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Immunomodulatory Effects of Bacterial Lysates Combined with Bronchoalveolar Lavage on Cytokine Regulation and T-cell Responses in *Pseudomonas aeruginosa*–colonized Bronchiectasis

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ABSTRACT

We aimed to determine the impact of combined bacterial lysates (BLs) and bronchoalveolar lavage (BAL) therapy on specific immune biomarkers (interleukin [IL]-6, IL-17, $CD4^+$, $CD8^+$), lung function (forced expiratory volume in 1 second [FEV_1], forced vital capacity [FVC], maximal expiratory flow at 25% of FVC [MEF25]), and clinical symptoms in bronchiectasis patients with chronic *P aeruginosa* colonization.

Sixty-five stable bronchiectasis patients were randomized to receive BAL plus oral BLs (treatment group) or BAL alone (control group). After 3 months, IL-6 and IL-17 levels in BAL fluid and serum, peripheral blood T-lymphocyte subsets, lung function indices, clinical symptom scores (CSS), and reinfection rates were assessed.

Compared with BAL alone, the combination therapy significantly reduced IL-6 and IL-17 concentrations in both BAL fluid and serum. $CD4^+$ and $CD8^+$ T-cell counts increased markedly in the treatment group, correlating positively with improved lung function metrics (FEV_1, MEF75%, MEF50%, MEF25%, diffusion capacity). Elevated IL-6 and IL-17 were inversely correlated with pulmonary function and positively associated with symptom severity and reinfection risk. Clinically, the treatment group demonstrated improved CSS, enhanced lung function, and reduced reinfections.

BLs combined with BAL exert potent immunomodulatory effects by enhancing T-cell responses and downregulating pro-inflammatory cytokines, thereby improving both immune regulation and clinical outcomes in *P aeruginosa*–colonized bronchiectasis. These findings suggest BLs represent a promising adjunctive immunotherapy in chronic airway infections.

Keywords: Bacterial lysate; Bronchiectasis; Bronchoalveolar lavage; Cytokines; Immunomodulation; *Pseudomonas aeruginosa*; T lymphocytes

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INTRODUCTION

Bronchiectasis, a chronic respiratory disease characterized by persistent bronchial dilation, often results from underlying infections and inflammation.[1] It represents a growing global health burden with increasing prevalence over the past decade.[2] The disease is typified by a heterogeneous presentation, with symptoms ranging from mild to severe, often associated with recurrent respiratory infections that can lead to a decline in lung function and quality of life.[3] The etiology of bronchiectasis is multifactorial, encompassing both genetic and environmental factors. A significant body of evidence highlights the role of *Pseudomonas aeruginosa* (PA) as a predominant pathogen in bronchiectasis, particularly in patients with advanced disease.[3,4] This bacterium's ability to form biofilms, evade host immunity, and rapidly develop antimicrobial resistance complicates treatment strategies.[4]

Recent studies have identified PA, along with *Haemophilus influenzae*, *Prevotella* species, and *Veillonella* species, as predominant bacterial strains during both the stable and acute treatment phases of bronchiectasis.[5] In China, similar findings demonstrate a high prevalence of PA colonization, positively correlating with airway inflammation.[2] PA is an opportunistic pathogen that can impair the immune system, exhibiting innate resistance to many antibiotics and rapidly developing resistance mutations against anti-infective therapies. Under recurrent infection pressures, PA can transition from a non-mucoid to a mucoid phenotype, facilitating persistent colonization within the airways and inducing chronic pulmonary inflammation.[6] The genetic diversity of PA infections in bronchiectasis is notable, with distinct evolutionary paths emerging compared to cystic fibrosis (CF).[7] These studies emphasize the necessity of examining the genomic diversity of PA isolates from bronchiectasis patients to inform targeted therapeutic approaches.

Given the complexities and challenges associated with managing bronchiectasis, particularly in the context of PA colonization, there is a critical need for novel therapeutic approaches. The standard of care (SOC) for patients with stable bronchiectasis typically includes airway clearance therapy, long-term antimicrobial therapy, and, importantly, bronchoalveolar lavage (BAL).[8] BAL is utilized to enhance airway hygiene by clearing mucus and pathogens, thereby improving clinical outcomes and potentially prolonging the time to the first acute exacerbation.[9] Its incorporation into the SOC is crucial for patients colonized by PA, as it directly addresses airway infection management. In addition to BAL, bacterial lysates (BLs), such as Broncho-Vaxom, are employed as immunomodulators in clinical practice to prevent recurrent respiratory infections and acute exacerbations of chronic bronchitis. These preparations consist of lyophilized BLs from 8 common respiratory pathogens (*H influenzae*, *Diplococcus pneumoniae*, *Klebsiella pneumoniae*, *Klebsiella ozaenae*, *Staphylococcus aureus*, *S pyogenes*, *Streptococcus viridans*, and *Neisseria catarrhalis*).[10] The mechanisms of action of BLs involve the recognition of pathogen-associated molecular patterns (PAMPs)—including peptidoglycans from Gram-positive bacteria, lipopolysaccharides (LPS) from Gram-negative bacteria (eg, *P aeruginosa*), and CpG motif-containing bacterial DNA—by specific Toll-like receptors (TLRs, primarily TLR2, TLR4, and TLR9) on dendritic cells; this TLR activation triggers downstream signaling cascades to activate the innate immune response, enhance non-specific immunity, and modulate adaptive immune balance, thereby reducing the frequency and severity of respiratory infection.[11] Recent studies have shown that BLs significantly reduce the frequency and duration of acute exacerbations in patients with bronchiectasis, providing a promising direction for the treatment of patients with stable PA colonization.[12]

