The Airway Microbiota Signatures of Infection and Rejection in Lung Transplant Recipients

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Research

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Abstract

Background

Infection and rejection are the two most common complications after lung transplantation (LT) and are associated with increased morbidity and mortality. The differential diagnosis of infection and rejection is sometimes difficult due to similar clinical manifestations. However, few studies have investigated the airway microbiota between lung transplant recipients (LTRs) with infection and rejection.

Results

A total of 181 sputum samples (event-free, n=47; infection, n=103; rejection, n=31) were collected from 59 LTRs. A distinct airway microbiota was observed among clinically stable (or event-free) recipients and those with infection or acute rejection after LT. Alpha and beta diversity were significantly different between event-free and rejection recipients and between infection and rejection recipients. Ten differential genera were identified by linear discriminant analysis effect size (LEfSe), with *Corynebacterium*, unclassified *Enterococcaceae* and unclassified *Lactobacillales* enriched in recipients with infection, and *Rothia*, *Granulicatella*, *Neisseria*, *Actinomyces*, *Leptotrichia*, *Lactobacillus* and unclassified *Aerococcaceae* more abundant in LTRs with acute rejection. Random forest analyses indicated that the combination of the 10 microbiota constituents and procalcitonin (PCT) and T-lymphocyte levels showed AUCs of 0.894, 0.955 and 0.913 to differentiate between event-free and infection, event-free and rejection, and infection and rejection recipients, respectively.

Conclusions

Our study is the first to compare the airway microbiota between LTRs with infection and acute rejection. The airway microbiota, especially combined with PCT and T-lymphocyte levels, showed satisfactory predictive efficiency in discriminating among clinically stable recipients and those with infection and acute rejection, suggesting that the airway microbiota was an indicator to differentiate between infection and acute rejection after LT.

Background

Lung transplantation (LT) is the only therapeutic option for patients with end-stage lung disease and short-term graft survival has improved over decades. However, the survival of lung transplant recipients (LTRs) is limited compared with that of other solid organ transplant recipients [1]. The low survival rate is predominantly due to infection- and rejection-related complications, which are the two major threats for LTRs in both early and long-term follow-up [1,2]. Severe acute rejection often occurs after LT, and various infections due to an immunosuppressed state and the unique anatomy and physiology of the transplanted lung also occur frequently [2]. As a result, rejection and infection affect and interact with each other, and balancing rejection and infection is the major challenge of LT [3]. Many studies have revealed the importance of the airway microbiota in local and systemic immunity, and airway microbiota

dynamics play a very important role in the development and pathophysiology of respiratory diseases [4-6]. This suggests that the airway microbiota may affect the immune response and therefore the balance between infection and rejection in LTRs.

Over the past decades, several studies have observed an altered airway microbiota in LTRs compared with that in healthy controls and pretransplant patients [7-9]. For example, Charlson *et al.* found that LTRs have lower microbial richness and diversity but a higher bacterial burden in bronchoalveolar lavage fluid (BALF) than control subjects [7]. Syed and colleagues demonstrated a similar alpha diversity and a distinct beta diversity of the airway microbiota between pre- and post-LT [8]. In addition, Pseudomonadaceae, Enterobacteriaceae and Staphylococcaceae were enriched in LTRs, while Prevotellaceae, Veillonellaceae and Streptococcaceae were frequently detected in nontransplant individuals [9]. Importantly, increasing evidence has indicated a close relationship between the airway microbiota and the disease progression and outcome of LTRs [10-12]. In summary, investigation of the airway microbiota may improve our understanding of the mechanisms involved in allograft dysfunction and may suggest potential therapies to improve survival for LTRs.

In addition to the immunosuppressed state, LTRs are more susceptible to infection because of direct exposure to the external environment, a defective cough reflex and damaged mucociliary clearance compared with those in other organ transplant patients [2,13]. Respiratory infection is the main cause of death within the first year after LT, with bacteria being the most frequent cause [13,14]. A recent study suggested a distinct airway microbiota in LTRs between respiratory infection and colonization without respiratory infection [15], and loss of airway microbial diversity can increase the risk of infection [9]. BALF neutrophilia was found to be associated with lower microbial diversity, indicating a correlation between the airway microbiota and infection after LT [16]. Allograft rejection is another common complication after LT and is associated with increased morbidity and mortality [3]. Previous studies have revealed a significant relationship between the airway microbiota and acute rejection. For example, low microbial diversity was associated with acute rejection [17], while microbiome phenotypes dominated by *Actinobacteria* reduced the risk of developing acute rejection [12]. Overall, these studies indicated a close relationship between the airway microbiota and infection and rejection of LTRs.

