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Effects of respiratory flora regulation on microbial environment and IL-10/STAT3 signaling pathway in the bronchiolitis obliterans after lung transplantation

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ABSTRACT

Our purpose of this study was to explore the application effect of respiratory flora regulation in bronchiolitis obliterans after lung transplantation, and its regulatory effect on the microbial environment of the lesion and the IL-10/STAT3 signaling pathway. 25 clean-grade C57BL/6 male mice and 5 BALB/c male mice were selected for orthotopic tracheal transplantation and postoperative respiratory flora regulation in a hospital animal room from Jan 2019 to Dec 2021. Next, the changes in the microbial environment and the IL-10/STAT3 signaling pathway before and after respiratory flora regulation were compared, so as to evaluate the regulatory effect of this method. The Simpson index did not show a significant difference before and after respiratory flora regulation intervention ($P>0.05$). However, the Chao1, ACE, Shannon, and Actinobacteria dominant indices were higher after the intervention. There were significant changes in the abundance of Proteobacteria, Bacteroidetes, Acidobacteria, Firmicutes, Propionibacterium, Corynebacterium, Staphylococcus, and Streptococcus after the intervention ($P<0.05$). Additionally, IL-10 and STAT3 levels were higher after the intervention and showed significant differences ($P<0.05$) compared to before. Regulating the respiratory tract flora can improve the microbial environment of bronchiolitis obliterans post-lung transplantation. This helps balance the respiratory flora, increase IL-10 and STAT3 levels, and aid in the recovery of inflammatory responses.

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Introduction

Lung transplantation is a therapeutic procedure that involves the replacement of one or both lungs in individuals suffering from severe functional damage caused by terminal benign lung diseases. This procedure is typically considered when conventional drugs and surgical treatments have proven ineffective, and the estimated survival rate for recipients is approximately 1-2 years (1, 2). Following lung transplantation, the healing and recovery of bronchial anastomosis, the surgical connection between the transplanted lung and the recipient's airway, is crucial. However, during this period, complications such as bronchiolitis obliterans can occur, posing a significant challenge to the success of the transplantation. Without timely intervention, these complications can increase the risk of rejection and negatively impact the overall outcome of lung transplantation (3).

The regulation of respiratory flora, also known as the respiratory microbiota, plays a vital role in reducing the risk of infection and allergy by improving the overall state of the respiratory system's microbial community. By modulating the composition and diversity of microorganisms residing in the respiratory tract, the regulation of respiratory flora can facilitate the elimination of inflammatory responses during the healing process of bronchial anastomosis (4). However, research investigating the regulation of respiratory flora in the context of bronchiolitis obliterans after lung transplantation remains scarce.

This study aims to address this gap in knowledge by conducting a comparative analysis of the practical effects of respiratory flora regulation in bronchiolitis obliterans following lung transplantation. The primary objective is to determine whether there are significant differences in the structure of respiratory flora before and after the implementation of this intervention. Additionally, the study seeks to explore the potential influences of changes in the flora structure and the interleukin-1β (IL-1β)/signal transducer and activator of transcription 3 (STAT3) signaling pathway on the development and progression of bronchiolitis obliterans.

By elucidating the impact of respiratory flora regulation on bronchiolitis obliterans, this research has the potential to provide valuable insights into the therapeutic strategies for improving the outcomes of lung transplantation. If the study findings demonstrate a positive correlation between respiratory flora regulation and the prevention or mitigation of bronchiolitis obliterans, it could pave the way for the development of novel interventions that target the respiratory microbiota to enhance the long-term success of lung transplantation procedures.

The following sections of this paper will provide a de-

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tailed description of the methods employed in the study, present the results and their interpretation, and discuss the implications of the findings. It is hoped that this investigation will contribute to a better understanding of the complex interactions between the respiratory flora, bronchiolitis obliterans, and the success of lung transplantation, ultimately leading to improved patient outcomes and prolonged survival rates.

Materials and Methods

General data

Totaling 25 clean-grade C57BL/6 male mice and 5 BALB/c male mice kept in a specific-pathogen-free (SPF)-grade animal room of the laboratory animal center in Tianjin Medical University General Hospital from January 2019 to December 2021 were selected for orthotopic tracheal transplantation, followed by postoperative regulation of respiratory flora. The 30 mice were aged 1-2 years old, with an average age of (1.52±0.32) years old, and they weighed 20-25 g, with an average weight of (22.54±0.64) g. This study was approved by the Animal Ethics Committee of Tianjin Medical University Animal Center.

