(E2). Co-cultures were seeded at a density of 25'000 and 12'500 cells/cm², respectively, in μ -Slide 8 well ibi-Treat slides (Ibidi, Martinsried, Germany), and were incubated for 6 days without medium change, in the presence or absence of bacteria, whereafter supernatants were removed and cells were fixed in 4% paraformaldehyde in PBS for 20 minutes, for subsequent immunostaining analysis.

Immunostaining, image acquisition and measurement of fluorescence area

Paraformaldehyde-fixed cells were permeabilized for 10 minutes in 0.2% Triton X-100 in PBS, and further incubated for 30 minutes in 1% bovine serum albumin (BSA; Sigma-Aldrich, St Louis, MO) in PBS to reduce nonspecific antibody binding. Primary antibodies (mouse anti-human Collagen type I, Sigma-Aldrich; and rabbit anti-human fibronectin, Abcam, Cambridge, UK) and secondary antibodies (goat anti-mouse IgG Cy3, Merck, Darmstadt, Germany; and goat anti-rabbit IgG AlexaFluor 488, Thermo Fisher Scientific) were incubated for 2 h and 1 h, respectively, in 1% BSA in PBS. Incubation with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, 10 μ g/mL in methanol; PanReac AppliChem, Darmstadt, Germany) was for 15 min. All steps were performed at room temperature. For image acquisition, we used a motorized inverted fluorescence microscope (Axio Observer Z1, Carl Zeiss, Oberkochen, Germany) with 20x objective. A consistent predetermined pattern of 8 images were acquired, both within and across experiments. The process was automated using the Mark&Find function in AxioVision imaging software (release 4.7.1, Carl Zeiss MicroImaging, Goettingen, Germany). The fluorescence area was quantified and cell nuclei were counted with ImageJ (version 1.50b) software (National Institutes of Health, Bethesda, MD).

Supplemental references

- E1. Ishii T, Wallace AM, Zhang X, Gosselink J, Abboud RT, English JC, et al. Stability of housekeeping genes in alveolar macrophages from COPD patients. *Eur Respir J* 2006;27:300-306.
- E2. Kumar P, Satyam A, Fan X, Collin E, Rochev Y, Rodriguez BJ, et al. Macromolecularly crowded in vitro microenvironments accelerate the production of extracellular matrix-rich supramolecular assemblies. *Sci Rep*. 2015 Mar 4;5:8729.
- E3. Stewart S, Fishbein MC, Snell GI, Berry GJ, Boehler A, Burke MM, et al. Revision of the 1996 working formulation for the standardization of nomenclature in the diagnosis of lung rejection. *J Heart Lung Transplant* 2007;26:1229-42.

Supplemental figure legends

FIG E1. Spearman correlation coefficient matrix based on expression levels of the selected 11 remodeling genes. Gene names are listed in Table 2. Data are presented as Spearman's rho with p-value, obtained by analyzing by quantitative PCR expression of the 11 selected genes in the total set of 187 BAL samples.

FIG E2. Cell viability determined in fibroblast-THP-1-derived macrophage co-cultures, either in the absence (no bacteria) or presence of bacterial mixtures, as indicated. Data were pooled from three independent experiments with duplicates. Values (mean $\pm$ SEM) were normalized to viability levels in the absence of bacteria. Statistical significance was determined using Friedman test and Dunn post hoc analysis.

Table E1. Oligonucleotide primers and probes for analysis of BAL cell profiling.