Although clinicians try to monitor these two complications carefully, the diagnostic options are limited. The diagnosis of infection requires clinicians to identify the source and carry out pathogen specific tests. The pathological assessment of transbronchial biopsies, which is the gold standard for the diagnosis of acute rejection after LT, is an invasive procedure with limited predictive value [18]. However, both of post-transplant infection and acute rejection may be accompanied by signs of similar clinical features, including cough, shortness of breath and radiological infiltrate, thus resulting in difficult differential diagnosis [19]. Considering the high morbidity and mortality of infection and rejection in LTRs and the bidirectional relationship between them, clinical decision-making depends on accurate diagnosis of both infection and rejection. Recent studies have revealed an association between the airway microbiota and infection or rejection after LT. However, few studies have investigated the specific microbial differences between infection and acute rejection recipients. Whether there are potential indicators to distinguish

between infection and acute rejection in LTRs remains unknown. In this cross-sectional study, we analyzed the airway microbial profiles associated with infection and acute rejection in LTRs.

Methods

Study population

A total of 181 sputum samples collected from 59 adult LTRs were enrolled in our study at the First Affiliated Hospital of Guangzhou Medical University (Guangzhou, China) between June 2017 and December 2019. Clinical information collected from the recipients included standardized medical record abstraction, including the demographic data, transplant data, laboratory examination and clinical diagnosis of the recipients (Table 1).

Adult LTRs (age >18 years) hospitalized in the First Affiliated Hospital of Guangzhou Medical University from June 2017 to December 2019 were included. The exclusion criteria included intubation or mechanical ventilation or other complications (e.g., bleeding, anastomotic complications, pneumothorax, etc.) at the time of sampling. The diagnostic criteria for respiratory infection were based on clinical (such as fever, cough, sputum production and radiographic infiltrate) and microbiological grounds [14,20]. Acute rejection was diagnosed by transbronchial lung biopsy according to the International Society for Heart and Lung Transplantation (ISHLT) criteria [21]. Clinically stable recipients (or event-free) were defined as having neither infection nor rejection. Each sample was assigned a diagnosis of infection, acute rejection or event-free independently by two experienced clinicians. In the case of disagreement, a third clinician was consulted (for more details, please see the Supplementary Material).

Data collection

For each enrolled subject, we collected demographic data, operation-related data, laboratory tests and pharmacological treatment. Except for operation-related data collected during or after LT, other data were collected at the time of sampling. Laboratory data, including cell counts/percentages in the blood and BALF, the biomarkers of inflammation (PCT) and microbiological data, were provided by the clinical laboratory of the First Affiliated Hospital of Guangzhou Medical University. The absolute count of total T lymphocytes (CD3⁺) in peripheral blood was determined by flow cytometry using the Cytomics FC500 cytometer. Serum PCT was measured using an electrochemical luminescence immunoassay. PGD was diagnosed and graded based on pulmonary edema on chest X-ray and the PaO₂/FiO₂ ratio according to the 2016 ISHLT consensus statement [22].

Sample collection, DNA extraction and 16S rRNA gene sequencing

Sputum samples were obtained by induced sputum of hypertonic or isotonic saline with salbutamol according to the Task Force on Induced Sputum of the European Respiratory Society [23]. All samples were immediately stored at -80°C for subsequent DNA extraction. The V3-V4 hypervariable region of the 16S rRNA gene was amplified by PCR, and the amplicons were sequenced using the Illumina NovaSeq

6000 platform (Guangzhou, China). Full details about the DNA amplification, purification and preparation for sequencing are provided in our previous study and in the Supplementary Material [24,25].

Subsequent sequence processing and analysis were performed using the Quantitative Insights into Microbial Ecology 2 (QIIME2) platform [26]. The barcode primers were removed if they contained ambiguous reads or mismatches in the primer sequences following the barcoded Illumina paired-end sequencing (BIPES) protocol [27]. We screened and removed chimeras using UCHIME in *de novo* mode to obtain high-quality sequence reads [28]. Representative sequences were aligned using Python Nearest Alignment Space Termination (PyNAST) against the Greengenes database [29]. The bioinformatics procedure is documented in <https://github.com/lichunxi0/Lung-transplant/tree/main>. Sequences were clustered into operational taxonomic units (OTUs) with 97% sequence similarity using USEARCH [30]. The 16S rRNA gene sequences were classified into specific taxa using the Ribosomal Database Project (RDP) classifier [31]. Additional details are provided in the Supplementary Material.

Statistical analysis

The Shannon index was used to evaluate the alpha diversity (within-sample diversity). Principal coordinate analysis (PCoA) based on the unweighted UniFrac distance matrix was performed to calculate beta diversity (dissimilarity between samples). Differential taxa between groups were identified using the linear discriminant analysis (LDA) effect size (LEfSe) method with a threshold set at 2.0 for the LDA score [32]. Redundancy analysis (RDA) was performed to evaluate the relationship between clinical characteristics and different groups. Random forest models were used to classify the LTRs with different diagnoses and evaluate the importance of indicator using the R “randomForest” package [33]. The performance of the model was evaluated using a 10-fold cross-validation approach and measured using a receiver operating characteristic (ROC) curve (“pROC” package). The area under the ROC curve (AUC) was determined to assess the ROC effect. Clinical characteristics were evaluated using SPSS (v20.0) software, and Figures were generated using GraphPad Prism (v7.0), Canoco (v5.0), TBtools (v1.051) and R (v2.1.1) software.