Methods

Establishment of mouse models

Pentobarbital sodium powder (Sigma) was prepared into 1% solution using sterile normal saline (Beijing Tiantan Biological Products Co., Ltd., Beijing, China). The mice were given water deprivation for 24 h before the operation and then injected with the mixture of pentobarbital sodium powder and normal saline (0.1 mg/10 g). Then they were fixed in the supine position on the operating table. An incision was made from the anterior cervical larynx to the mediastinum to separate the trachea with the aid of an operating microscope (SM-2000L, Shanghai EDER Medical Technology Inc., Shanghai, China). After the incised end was sterilized, the trachea was washed with 4°C normal saline, and divided into two trachea segments with five tracheal rings as the donor trachea. The anesthesia mode of the recipient mice was the same as that of the donor mice. An incision was made in the neck, and the trachea was reserved. The 3rd and 8th tracheal rings were each stitched with a 7-0 thread for pulling, and the 5th and 6th tracheal rings were excised. Later, the donor trachea was continuously anastomosed with the distal trachea of the recipient mice using 7-0 Proline end to end, and the proximal tracheal cartilage was intermittently anastomosed. Later, the position of the transplanted trachea was aligned. Once there was no blood leakage or air leakage after confirmation, the neck skin was sutured layer by layer. After the operation, the respiratory flora was regulated.

Regulation of respiratory flora

The respiratory flora was regulated as follows: 1) Pathogenic microorganisms were detected, and targeted therapy was performed with antibacterial agents such as levofloxacin and cefaclor. 2) Treatment with specific probiotic strains at a certain dosage was adopted to detect the respiratory flora. After direct treatment with related agents and probiotics for one week, the respiratory tissue samples were collected.

Detection methods

The Respiratory Flora was detected according to the following procedures: The respiratory tissue samples were collected into a 2 mL sterile microcentrifuge tube (Eppendorf) and stored in a liquid nitrogen bottle (-80°C) for examination. Under aseptic conditions, the samples to be tested were ground and homogenized, followed by deoxyribonucleic acid (DNA) extraction. Then the DNA was subjected to polymerase chain reaction (PCR) amplification (forward primer: 5'-AYTGGGYDTAAAGNG-3', and reverse primer: 5'-TACNVGGGTATCTAATCC-3'). Subsequently, the high-throughput sequencing of the V4 region of the bacterial 16S rDNA gene was performed by the Illumina MiSeq sequencing platform. After the removal of interrogative sequences, the remaining sequences were divided using the sequence comparison tool UCLUST (merging of sequences with the similarity of 97% and division of operational taxonomic unit (OTU)). OTU representative sequences were compared with the Greengenes database using QIIME software, and the α-diversity indexes (Simpson, Chao1, ACE, Shannon and dominance of Actinobacteria, Shanghai, China) were calculated. In addition, the four common respiratory phyla, *Proteobacteria*, *Bacteroidetes*, *Acidobacteria*, *Firmicutes*, *Propionibacterium*, *Corynebacterium*, and *Staphylococcus* were counted.

In order to examine the IL-10/STAT3 signaling pathway, whole blood samples were collected and centrifuged with a centrifuge (LXJ-II, Shanghai Medical Instruments Co., Ltd., Shanghai, China) at 15 cm × 3000 r/min for 10 min to separate the serum for later use. 1) IL-10 kit (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) was utilized for enzyme-linked immunoassay. Specifically, the samples were loaded, evenly mixed and incubated at 37°C for 1 h. Then washing solution was added and sucked dry by vibration (repeated 3 times). Next, the samples were incubated with streptavidin with affinity at 37°C for 30 min, washed by washing solution and sucked dry *via* vibration (repeated 3 times). Later, substrates A and B were added for sample incubation at 37°C for 10 min, and the reaction was terminated by stop buffer. Finally, a fluorescence microplate reader (Synergy HT, Bio-Tek, Biotek Winooski, VT, USA) was employed to detect the level of IL-10 at the wavelength of 450 nm. 2) TRIzol (total RNA isolation) kit (Shanghai Shenergy Biocolor BioScience & Technology Co., Ltd., Shanghai, China) was used for cell RNA extraction, and the samples with the $OD_{260/280}$ of 1.8-2.0 were available and stored at -80°C for later testing. Then 6 μL of the samples to be tested were reversely transcribed into complementary DNA (cDNA) samples using the cDNA first strand synthesis kit (Fermentas, Lithuania), and store at -20°C for subsequent testing. After that, the PCR amplification of STAT3 messenger RNAs (mRNAs) was conducted using the corresponding primers and probes synthesized by Shanghai Sangon Biotech Co., Ltd. (probe: 5'-FAM-GCCTTCTTTCAGAGCCATCAT-TAMRA-3', forward primer: 5'-CCTGCAAGAGTCGAATGTTCTC-3', and reverse primer: 5'-TATCAGCACAATCTACGAAGAAT-CAAGCAGT-3') and the LightCycler instrument (Roche, Basel, Switzerland) The reaction system (25 μL) consisted of 2 μL of the samples, 2.5 μL of 10×buffer, 1.5 μL of 25 mmol/L $MgCl_2$, 0.5 μL of 10 mmol/L 4×dNTPs, 0.1 μL of 100 μmol/L forward primers, 0.1 μL of 100 μmol/L reverse primers and 0.1 μL of 100 μmol/L probes, 0.25 μL of 5 U/

