**The airway microbiota signatures of infection and rejection in lung transplant recipients**

**Supplemental material**

**Methods**

**Diagnostic criteria**

In order to examine the difference in the sputum microbiota among different LTRs, all recipients were divided into three groups depending on the post‐LT evolution: Event-free, infection and rejection. The diagnostic criteria of respiratory infection in LTRs are summarized as follows [1]:

(1). Signs/symptoms: at least one of the following: (a). fever >38°C or hypothermia <36.5°C with no other recognized source; (b). leukocyte count <4000 or >15000 /mm3; (c). purulent secretions; (d). new onset or worsening cough, dyspnea, tachypnea or plural rub, rales or bronchial breath sounds; (e). worsening gas exchange (O2 desaturation, PaO2/FiO2<240) increased the O2 requirement and increased the ventilation demand; and (f). pleural effusion.

(2). Radiology: new/worsening radiographic infiltrate on chest X-ray or CT scan.

(3). Microbiology: at least one of the following: (a). positive growth in blood culture unrelated to other sources; (b). positive growth of pleural fluid; (c). positive respiratory culture (sputum, bronchial secretions, BALF, or bronchial sterile brushing); and (d). >5% of BALF-obtained cells contained intracellular bacteria on direct microscopic examination.

As a supplement, markers such as neutrophil proportion and PCT may help in the diagnosis of infection [2,3]. It is noteworthy that positive bacterial culture could be due 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 by 2 clinicians as colonized bacteria. Thereafter the recipients with colonization were divided into groups according to the criteria of rejection and event-free. In addition, if fungal infection was suspected, other tests such as galactomannan, 1,3‐β‐D‐glucan assay or even PCR, could be performed, although their effect is limited [4]. Finally, bacterial infection is necessary, LTRs who were infected with only fungi or viruses were excluded.

Acute allograft rejection presents with nonspecific features, such as shortness of breath, cough with or without sputum production and even low-grade fever. According to the ISHLT criteria, pathologic findings in transbronchial biopsy are the gold standard for the diagnosis of acute rejection after LT [5]. The diagnosis of acute rejection is based on perivascular and interstitial mononuclear infiltrates. The severity grade of acute rejection is based on the intensity of mononuclear cell infiltrates and extension into the adjacent interstitium. The vascular component ranges from grade A0 (no rejection) to grade A4 (severe rejection). The airway component ranges from B0 (no rejection) to B2R (high grade). The detailed ISHLT acute rejection grading schema is summarized in Table S2. Moreover, the cytology of BALF and peripheral blood, such as eosinophil count, lymphocyte count and basophil count, may suggest a tendency to acute rejection [6].

Eleven recipients were continually included in our study, and they were sampled in two clinical statuses (same or different) at different time periods. Among the 11 recipients, one recipient was grouped as event-free, and five recipients were divided into the infection group at two sampling periods. In addition, one recipient was diagnosed with event-free and rejection; two recipients were diagnosed with event-free and infection; and two recipients were diagnosed with infection and rejection in two sampling period (Figure 1).

**Sputum sample processing for DNA extraction**

Frozen sputum samples were thawed under ventilation for 15 min, and genomic DNA extraction was performed using a Bacterial DNA Extraction Mini Kit (Mabio, Guangzhou, China) according to the manufacturer’s instructions. DNA was quantified with a NanoDrop One (Thermo Scientific, USA). PCR amplification was performed using specific primers with barcodes and Takara Premix Taq® Version 2.0 (Takara Biotechnology Co., Dalian, China), using genomic DNA as a template. The V3-V4 hypervariable region of the 16S rRNA gene was amplified by PCR with the forward primer: 338F 5′-ACTCCTACGGGAGGCAGCA -3′ and reverse primer: 806R 5′-GGACTACHVGGGTWTCTAAT-3′. PCR amplification was performed following the cycling protocol [Bio-Rad S1000 (Bio-Rad Laboratory, CA)] initial denaturation step at 94°C for 5 min; 30 cycles at 94°C for 30 s, 52°C for 30 s and 72°C for 30 s; and a final extension at 72°C for 10 min. Three replicates were performed for each sample, and PCR products from the same sample were mixed. The amplification reaction system included 50 ng of genomic DNA template; 25 μL of 2x Premix Taq; 1 μL of 10 μmol/L primer 338F; 1 μL of 10 μmol/L primer 806R; and nuclease-free water added to 50 μL. The PCR products were detected using 1% agarose gel electrophoresis (AGE). All the amplification products were stored at -80°C for subsequent sequencing.