Results

After sequencing, a total of 181 sputum samples from 59 LTRs were included for subsequent analysis (Fig. 1). We divided the recipients into three groups according to the presence or absence of pulmonary infection and acute rejection at sampling: clinically stable (or event-free, n=47) recipients, recipients with infection (n=103) and recipients with rejection (n=31). The clinical characteristics of the LTRs are presented in Table 1.

Clinical characteristics, such as laboratory parameters, hospital stay, intensive care unit (ICU) stay, primary graft dysfunction (PGD) grade and pulmonary function, are regarded as factors that influence both clinical diagnosis and the airway microbiota. To assess the contribution of clinical variables to microbial community composition and clinical diagnosis, RDA was performed at the OTU level. The results showed that the event-free, infection and rejection groups could be distinguished from each other,

and several clinical characteristics, such as hospital stay, ICU stay and serum PCT, were highly positively associated with the airway microbiota of infection recipients, while T lymphocytes were positively related to rejection recipients (Fig. S1).

The airway microbial community in LTRs with different transplant outcomes

First, we compared the airway microbial diversity among LTRs with different clinical diagnoses. Alpha diversity was significantly different between the event-free and rejection and between the infection and rejection groups ($P<0.011$, $P=0.001$, respectively), with the highest Shannon index in the rejection group (Fig. 2A). PCoA based on the unweighted UniFrac distance matrix showed a distinct beta diversity among the 3 transplant groups ($P=0.001$, $R^2=0.13$, Fig. 2B), as well as between the event-free and rejection groups and between the infection and rejection groups ($P=0.004$, $R^2=0.03$; $P=0.001$, $R^2=0.02$; respectively, Fig. 2D, E). However, the event-free and rejection groups were not clearly separated in alpha (Shannon, $P=0.136$) and beta diversity (unweighted UniFrac, $P=0.35$, $R^2=0.01$, Fig. 2C).

Additionally, a heat map of the 28 dominant genera (relative abundance $>1\%$ in at least one group) showed a different microbial profile among the 3 groups (Fig. 3A). A Venn plot was drawn at the family, genus and OTU levels with relative abundance $>1\%$ among the different groups (Fig. 3B, C, D). The results showed that 13 families, 13 genera and 8 OTUs were shared by the event-free, infection and rejection recipients. Several microbial taxa were unique to the 3 groups, including 1 family, 2 genera and 6 OTUs in the event-free group; 2 families, 3 genera and 8 OTUs in the infection group; and 1 family, 1 genus and 5 OTUs in the rejection group. This finding indicated that the 3 groups not only shared a common microbiota but also had their own unique taxa which may associated with the pathogenesis of both diseases.

Changes in the airway microbiota during infection and rejection of LTRs

The top 6 most abundant phyla of the airway microbiota detected in our study were Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria and Fusobacteria. The 28 dominant genera with an average relative abundance greater than 1% in at least one group (mainly *Streptococcus*, *Rothia*, *Enterococcus*, *Haemophilus* and *Granulicatella*) accounted for up to 90% of the total genera. The microbial composition of the airway microbiota at the phylum level and the genus level was distinct among the event-free, infection and rejection recipients (Fig. 4A, B).

Moreover, LEfSe on all bacterial taxa was used to identify the different microbiota constituents among the 3 transplant groups (LDA score >2.0 , Fig. S2). To more clearly explore microbial differences among the 3 groups, we selected the above 28 dominant genera to build the LEfSe plot, and a total of 10 distinguished genera were identified (Fig. 4C). Three genera, namely, *Corynebacterium*, unclassified *Enterococcaceae* and unclassified *Lactobacillales* were greatly enriched in the infection group, and 7 genera, namely, *Rothia*, *Granulicatella*, *Neisseria*, *Actinomyces*, *Leptotrichia*, *Lactobacillus* and unclassified *Aerococcaceae*, were significantly enriched in the rejection group, while no differentially

abundant genera were found in clinically stable recipients. Table S1 compares the relative abundance and prevalence of the 10 genera in the 3 groups.

The prediction efficiency of the airway microbiota and clinical features for different clinical diagnoses of LTRs

Finally, we attempted to evaluate whether there were useful adjunctive indicators for the discrimination of different transplant groups. Generally, increases in serum PCT and peripheral blood T-lymphocyte levels are associated with infection and acute rejection after LT, respectively [34,35]. In our study, PCT and T-lymphocyte levels were relatively high in the recipients with infection and rejection, respectively, but the differences were not statistically significant (Fig. 5A, B). Therefore, random forest analysis was performed using individual airway microbiota constituents alone or in combination with clinical variables (PCT and T-lymphocyte levels) to investigate their prediction efficiencies in LTRs with different clinical diagnoses. First, the ROC curve was determined using the above 10 potential indicators identified by LEfSe, with AUC values of 0.697 (95%CI: 68.45-71.03%), 0.854 (95%CI: 84.13-86.63%) and 0.822 (95%CI: 81.08-83.28%) to distinguish between the event-free and infection, event-free and rejection, and infection and rejection groups, respectively (Fig. 5C). In comparison, the model was built based on the combination of the 10 genera and PCT and T lymphocyte levels. The results revealed an improved performance, and the corresponding AUCs reached 0.894 (95% CI: 88.54-90.34%), 0.955 (95% CI: 94.78-96.24%) and 0.913 (95% CI: 90.28-92.31%, Fig. 5D). These results indicated that the airway microbiota, especially combined with PCT and T lymphocyte levels, was a reliable indicator of infection and acute rejection in LTRs.