μL Taq enzymes, and PCR-grade water at a volume made up to 25 μL. The reaction conditions were as follows: 40 cycles of pre-denaturation at 95°C for 3 min, denaturation at 95°C for 15 s, annealing, extension and amplification at 60°C for 45 s. At last, fluorescence signals were collected, and the expression level of STAT3 was calculated by the $2^{-\Delta\Delta Ct}$ method.

Observational indexes

The microbial environment of the lesion and the levels of IL-10 and STAT3 before and after the intervention with respiratory bacterial flora regulation were compared.

Statistical analysis

Data were statistically analyzed using Statistical Product and Service Solutions (SPSS) 23.0 software (IBM, Armonk, NY, USA). Measurement data were expressed by (x±s) and compared between groups *via* independent-samples *t*-test. Count data were expressed by percentage (%), and compared *by* x^2 test. $P<0.05$ indicated that the data difference was statistically significant.

Results

Analyses of the lesion microbial environment

The microbial environment within the lesion was assessed to investigate the effects of the respiratory flora regulation method. The comparison of the Simpson index before and after intervention revealed no significant difference ($P > 0.05$), indicating that the overall microbial diversity within the lesion did not show a noticeable change following the implementation of the respiratory flora regulation method. However, further analysis uncovered significant alterations in the composition and relative abundance of specific microbial taxa.

After the intervention, the Chao1, ACE, Shannon, and Actinobacteria dominant indexes were found to be higher compared to their respective values before the intervention, and these differences were statistically significant ($P < 0.05$). This suggests that the respiratory flora regulation method positively impacted the richness and diversity of the microbial community within the lesion (Table 1). Specifically, the relative abundance of major phyla, including Proteobacteria, Bacteretes, and Acidobacteria, was significantly lower after the intervention compared to before the intervention. Conversely, phyla such as Firmicutes, Propionibacterium, Corynebacterium, Staphylococcus, and Streptococcus exhibited higher relative abundance after the intervention. These changes in microbial composition indicate that the respiratory flora regulation method induced a favorable shift in the structure of the microbial community within the lesion, which could potentially influence the healing process of bronchial anastomosis (Table 2).

Analyses of IL-10 and STAT3 levels

In addition to examining the microbial environment, the levels of IL-10 and STAT3 were assessed before and after the intervention. The results showed that the levels of IL-10 and STAT3 were significantly higher after the intervention compared to before the intervention ($P < 0.05$). This finding indicates that the respiratory flora regulation method had a positive impact on the immune response within the lesion, potentially leading to a reduction in inflammation. The increase in IL-10 levels suggests an enhanced anti-inflammatory response, while the upregulation of STAT3 indicates the activation of downstream signaling pathways involved in immune regulation. These results suggest that the respiratory flora regulation method not only influences the microbial community but also modulates the immune response, which may contribute to the healing and recovery process of bronchial anastomosis (Table 3).

These results provide valuable insights into the effects of respiratory flora regulation on bronchiolitis obliterans following lung transplantation. Although no significant changes were observed in the overall microbial diversity, the alterations in microbial composition and the upregulation of IL-10 and STAT3 levels suggest potential mechanisms through which the respiratory flora regulation method influences the development and progression of bronchiolitis obliterans. The favorable shift in the microbial community structure and the modulation of the immune response may contribute to reducing the risk of complications, such as rejection, and improving the overall outcomes of lung transplantation. However, further research is necessary to unravel the precise underlying mechanisms and long-term implications of respiratory flora regulation in the context of bronchiolitis obliterans after lung transplantation.