Oligonucleotide Name	NCBI Gene ID	NCBI Reference Sequence	5' label	Sequence
CHI3L1_forward			-	5'-GATTTTCATGGAGCCTGGCG-3'
CHI3L1_reverse	1116	NM_001276.2	-	5'-CCCCACAGCATAGTCAGTGTT-3'
CHI3L1_probe			Tx Red	5'-ACAGGCCATCACAGTCCCCTGTTCCGA-3'
COL6A2_forward			-	5'-AGCTCTACCGCAACGACTAC-3'
COL6A2_reverse	1292	NM_001849.3	-	5'-CACCTTGTAGCACTCTCCGT-3'
COL6A2_probe			FAM	5'-ACTCCACCGAGATCGACCAGGACACCA-3'
MMP7_forward			-	5'-AAGTGGTCACCTACAGGATCG-3'
MMP7_reverse	4316	NM_002423.4	-	5'-TCAGCAGTTCCCCATACT-3'
MMP7_probe			Cy5	5'-ACATGTGGGGCAAAGAGATCCCCCTGC-3'
MMP9_forward			-	5'-GTACTCGACCTGTACCAGCG-3'
MMP9_reverse	4318	NM_004994.2	-	5'-AACAACTGTATCCTTGGTCCG-3'
MMP9_probe			HEX	5'-ACAGCGACAAGAAGTGGGGCTTCTGCC-3'
IGF1_forward			-	5'-GCTTTTATTCAACAAGCCCACAG-3'
IGF1_reverse	3479	NM_001111283.2	-	5'-GCCTCCTTAGATCACAGCTCC-3'
IGF1_probe			HEX	5'-ACAGGCATCGTGGATGAGTGCTGCTTCC-3'
IGFBP2_forward				5'-AACCTCAAACAGTGCAAGATGTC-3'
IGFBP2_reverse	3485	NM_000597.2		5'-GTAGAAGAGATGACACTCGGGG-3'
IGFBP2_probe			Tx Red	5'-AGCGTGGGGAGTGCTGGTGTGTGAA-3'
SPP1_forward				5'-TAAATTCTGGGAGGGCTTGGTT-3'
SPP1_reverse	6696	NM_001040060.1		5'-CATGGTAGTGAGTTTCTTGGTC-3'
SPP1_probe			Cy5	5'-AGGCCAGTTGCAGCCTTCTCAGCCA-3'
THBS1_forward				5'-GGAGGAGGGGTACAGAAACG-3'
THBS1_reverse	7057	NM_003246.3		5'-CAGGCATCCATCAATTGGACAG-3'
THBS1_probe			FAM	5'-ACCCAGTTTGGAGGCAAGGACTGCGT-3'
FN1_forward			-	5'-GGCTGGAGCCGGGCATTGAC-3'
FN1_reverse	2335	NM_212482.1	-	5'-GGGAGGAGGAACAGCCGTTTGTT-3'
FN1_probe			Tx Red	5'-TGTAGTAGGGGCACTCTCGCCGCCA-3'
MMP12_forward				5'-TGCCCGTGGAGCTCATGGAGAC-3'
MMP12_reverse	4321	NM_002426.4		5'-CCTCCAATGCCAGATCCAGGTCCAA-3'
MMP12_probe			HEX	5'-AGCATGGGCTAGGATTCCACCTTTGCCATCA-3'
PDGFD_forward				5'-CCTCAGGCGAGATGAGAGCAATCAC-3'
PDGFD_reverse	80310	NM_025208.4		5'-TTCCTGGGGTAGCTGTTCCGGA-3'
PDGFD_probe			Cy5	5'-TGCACGTAGCCGTTTCCTTTCACCTGG-3'
GNB2L1_forward			-	5'-CCAGCAGCAAGGCAGAACCACC-3'
GNB2L1_reverse	10399	NM_006098.4	-	5'-ACACTCGCACCAGGTTGTCCG-3'
GNB2L1_probe			Cy5.5	5'-TGCACCTCCCTGGCCTGGTCTGCT-3'

Abbreviations: Tx Red = Texas Red; FAM = Fluorescein; HEX = Hexachloro-Fluorescein; Cy = Cyanine.

Note: The primers and probes specific for (CHI3L1, COL6A2, MMP7, MMP9), (IGF1, IGFBP2, SPP1, THBS1) and (FN1, MMP12, PDGFD) were collectively used in multiplex-1, -2 and -3, respectively. The primers and probe specific for GNB2L1, our reference gene (E1), were added into each multiplex.

Table E2. Timing of BAL sample collection in patients with multiple lavages.

	0.5-3*	3-6	6-12	12-24	Total samples
Pat. 1†		1	1		2
Pat. 2	2	1			3
Pat. 3	2	2	2		6
Pat. 4	2	1			3
Pat. 5		2			2
Pat. 6	2	2			4
Pat. 7	2	1			3
Pat. 8	2	2	2		6
Pat. 9	2				2
Pat. 10	2	1			3
Pat. 11	2	2			4
Pat. 12	2				2
Pat. 13	1	2	1		4
Pat. 14			1	1	2
Pat. 15			1	1	2
Pat. 16	1	1			2
Pat. 17	2	1	1		4
Pat. 18	2	2	1		5
Pat. 19	1	1			2
Pat. 20	1	1	1		3
Pat. 21	2				2
Pat. 22		2	2	1	5
Pat. 23	2	1	1		4
Pat. 24				2	2
Pat. 25	2	2	1		5
Pat. 26	2	2	1		5
Pat. 27	1	2	1		4
Pat. 28	2	1	1	1	5
Pat. 29				2	2
Pat. 30		1	1		2
Pat. 31		1	1		2
Pat. 32		1	1		2
Pat. 33		1	1		2
Pat. 34		1	1		2

*Time window post-transplantation (months)

†Patient code

Table E3. Sample distribution based upon transbronchial biopsy evaluation*†.