Discussion

In this study, we explored the relationship between airway microbiota and infection and acute rejection of LTRs. A significantly different airway microbiota was observed among event-free, infection and rejection recipients. The ten most differential genera identified by LEfSe were considered to be potential indicators for infection and acute rejection diagnosis in LTRs, including *Corynebacterium*, unclassified *Enterococcaceae*, unclassified *Lactobacillales*, *Rothia*, *Granulicatella*, *Neisseria*, *Actinomyces*, *Leptotrichia*, *Lactobacillus* and unclassified *Aerococcaceae*. Furthermore, a combination of the 10 genera and PCT and T lymphocyte levels indicated great discrimination for different clinical diagnosis, suggesting that the airway microbiota may be a useful indicator for infection and acute rejection diagnosis in LTRs.

Previous studies have found lower microbial richness and diversity but a higher bacterial burden in the lungs of LTRs than in those of control subjects [7], as well as a similar alpha diversity and a distinct beta diversity in the airways before and after LT [8]. However, few studies have reported microbial differences among clinically stable, infection and rejection LTRs. In this study, we found different alpha and beta diversities between event-free and rejection groups and between infection and rejection groups, but no marked difference in either alpha or beta diversity was found between event-free and infection recipients. Venn plots indicated both similarities and differences among the 3 transplant groups. Microbiota

composition analysis revealed microbial changes following different diagnoses of LTRs. Moreover, LEfSe identified 3 and 7 genera that were specifically enriched in infection and rejection recipients respectively. *Corynebacterium*, unclassified *Enterococcaceae* and unclassified *Lactobacillales* were enriched in infection recipients, while *Rothia*, *Granulicatella*, *Neisseria*, *Actinomyces*, *Leptotrichia*, *Lactobacillus* and unclassified *Aerococcaceae* were more abundant in rejection LTRs. Taken together, these results suggest a close association between the airway microbiota and infection and acute rejection in LTRs.

Among the 10 most differential genera, *Corynebacterium* is considered a proinflammatory bacterium associated with catabolic remodeling in the transplanted lung [36]. *Enterococcus*, the major genus of the family Enterococcaceae, is one of the most frequent pathogens during the first year after LT [37]. Our results showed significantly elevated abundances of *Corynebacterium* and unclassified *Enterococcaceae* in recipients with infection, which may therefore support their more important roles in transplant infection. In addition, *Rothia*, *Granulicatella*, *Neisseria*, *Actinomyces*, *Leptotrichia*, *Lactobacillus* and unclassified *Aerococcaceae* have been found to be associated with asthma [38-44], suggesting an interaction between these microbiota constituents and the immune system in the lung. In the current study, these 7 bacterial genera were enriched in recipients with acute rejection, indicating that these genera may be involved in the immune response in acute rejection. However, few studies have reported the role of these 7 bacterial genera in allograft lung rejection, and the mechanism remains unknown.

Infection and rejection are very common complications associated with increased morbidity and mortality following LT [13]. Unfortunately, the differential diagnosis of infection and rejection is sometimes difficult due to similar clinical manifestations [19,45]. Several studies have suggested that upregulated PCT levels reflect the presence of infective complications after solid organ transplantation and LT [34,45]. T-lymphocyte is regarded as the major immune lymphocytes responsible for lung allograft rejection [35,46,47]. An increase in peripheral blood T-lymphocyte counts was observed during acute lung rejection [48]. However, in our results, infection and rejection recipients showed only a slight increase in PCT and T lymphocyte levels compared with those in the other 2 groups ($P>0.05$). Therefore, we attempted to seek another indicator to differentiate infection from rejection. We performed random forest analyses and achieved a satisfactory prediction effect of the airway microbiota, especially with the above 10 differential genera identified by LEfSe (*Corynebacterium*, unclassified *Enterococcaceae* and unclassified *Lactobacillales*, *Rothia*, *Granulicatella*, *Neisseria*, *Actinomyces*, *Leptotrichia*, *Lactobacillus* and unclassified *Aerococcaceae*), for distinguishing between different clinical diagnoses (event-free vs. infection, AUC=0.697; event-free vs. rejection, AUC=0.854; infection and rejection, AUC=0.822). Furthermore, a better classification efficacy of the combination of the 10 microbiota constituents and PCT and T lymphocyte levels was assessed: event-free vs. infection, AUC=0.894; event-free vs. rejection AUC=0.955; and infection and rejection, AUC=0.913. Overall, these findings further confirmed the importance of the 10 airway microbiota constituents in transplant infection and rejection, and provided important evidence that the airway microbiota was potentially helpful in predicting infection and acute rejection in LTRs. Nevertheless, biomarker-based models still need to be confirmed with larger samples.