**16S rRNA gene sequencing**

First, the barcode primers were trimmed and filtered if they contained ambiguous reads or mismatches in the primer regions following the barcoded Illumina paired-end sequencing (BIPES) protocol [7]. Next, we screened and removed chimeras using UCHIME in de novo mode to obtain high-quality sequence reads of the 16S rRNA gene [8]. All samples were normalized to 13,000 sequences to avoid deviation caused by the effects of different sequencing depths. Eight samples were excluded from the 16S V3-V4 data analysis after normalization. The taxonomy of representative 16S rRNA gene sequences was determined using Python Nearest Alignment Space Termination (PyNAST) with the Greengenes 13\_8 database as the reference, and multiple alignments of representative sequences were performed using PyNAST [9]. Representative 16S rRNA gene sequences were classified into specific taxa using the Ribosomal Database Project (RDP) classifier [10]. The operational taxonomic units (OTUs) were assigned by clustering the reads with 97% sequence similarity using USEARCH [11].

**SUPPLEMENT REFERENCES**

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**SUPPLEMENTATY TABLES**

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/m2) | 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#§ | | | | |
| *Acinetobacter baumannii* | 24 (13.3%) | 9 (19.1%) | 15 (14.6%) | 0 (0.0%) |
| *Enterobacter* sp. | 2 (1.1%) | 0 (0.0%) | 2 (1.9%) | 0 (0.0%) |
| *Enterococcus* sp. | 14 (7.7%) | 2 (4.3%) | 12 (11.7%) | 0 (0.0%) |
| *Klebsiella pneumoniae* | 31 (17.1%) | 14 (29.8%) | 8 (7.8%) | 9 (29.0%) |
| *Pseudomonas aeruginosa* | 29 (16.0%) | 0 (0.0%) | 20 (19.4%) | 9 (29.0%) |
| *Staphylococcus* sp. | 27 (14.9%) | 6 (12.8%) | 20 (19.4%) | 1 (3.2%) |
| *Stenotrophomonas maltophilia* | 43 (23.8%) | 12 (25.5%) | 24 (23.3%) | 7 (22.6%) |
| *Haemophilus influenzae* | 5 (2.8%) | 0 (0.0%) | 5 (4.9%) | 0 (0.0%) |
| *Aspergillus* sp. | 15 (8.3%) | 0 (0.0%) | 15 (14.6%) | 0 (0.0%) |
| *Candida* 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.