	Grade	Low	Ana-bolic	Inter-mediate	Cata-bolic	Firmi./Proteo./Actino. dysbiosis	No dysbiosis	Bact. dysbiosis
Acute rejection	A0	28 80%	24 80%	57 86.4%	13 81.3%	20 87%	84 81.5%	24 92.3%
	A1 (minimal)	5 14.3%	6 20%	7 10.6%	3 18.7%	3 13.0%	16 15.6%	1 3.8%
	A2 (mild)	2 5.8%	0 0%	2 3%	0 0%	0 0%	3 2.9%	1 3.8%
Airway inflammation	B0	31 88.6%	22 84.6%	57 89.1%	11 78.6%	13 72.2%	69 84.1%	20 95.2%
	B1-B2 (low grade)	4 11.4%	4 15.4%	7 10.9%	3 21.4 %	5 27.8%	13 15.9%	1 4.8%

Abbreviations: Actino. = Actinobacteria; Bact. = Bacteroidetes; Firmi. = Firmicutes; Proteo. = Proteobacteria

*Grading of pulmonary allograft rejection according to guidelines of the International Society for Heart and Lung Transplantation (E3)

† Data presented as n (%) within each BAL cell gene expression profile or microbiota composition group

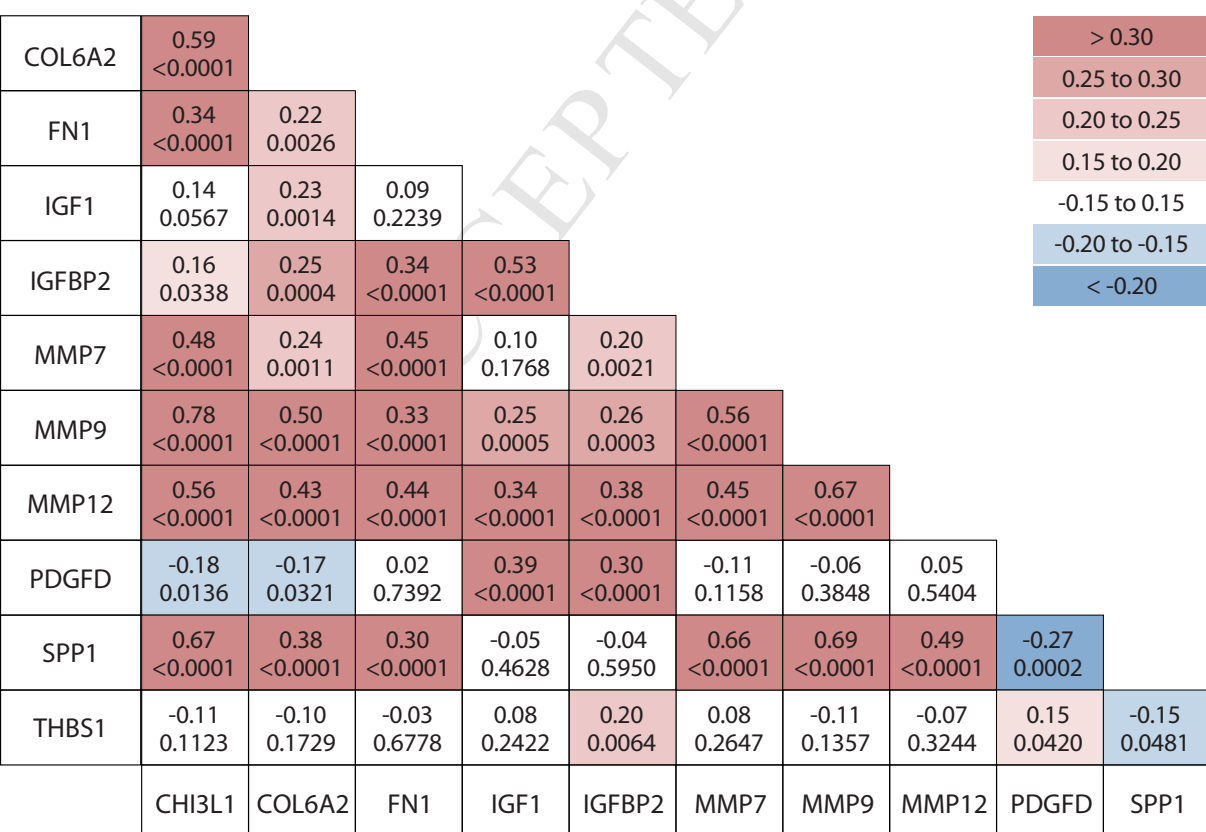


Figure E1

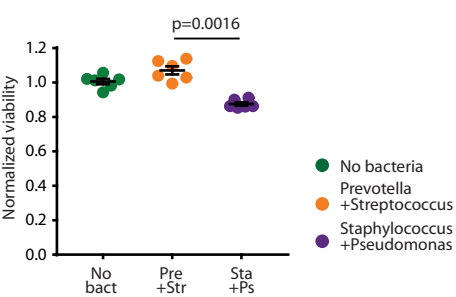


Figure E2