Notably, our study is the first to compare the airway microbial profiles between recipients with infection and rejection after LT, as well as to evaluate the use of airway microbiota in differentiating between LTRs with infection and rejection. Another strength of this study is a relatively large number of LTRs and the first exploration of the airway microbiota in Chinese LTRs using high-throughput technology [12,49]. A major limitation is the cross-sectional design that provides only the possible relationships between the airway microbiota and infection and rejection in LTRs. Larger studies are needed to repeat and confirm these findings, and future *in vivo* and *in vitro* experiments are needed to determine the microbial-mediated mechanism. Moreover, the dynamic changes in the airway microbiota may be more reliable and meaningful.

Conclusion

In summary, we conducted one of the first studies to explore the relationship between the airway microbiota and infection and acute rejection of LTRs. *Corynebacterium*, unclassified *Enterococcaceae*, and unclassified *Lactobacillales* were enriched in patients with infection, while *Rothia*, *Granulicatella*, *Neisseria*, *Actinomyces*, *Leptotrichia*, *Lactobacillus* and unclassified *Aerococcaceae* were enriched in recipients with acute rejection. The combination of airway microbiota with PCT and T-lymphocyte levels may complement the deficiencies of the diagnosis of acute rejection and infection in LTRs and thus improve the treatment and clinical outcomes of patients after LT. In the future, it seems important to understand the detailed role of the airway microbiota in the mechanism and development of infection and acute rejection after LT.

Abbreviations

AUC: area under the ROC curve; BALF: bronchoalveolar lavage fluid; BIPES: barcoded Illumina paired-end sequencing; ICU: intensive care unit; ISHLT: International Society for Heart and Lung Transplantation; LEfSe: linear discriminant analysis effect size; LT: lung transplantation; LTRs: lung transplant recipients; OTU: Operational taxonomic unit; RDA: Redundancy analysis; PCoA: Principal coordinate analysis; PCT: procalcitonin; PGD: primary graft dysfunction; PyNAST: Python Nearest Alignment Space Termination; QIIME2: Quantitative Insights into Microbial Ecology 2; RDP: Ribosomal Database Project; ROC: receiver operating characteristic.

Declarations

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Availability of data and materials

The raw sequencing data were deposited in ENA (accession number PRJEB40386).

Ethics approval and consent to participate

This study was approved by the ethics committee of the First Affiliated Hospital of Guangzhou Medical University (no 2017-22). All patients provided written informed consent, in accordance with the Declaration of Helsinki.

Authors' Contributions

J.S, CX.L, HY.L, CX.Y, CR.J and JX.H designed the experiments. CX.L, ZX.Y, A.C, QY.L, YH.C, YX.L and JB.P collected the samples and performed the experiments. J.S, CX.L, HY.L and K.L analysed the microbial data. CX.L, HY.L, J.S, GX.Z, CX.Y, CR.J and JX.H prepared the manuscript and had primary responsibility for its final content. All authors contributed to the article and approved the submitted version.

Consent for publication

Consent was given by subjects to release personal data for publication as needed.

Competing interests

The authors declare no competing interests.

References

1. Chambers DC, Cherikh WS, Harhay MO, Hayes D, Jr., Hsich E, Khush KK, et al. The international thoracic organ transplant registry of the international society for heart and lung transplantation: thirty-sixth adult lung and heart-lung transplantation Report-2019; Focus theme: Donor and recipient size match. *J Heart Lung Transplant*. 2019;38:1042-55.
2. Burguete SR, Maselli DJ, Fernandez JF, Levine SM. Lung transplant infection. *Respirology*. 2013;18:22-38.
3. Yusen RD, Edwards LB, Kucheryavaya AY, Benden C, Dipchand AI, Goldfarb SB, et al. The registry of the international society for heart and lung transplantation: thirty-second official adult lung and heart-lung transplantation report–2015; focus theme: early graft failure. *J Heart Lung Transplant*. 2015;34:1264-77.
4. Pulvirenti G, Parisi GF, Giallongo A, Papale M, Manti S, Savasta S, et al. Lower airway microbiota. *Frontiers in pediatrics*. 2019;7:393.