Discussion

Lung transplantation is a four-stage procedure and can be subdivided into single lung transplantation, double lung transplantation, heart-lung transplantation and living lung transplantation in light of different operation modes. According to a study, patients receiving double lung transplantation had a longer median survival time than those undergoing single lung transplantation (6.6 years *vs.* 4.6 years) (5). This may be because the autologous lung on the other side of a single lung transplant can be a source of very serious infection, increasing the risk of inflammatory and infectious disease after lung transplantation, which can have a serious impact on the donor lung and the quality of life of the patient after transplantation. As a result, the proportion of double lung transplants has been increasing year on year in recent years (6). However, in practice, the risk of developing occlusive bronchiolitis after lung transplantation is still generally high and has a direct impact on prognosis. Hence, at present, the approach to reducing the risk of bronchiolitis obliterans after lung transplantation and elevating the long-term survival rate of lung transplantation patients has become the focus of clinical research.

Respiratory flora regulation contributes to the maintenance of a stable and balanced state by regulating the microecological environment of respiratory flora, to prevent and treat respiratory inflammation and infectious diseases. The study results indicated that respiratory flora regulation improves the microecological environment of respiratory flora. This may be attributed to the fact that some pathogenic bacteria, such as *Streptococcus pneumoniae* and *Staphylococcus aureus*, colonized on respiratory mucosa in infants and young children will not excessively invade and damage the body and will not induce cough, expectoration and other symptoms under normal conditions of the body (7,8). Once the homeostasis of the respiratory mucosa is altered, pathogenic bacteria that colonise the res-

Table 1. Comparative microbial diversity ($\bar{x}\pm s$).

Group	Simpson	Chao1	ACE	Shannon	Actinobacteria dominant
Pre-intervention	0.93 ± 0.06	354.20 ± 13.78	407.60 ± 16.21	5.02 ± 0.07	6.23 ± 0.07
Post Intervention	0.92 ± 0.04 *	501.40 ± 14.75 [1]	539.80 ± 17.75 [1]	5.56 ± 0.09 [1]	7.04 ± 0.13 [1]

Note: [1] refers to comparison with pre-intervention $P < 0.05$; * refers to comparison with pre-intervention $P > 0.05$.

Table 2. Comparison of relative abundances among major phyla ($\bar{x}\pm s$).

Group	Proteobacteria	Bacteroidetes	Acidobacteria	Firmicutes	Propionibacterium	Corynebacterium	Staphylococcus	Streptococcus
Pre-intervention	20110.54 ± 1054.56	4243.54 ± 105.43	996.84 ± 87.84	3305.83 ± 402.86	4325.54 ± 253.54	883.82 ± 109.44	1085.32 ± 108.43	3872.85 ± 317.33
Post Intervention	33252.82 ± 988.86 [1]	1957.85 ± 98.54[1]	256.97 ± 60.43[1]	13856.88 ± 428.85[1]	6747.56 ± 275.73[1]	1085.38 ± 172.53[1]	1476.88 ± 122.85[1]	6873.48 ± 351.64[1]

Note: [1] refers to comparison with pre-intervention $P < 0.05$; * refers to comparison with pre-intervention $P > 0.05$.

Table 3. Analysis IL-10, STAT3 Levels ($\bar{x}\pm s$).

Group	IL-10 (pg/ml)	STAT3 (2^{-Ct})
Pre-intervention	174.32 ± 12 .85	0.51 ± 0.09
Post Intervention	275.89 ± 14 .94 [1]	0.82 ± 0.12 [1]

Note: [1] refers to comparison with pre-intervention $P < 0.05$; * refers to comparison with pre-intervention $P > 0.05$.

piratory mucosa can cause damage to the respiratory tract and induce an inflammatory response, leading to typical symptoms such as coughing and sputum. Moreover, the dyshomeostasis of respiratory mucosa will also affect the internal environment stability and immune homeostasis regulation, leading to higher risks of pathogenic bacteria infections as well as infectious and inflammatory diseases. Owing to the involvement of the anastomosis of many blood vessels and bronchi, lung transplantation inevitably affects the homeostasis of respiratory mucosa, resulting in the imbalance of Respiratory Flora, increasing the risk of pathogenic bacteria infections, and ultimately triggering inflammatory diseases such as bronchiolitis obliterans (9). The implementation of the respiratory flora modulation method, after the implementation of pathogenic microbial testing, the adoption of targeted antibacterial drug treatment can effectively inhibit the growth and reproduction of pathogenic bacteria in the respiratory tract. It can effectively remove the pathogenic bacteria, control and reduce the damage caused by pathogenic bacteria to the lungs and bronchi, reduce the level of inflammatory factors and eliminate the inflammatory response. Probiotic preparations are then given according to the condition to promote the balance of the respiratory flora and at the same time, the benign flora is recolonised, facilitating the formation of a barrier in the respiratory mucosa. It can effectively inhibit the proliferation and spread of pathogenic bacteria and can form a mutual antagonistic effect with respiratory pathogenic bacteria, facilitating the recovery of respiratory health. In conclusion, the regulation of respiratory flora improves the balance of respiratory flora, and restores the "balance and antagonism" between benign flora and pathogenic bacteria, contributing to the prevention and treatment of inflammatory diseases of the respiratory tract.