Hence, this randomized controlled trial aimed to evaluate the efficacy of combining BAL with BLs in patients with PA-colonized bronchiectasis by specifically assessing its impact on key parameters: modulation of the airway immune microenvironment through reductions in pro-inflammatory cytokines (IL-6, IL-17), enhancement of systemic cellular immunity via changes in peripheral T lymphocyte counts ($CD4^+$, $CD8^+$), and improvement of clinical outcomes as measured by lung function (FEV_1, FVC, MEF25) and symptom scores.

MATERIALS AND METHODS

Study Subjects and Inclusion/Exclusion Criteria

This study was conducted from August 2019 to December 2023 and included 67 inpatients with PA colonization and bronchiectasis at Dongguan Binhaiwan Central Hospital. It is important to clarify that this study

did not involve healthy controls. All participants provided informed consent and signed written consent forms. This study was conducted in accordance with the Declaration of Helsinki and approved by Dongguan Binhaiwan Central Hospital Ethics Committee (Project ethics approval number: L2022001). Two patients withdrew from the study before randomization: one due to death and the other due to refused bronchofiberscopy. The final sample size was 65 patients, who were randomly divided into a treatment group (33 patients) and a control group (32 patients) (Figure 1). Baseline characteristics of the 2 groups showed no significant differences (p>0.05). The inclusion criteria were as follows: (1) history of bronchiectasis with chronic cough and purulent sputum; (2) medical records indicating a stable condition but with 2 consecutive positive sputum cultures for PA within 1 year; (3) confirmed bronchiectasis via chest HRCT; and (4) normal liver and kidney function. The exclusion criteria were: (1) presence of other progressive underlying diseases or severe complications; (2) pregnant women; (3) allergy to BLs; and (4) patients who had been diagnosed with COVID-19 or had any COVID-19-related complications during the study period. This was an open-label study. However, data analysts for laboratory endpoints were blinded to group allocation.

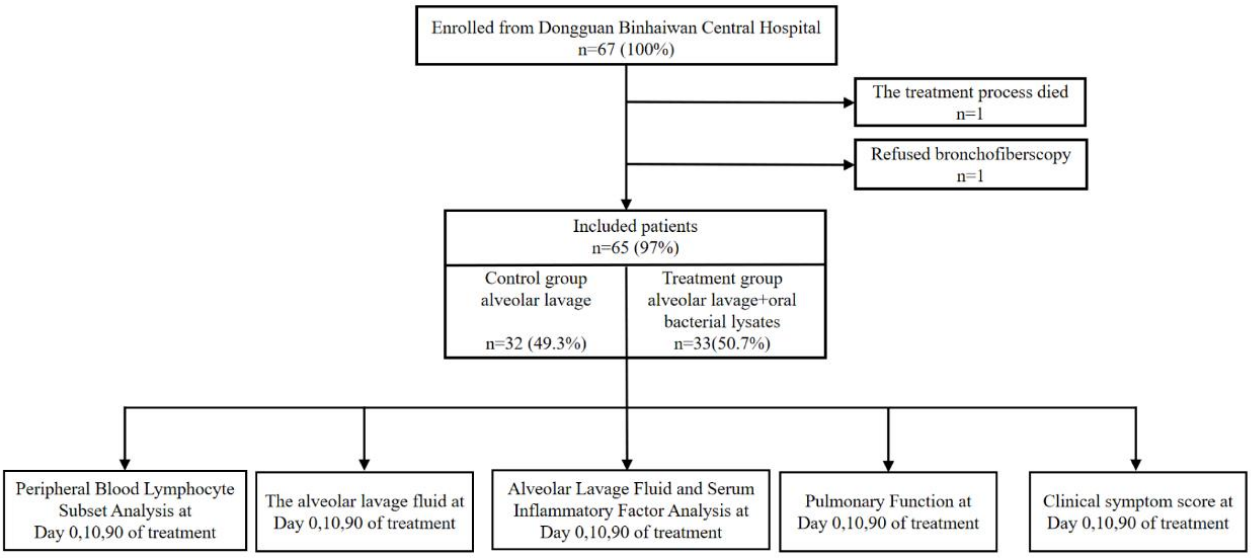


Figure 1. Flow diagram of patient selection, illustrating the inclusion and exclusion criteria, the number of patients assessed for eligibility, and the final subjects for both treatment and control groups.