5. Lynch SV. The lung microbiome and airway disease. *Ann Am Thorac Soc*. 2016;13 Suppl 2:S462-S5.
6. Wu BG, Segal LN. Lung microbiota and its impact on the mucosal immune phenotype. *Microbiol Spectr*. 2017;5.
7. Charlson ES, Diamond JM, Bittinger K, Fitzgerald AS, Yadav A, Haas AR, et al. Lung-enriched organisms and aberrant bacterial and fungal respiratory microbiota after lung transplant. *Am J Respir Crit Care Med*. 2012;186:536-45.
8. Syed SA, Whelan FJ, Waddell B, Rabin HR, Parkins MD, Surette MG. Reemergence of lower-airway microbiota in lung transplant patients with cystic fibrosis. *Ann Am Thorac Soc*. 2016;13:2132-42.
9. Dickson RP, Erb-Downward JR, Freeman CM, Walker N, Scales BS, Beck JM, et al. Changes in the lung microbiome following lung transplantation include the emergence of two distinct *Pseudomonas* species with distinct clinical associations. *PLoS One*. 2014;9:e97214.
10. Willner DL, Hugenholtz P, Yerkovich ST, Tan ME, Daly JN, Lachner N, et al. Reestablishment of recipient-associated microbiota in the lung allograft is linked to reduced risk of bronchiolitis obliterans syndrome. *Am J Respir Crit Care Med*. 2013;187:640-7.
11. Mitchell AB, Glanville AR. The human respiratory microbiome: implications and impact. *Semin Respir Crit Care Med*. 2018;39:199-212.
12. Schott C, Weigt SS, Turturice BA, Metwally A, Belperio J, Finn PW, et al. Bronchiolitis obliterans syndrome susceptibility and the pulmonary microbiome. *J Heart Lung Transplant*. 2018;37:1131-40.
13. Costa J, Benvenuto LJ, Sonett JR. Long-term outcomes and management of lung transplant recipients. *Best Pract Res Clin Anaesthesiol*. 2017;31:285-97.
14. Nosotti M, Tarsia P, Morlacchi LC. Infections after lung transplantation. *J Thorac Dis*. 2018;10:3849-68.
15. Shankar J, Nguyen MH, Crespo MM, Kwak EJ, Lucas SK, McHugh KJ, et al. Looking beyond respiratory cultures: microbiome-cytokine signatures of bacterial pneumonia and tracheobronchitis in lung transplant recipients. *Am J Transplant*. 2016;16:1766-78.
16. Dickson RP, Erb-Downward JR, Prescott HC, Martinez FJ, Curtis JL, Lama VN, et al. Cell-associated bacteria in the human lung microbiome. *Microbiome*. 2014;2:28.
17. Spence CD, Vanaudenaerde B, Einarsson GG, McDonough J, Lee AJ, Johnston E, et al. Influence of azithromycin and allograft rejection on the post-lung transplant microbiota. *J Heart Lung Transplant*. 2020;39:176-83.
18. Arcasoy SM, Berry G, Marboe CC, Tazelaar HD, Zamora MR, Wolters HJ, et al. Pathologic interpretation of transbronchial biopsy for acute rejection of lung allograft is highly variable. *Am J Transplant*. 2011;11:320-8.
19. Dabbs ADV, Hoffman LA, Iacono AT, Zullo TG, McCurry KR, Dauber JH. Are symptom reports useful for differentiating between acute rejection and pulmonary infection after lung transplantation? *Heart Lung*. 2004;33:372-80.

20. Grief SN, Loza JK. Guidelines for the evaluation and treatment of pneumonia. *Prim Care*. 2018;45:485-503.
21. 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.
22. Snell GI, Yusen RD, Weill D, Strueber M, Garrity E, Reed A, et al. Report of the ISHLT Working Group on Primary Lung Graft Dysfunction, part I: Definition and grading—A 2016 Consensus Group statement of the International Society for Heart and Lung Transplantation. *The Journal of Heart and Lung Transplantation*. 2017;36:1097-103.
23. Guiot J, Demarche S, Henket M, Paulus V, Graff S, Schleich F, et al. Methodology for Sputum Induction and Laboratory Processing. *J Vis Exp*. 2017.
<https://www.ncbi.nlm.nih.gov/pubmed/29286433>.
24. Su J, Liu HY, Tan XL, Ji Y, Jiang YX, Prabhakar M, et al. Sputum bacterial and fungal dynamics during exacerbations of severe COPD. *PLoS One*. 2015;10:e0130736.
25. Liu HY, Zhang SY, Yang WY, Su XF, He Y, Zhou HW, et al. Oropharyngeal and sputum microbiomes are similar following exacerbation of chronic obstructive pulmonary disease. *Front Microbiol*. 2017;8:1163.
26. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol*. 2019;37:852-7.
27. Zhou HW, Li DF, Tam NF, Jiang XT, Zhang H, Sheng HF, et al. BIPES, a cost-effective high-throughput method for assessing microbial diversity. *ISME J*. 2011;5:741-9.
28. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics*. 2011;27:2194-200.
29. Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R. PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics*. 2010;26:266-7.
30. Westcott SL, Schloss PD. De novo clustering methods outperform reference-based methods for assigning 16S rRNA gene sequences to operational taxonomic units. *PeerJ*. 2015;3:e1487.
31. Whelan FJ, Surette MG. A comprehensive evaluation of the sl1p pipeline for 16S rRNA gene sequencing analysis. *Microbiome*. 2017;5:100.
32. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. *Genome Biol*. 2011;12:R60.
33. Liaw A, Wiener M. Classification and Regression by randomForest. *R News*. 2002;23.
34. Desmard M, Benbara A, Boudinet S, Mal H, Dehoux M, Thabut G, et al. Post-operative kinetics of procalcitonin after lung transplantation. *The Journal of Heart and Lung Transplantation*. 2015;34:189-94.
35. Wilkes DS, Egan TM, Reynolds HY. Lung transplantation: opportunities for research and clinical advancement. *Am J Respir Crit Care Med*. 2005;172:944-55.