In addition, the data from this study showed that the levels of IL-10 and STAT3 were higher after the respiratory flora modulation intervention than before (P<0.05), suggesting that the respiratory flora modulation method can promote the recovery and improvement of the inflammatory response. The possible reasons for this are as follows: Over the past few years, with the in-depth study of molecular biology, it has been found that IL-10 is an important immunomodulatory factor, which is mainly produced by activated astrocytes and microglia, and is closely related to the inflammatory response, autoimmune diseases and tumor development (10, 11). Under normal conditions, glial cells have a role in neuronal fixation, nutrient transport, repair and phagocytosis, and regulation of the extracellular environment; an increase in IL-10 levels activates the glial cell-mediated inflammatory response. It has been found that STAT3 is a downstream oncogenic mediator of the JAK-STAT signaling pathway and has a major role in regulating cell cycle progression and anti-apoptosis. Once STAT3 is continuously activated and persistently highly expressed, it may induce cancer development. Under normal conditions, inactivated STAT3 is present in the cytoplasm, and when IL-10 binds to its cell surface homologous receptor, it activates STAT3, causing STAT3 phosphorylation and translocation to the nucleus, resulting in an anti-inflammatory cascade response that promotes abnormal cell proliferation and differentiation, inhibits apoptosis, aggravates respiratory mucosal epithelial cell damage, and promotes the release of large amounts of inflammatory factors. A positive feedback response is formed between IL-10 and STAT3. The implementation of the respiratory flora control method improves the balance of respiratory flora, controls and reduces the infestation of pathogenic bacteria on the respiratory tract, reduces the degree of damage to respiratory mucosal epithelial cells, reduces the amount of inflammatory factors being released, and reduces the increase of IL-10 level. As IL-10 levels increase, STAT3 levels are regulated and improved, and the body's immune system engulfs and clears damaged cells in a timely manner, effectively promoting the recovery of respiratory health.

In this process, dendritic cells (DCs), the primary antigen-presenting cells in the body, are activated by microorganisms or microflora fractions, and activated DCs secrete cytokines required for T-cell initiation and polarization (12,13). Activated CD4+ T cells become the most appropriate type of helper T cells (Th1, Th2, Th17) to promote inflammation or facilitate the immune rejection response. Of course, suppressive cytokines, such as IL-10, may also be produced to become regulatory T cells that suppress the body's response and induce immune tolerance, which is considered to be a spatiotemporal adaptation of DCs (14). In this study, we initially explored the possibility of using the microbial environment to adjust the spatiotemporal adaptation of DCs and to induce immune tolerance. However, we lack a corresponding understanding of the intrinsic and extrinsic mechanisms by which the microbial environment regulates the spatiotemporal adaptation of DCs. Cytokines such as IL-10 and its mediated signaling pathways may be one of the mechanisms, while TGF-β, macrophage-DCs networks, and different tissue microenvironments may be involved in the spatiotemporal adaptation of DCs. And the process of bronchiolitis obliterans is a decision-making process of multiple immunocytes, not only including DCs but also macrophage, T cells and other immune cells. All of these immune cells existed to some extent spatiotemporal heterogeneity and were related to multiple signal pathways. The microbial environment plays an important role in the process.

Our exploration of these mechanisms will undoubtedly help us to find new solutions to the problems of transplantation immunity, tumor immunity and infectious diseases, among others.

The implementation of respiratory flora modulation in occlusive fine bronchiectasis after lung transplantation can improve the microbial environment of the lesion, regulate the respiratory flora homeostasis, elevate IL-10 and STAT3 levels, and promote the recovery of the inflammatory response.

Ethical compliance

This study was approved by the Animal Ethics Committee of Tianjin Medical University Animal Center.

Data availability

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflict of interests

The authors declared no conflict of interest.

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