Table S1. The relative abundance (RA%) and prevalence (P%) of the 16 microbial genera identified by LEfSe among the 3 groups.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Genus | Event-free | | Infection | | Rejection | | *P* value (RA% between groups) | | |
| RA% | P% | RA% | P% | RA% | P% | Event-free vs. Infection | Event-free vs. Rejection | Infection vs. Rejection |
| *Actinomyces* | 2.61 | 97.87 | 2.50 | 99.03 | 5.07 | 100.00 | 0.201 | 0.003 | 0.003 |
| *Corynebacterium* | 0.42 | 76.60 | 4.80 | 85.44 | 0.11 | 100.00 | 0.012 | 0.091 | 0.597 |
| *Granulicatella* | 0.64 | 100.00 | 3.47 | 100.00 | 6.37 | 100.00 | <0.001 | <0.001 | 0.001 |
| *Lactobacillus* | 3.22 | 97.87 | 0.81 | 99.03 | 3.49 | 100.00 | 0.353 | 0.017 | 0.024 |
| *Leptotrichia* | 0.59 | 31.91 | 0.48 | 37.86 | 2.15 | 58.06 | 0.474 | 0.011 | 0.013 |
| *Neisseria* | 0.99 | 76.60 | 1.21 | 72.82 | 4.05 | 80.65 | 0.651 | 0.025 | 0.008 |
| *Rothia* | 4.57 | 97.87 | 6.90 | 99.03 | 17.61 | 100.00 | 0.04 | <0.001 | <0.001 |
| unclassified *Aerococcaceae* | 1.53 | 97.87 | 1.39 | 95.15 | 1.71 | 100.00 | 0.002 | <0.001 | <0.001 |
| unclassified *Enterococcaceae* | 0.24 | 100.00 | 1.41 | 97.09 | 0.42 | 100.00 | 0.026 | 0.001 | 0.354 |
| unclassified *Lactobacillales* | 0.23 | 89.36 | 1.24 | 97.09 | 0.41 | 100.00 | 0.012 | 0.001 | 0.301 |

*P* values are represented using the Wilcoxon rank-sum test.

Table S2 Pathologic grading of acute cellular rejection.

|  |  |  |  |
| --- | --- | --- | --- |
| Category of rejection | ISHLT grade | Severity | Histologic appearance |
| Grade A:  Acute rejection | A0 | None | Normal pulmonary parenchyma without evidence of mononuclear cell infiltration, hemorrhage, or necrosis. |
| A1 | Minimal | Scattered and infrequent perivascular mononuclear infiltrates, venules  cuffed by small and round plasmacytoid cells, and transformed lymphocytes forming rings of 2-3 cell thickness within perivascular adventitia. |
| A2 | Mild | More frequent perivascular mononuclear infiltrates seen, endothelialitis. Perivascular interstitium can be expanded by mononuclear cells, but no infiltration into adjacent alveolar septa or airspaces. |
| A3 | Moderate | Cuffing of venules and arterioles by dense perivascular mononuclear cell infiltrates extending into perivascular and peribronchial alveolar septa and airspace. Eosinophils and neutrophils are common. Can involve endothelialitis. |
| A4 | Severe | Diffuse perivascular, interstitial, and airspace infiltrates of mononuclear  cells with prominent alveolar pneumocyte damage and endothelialitis. |
| Grade B:  Lymphocytic bronchiolitis (small airways) | B0 | None | No evidence of bronchiolar inflammation. |
| B1 | Low grade | Mononuclear cells in bronchiolar submucosa; no evidence of epithelial damage or intraepithelial lymphocytic infiltration. |
| B2 | High grade | Greater number of eosinophils and plasmacytoid cells. Evidence of epithelial damage (necrosis and metaplasia), marked intraepithelial lymphocytic infiltration. |
| BX | Upgradeable | No small airways identified on tissue biopsy or overt evidence of infection. |

**Supplementary figure legends**



Figure S1 RDA shows the relationship between the airway microbiota and clinical variables of LTRs. The solid triangle represents the clinical diagnosis, and the black arrow represents a clinical variable. The clinical variables with nonobvious associations with any one of the diagnoses (*P*>0.05) are not presented. WBC, white blood cell; NEU, neutrophils; BMI, body mass index; ICU, intensive care unit; APACHE, acute physiology and chronic health evaluation; SOFA, sequential organ failure assessment; ECMO, extracorporeal membrane oxygenation; PGD, primary graft dysfunction; VC, vital capacity; FVC, forced vital capacity; FEV1, forced expiratory volume in the first second.



Figure S2 LEfSe identified the differentially abundant microbial taxa among the Event-free, infection and rejection groups (LDA score >2.0).