36. Mouraux S, Bernasconi E, Pattaroni C, Koutsokera A, Aubert JD, Claustre J, et al. Airway microbiota signals anabolic and catabolic remodeling in the transplanted lung. *J Allergy Clin Immunol.* 2018;141:718-29 e7.
37. Kovats Z, Sütto Z, Muraközy G, Bohacs A, Czebe K, Lang G, et al. Airway pathogens during the first year after lung transplantation: a single-center experience. *Transplant Proc.* 2011;43:1290-1.
38. Sverrild A, Kiilerich P, Brejnrod A, Pedersen R, Porsbjerg C, Bergqvist A, et al. Eosinophilic airway inflammation in asthmatic patients is associated with an altered airway microbiome. *J Allergy Clin Immunol.* 2017;140:407-17 e11.
39. Park JS, Kim J, Yoo H, Son JH, Chang HS. Relationship of microbial profile with airway immune response in eosinophilic or neutrophilic inflammation of asthmatics. *J Allergy Clin Immunol.* 2020;145:AB16.
40. Chiu CY, Chan YL, Tsai YS, Chen SA, Wang CJ, Chen KF, et al. Airway Microbial Diversity is Inversely Associated with Mite-Sensitized Rhinitis and Asthma in Early Childhood. *Sci Rep.* 2017;7:1820.
41. Chun Y, Do A, Grishina G, Grishin A, Fang G, Rose S, et al. Integrative study of the upper and lower airway microbiome and transcriptome in asthma. *JCI Insight.* 2020;5.
42. Durack J, Lynch SV, Nariya S, Bhakta NR, Beigelman A, Castro M, et al. Features of the bronchial bacterial microbiome associated with atopy, asthma, and responsiveness to inhaled corticosteroid treatment. *J Allergy Clin Immunol.* 2017;140:63-75.
43. Ta LDH, Yap GC, Tay CJX, Lim ASM, Huang CH, Chu CW, et al. Establishment of the nasal microbiota in the first 18 months of life: Correlation with early-onset rhinitis and wheezing. *J Allergy Clin Immunol.* 2018;142:86-95.
44. Taylor SL, Leong LEX, Choo JM, Wesselingh S, Yang IA, Upham JW, et al. Inflammatory phenotypes in patients with severe asthma are associated with distinct airway microbiology. *J Allergy Clin Immunol.* 2018;141:94-103.e15.
45. Sammons C, Doligalski CT. Utility of procalcitonin as a biomarker for rejection and differentiation of infectious complications in lung transplant recipients. *Ann Pharmacother.* 2014;48:116-22.
46. Yamada Y, Brüstle K, Jungraithmayr W. T helper cell subsets in experimental lung allograft rejection. *J Surg Res.* 2019;233:74-81.
47. Gauthier JM, Li W, Hsiao H-M, Takahashi T, Arefanian S, Krupnick AS, et al. Mechanisms of Graft Rejection and Immune Regulation after Lung Transplant. *Annals of the American Thoracic Society.* 2017;14:S216-S9.
48. Meloni F, Morosini M, Solari N, Bini F, Vitulo P, Arbustini E, et al. Peripheral CD4+ CD25+ Treg cell expansion in lung transplant recipients is not affected by calcineurin inhibitors. *Int Immunopharmacol.* 2006;6:2002-10.
49. Mitchell AB. The lung microbiome and transplantation. *Current Opinion in Organ Transplantation.* 2019;24:305-10.

Table 1 Patient Characteristics.

	Total	Event-free	Infection	Rejection
Patients/samples	59/181	14/47	39/103	11/31
Sex (male)	49 (83.1%)	13 (92.9%)	31 (79.5%)	9 (81.8%)
Age, years (mean±SD)	57.2±12.8	60.5±13.1	57.0±12.1	53.9±15.7
Type of transplant				
Double	17 (28.8%)	3 (21.4%)	15 (38.5%)	1 (9.1%)
Single	41 (69.5%)	11 (78.6%)	24 (61.5%)	9 (81.8%)
Heart-lung transplant	1 (1.7%)	1 (7.1%)	0 (0.0%)	1 (9.1%)
Time post-transplant (days)	284.2±484.6	51.6±206.0	161.1±366.7	87.3±164.5
BMI (kg/m ²)	20.4±3.7	20.6±3.2	19.8±3.5	22.1±4.0
History of smoking, yes	36 (61.0%)	11 (73.3%)	24 (54.5%)	7 (63.6%)
PGD grade	2.0±1.1	1.6±1.1	2.2±1.0	4.7±2.9
Pretransplant diagnosis				
COPD	18 (30.5%)	4 (28.6%)	14 (35.9%)	2 (18.2%)
ILD	32 (54.2%)	10 (71.4%)	17 (43.6%)	8 (72.7%)
Other	9 (15.3%)	0 (0.0%)	8 (20.5%)	1 (9.1%)
Laboratory parameters [#]				
PCT (µg/L)	0.2±0.5	0.1±0.1	0.3±1.0	0.1±0.1
Blood T lymphocyte (/UL)	298.0±261.0	315.2±236.1	450.3±407.2	280.6±142.6
Positive culture ^{#§}				
<i>Acinetobacter baumannii</i>	24 (13.3%)	9 (19.1%)	15 (14.6%)	0 (0.0%)
<i>Enterobacter</i> sp.	2 (1.1%)	0 (0.0%)	2 (1.9%)	0 (0.0%)
<i>Enterococcus</i> sp.	14 (7.7%)	2 (4.3%)	12 (11.7%)	0 (0.0%)
<i>Klebsiella pneumoniae</i>	31 (17.1%)	14 (29.8%)	8 (7.8%)	9 (29.0%)
<i>Pseudomonas aeruginosa</i>	29 (16.0%)	0 (0.0%)	20 (19.4%)	9 (29.0%)
<i>Staphylococcus</i> sp.	27 (14.9%)	6 (12.8%)	20 (19.4%)	1 (3.2%)
<i>Stenotrophomonas maltophilia</i>	43 (23.8%)	12 (25.5%)	24 (23.3%)	7 (22.6%)
<i>Haemophilus influenzae</i>	5 (2.8%)	0 (0.0%)	5 (4.9%)	0 (0.0%)
<i>Aspergillus</i> sp.	15 (8.3%)	0 (0.0%)	15 (14.6%)	0 (0.0%)

<i>Candida</i> sp.	5 (2.8%)	0 (0.0%)	5 (4.9%)	0 (0.0%)
Blood CMV DNA	11 (6.1%)	0 (0.0%)	11 (10.7%)	0 (0.0%)
Antibiotics [#]				
Meropenem/Vancomycin	90 (49.7%)	20 (42.6%)	66 (64.1%)	4 (12.9%)
Piperacillin/Cefoperazone	72 (39.8%)	19 (40.4%)	34 (33.0%)	19 (61.3%)
TMP/SMX	29 (16.0%)	1 (2.1%)	24 (23.3%)	4 (12.9%)
Azithromycin	4 (2.2%)	1 (2.1%)	3 (3.0%)	0 (0.0%)
Immunosuppression [#]				
Glucocorticoid	181 (100%)	47 (100.0%)	103 (100.0%)	31 (100.0%)
Tacrolimus	163 (90.1%)	39 (83.0%)	93 (90.3%)	31 (100.0%)
Mycophenolate mofetil	158 (87.3%)	46 (97.9%)	89 (86.4%)	23 (74.2%)

Data are the mean±SD or n (%) as appropriate. BMI, body mass index; COPD, chronic obstructive pulmonary disease; ILD, interstitial lung disease; PGD, primary graft dysfunction; CMV, cytomegalovirus; TMP/SMX, trimethoprim/sulfamethoxazole.

[#]At sampling.

[§]Positive bacterial culture could be to the presence of respiratory pathogens or colonized bacteria. If there was no clear clinical evidence for respiratory infection or no previous culture for reference, the microorganisms in sputum were defined as colonized bacteria.

Figures

Figure 1

(A) Study flow chart. (B) Dots represent sputum samples collected from each of the 59 patients after LT. Patients 1-18 had COPD, patients 19-50 had ILD, and patients 51-59 were diagnosed with other lung diseases. *Patients who were sampled during two hospitalizations and diagnosed with two clinical statuses (same or different).

Figure 2

The airway microbial diversity of the event-free, infection and rejection recipients. (A). Alpha diversity (Shannon index) among the 3 transplant groups. The horizontal lines in the box plots represent median values; upper and lower ranges of the box represent the 75% and 25% quartiles. (B). Beta diversity (PCoA-based unweighted UniFrac distance matrix) among the 3 transplant groups. (C, D, E). Beta diversity (PCoA-based unweighted UniFrac distance matrix) between each pair of groups. The blue, red and green colors represent the event-free, infection and rejection groups, respectively. P values are represented using the Wilcoxon rank-sum test.

Figure 3

The airway microbial composition of the event-free, infection and rejection recipients. (A). Heat map of the 28 dominant genera (with average relative abundances >1% in at least 1 group) in the event-free, infection and rejection groups. (B). The Venn diagram demonstrates the unique and shared airway microbial numbers at the family level, genus level and OTU level among the different transplant groups.

Figure 4

The airway microbial differences among the event-free, infection and rejection recipients. (A). Comparison of bacterial communities of the airway microbiota based on the most abundant phyla and genera (average relative abundances >1% in at least one group) in LTRs with different clinical diagnoses. (B). LEfSe identified the most differentially abundant genera (with average relative abundances >1% in at least one group) among the 3 groups (LDA score >2.0).

Figure 5

Comparison of (A) PCT and (B) T lymphocyte levels among transplant groups. P values are represented using the t test. The ROC curve using (C) the airway microbiota alone and (D) the combination of the airway microbiota and PCT and T lymphocyte levels to differentiate among different transplant groups.

Supplementary Files

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