Treatment Protocol

Patients were randomly assigned to one of 2 groups through a simple randomization method, with participation in the study being entirely voluntary after obtaining informed consent. The observation group received alveolar lavage. The lavage procedure involved fasting and water restriction for 4 hours, local anesthesia with 2% lidocaine, and surface anesthesia of the throat via nebulization or spray. During the procedure, patient vital signs, including ECG, respiratory rate, and blood gas analysis, were closely monitored, and low-flow oxygen was administered. A fiberoptic bronchoscope was inserted transnasally or transorally; secretions were aspirated from the airway, and 5 mL of sterile 37°C saline was injected twice into the bronchi using a disposable syringe through the suction port, avoiding coughing. The lavage fluid was then collected via negative pressure suction and sent to the laboratory. If arterial oxygen saturation (SaO_2) dropped below 80%, the bronchoscope was withdrawn, and high-concentration oxygen or non-invasive mechanical ventilation was provided until SaO_2 rose above 90%. Lavage was performed on day 0, day 10, and day 90 of treatment. The treatment group also received oral BLs (Broncho-Vaxom) at a dosage of 7 mg daily for 10 days each month for 3 months (product by OM PHARMA SA, Switzerland, National Drug Approval Number: SJ20150041). This dosage regimen is supported by evidence indicating that administration of BLs in this manner enhances immune responses and reduces the

frequency of respiratory infections.[13] BLs serve as immunomodulators to enhance the immune response against respiratory infections, and Broncho-Vaxom is approved for the prevention of recurrent respiratory infections and is indicated for use in patients with a history of chronic bronchitis, bronchiectasis, and other respiratory conditions.[11] The control group received only alveolar lavage as described above.

Randomization

Eligible patients were randomly assigned in a 1:1 ratio to either the combination therapy group (BAL + oral BLs) or the control group (BAL alone). The randomization sequence was generated by an independent statistician using a computer-based random number generator (Microsoft Excel 2019). To ensure allocation concealment, the assignments were placed in sequentially numbered, opaque, sealed envelopes (SNOSE). The envelopes were opened by the enrolling clinician only after the patient had signed the informed consent form and completed all baseline evaluations.

Sample Collection

Alveolar lavage fluid (ALF) and peripheral venous blood were collected from all participants at baseline (day 0), day 10, and day 90 of treatment: 5 mL of ALF was collected each time following the standardized BAL procedure in the "Treatment Protocol" section; 10 mL of peripheral venous blood was collected each time and split into two 5-mL sterile tubes—one with heparin sodium for T lymphocyte subset analysis, and the other processed for serum separation (via centrifugation) to detect IL-6 and IL-17. All samples were placed on ice immediately after collection and transported to the laboratory within 1 hour for further processing to ensure biological marker stability.

Peripheral Blood Lymphocyte Subset Analysis

Peripheral blood lymphocyte subsets were analyzed on day 0, day 10, and day 90 of treatment using the BD Multitest™ 6-color TBNK panel (BD Biosciences, Anhui, China)-comprising anti-human monoclonal antibodies CD3 FITC, CD16 PE+CD56 PE, CD45 PerCP-Cy™5.5, CD4 PE-Cy™7, CD19 APC, CD8 APC-Cy™7-and matched isotype controls, with the following gating strategy: first, viable cells were gated via forward scatter (FSC) vs side scatter (SSC) to exclude cellular debris; next, $CD45^+$ leukocytes were isolated to target immune cells; within $CD45^+$ cells, $CD3^+$ T lymphocytes were identified (excluding $CD19^+$ B cells and $CD16^+CD56^+$ NK cells); finally, $CD3^+$ T cells were further gated into $CD4^+$ T cells ($CD3^+CD4^+$) and $CD8^+$ T cells ($CD3^+CD8^+$) for quantification. For sample processing, approximately 2 mL of peripheral blood was collected into heparin anticoagulant tubes, centrifuged at 1000*g* for 10 minutes, and 50 μL of mononuclear cells from the middle layer was transferred to sterile polypropylene vacutainer tubes (Eppendorf, UK); the cells were stained with the aforementioned antibody panel, incubated at room temperature in the dark for 30 minutes, treated with 1000 μL of red blood cell lysis buffer for 25 minutes, washed with phosphate-buffered saline (PBS), and resuspended in 500 μL of PBS before analysis using a BD FACS Aria 7 flow cytometer (Heidelberg, Germany), with data visualized and analyzed via FlowJo software (ver 10, BD Biosciences, UK).

Alveolar Lavage Fluid and Serum Inflammatory Factor Analysis

Bronchoalveolar lavage fluid (BALF) was prepared by performing the lavage procedure as previously described.[14] Following the procedure, the collected BALF was transported to the laboratory in sterile conditions. The BALF samples were then transferred into 15-mL vacutainer tubes (Eppendorf, UK) and centrifuged for 20 minutes at 4°C and 1000*g* to separate the supernatant from cellular debris. The supernatant was carefully collected using a new 15-mL vacutainer tube for subsequent analysis. Enzyme-linked immunosorbent assay (ELISA) was used to detect interleukin-6 (IL-6) and interleukin-17 (IL-17) in serum and BALF using kits from Ray Biotech, USA. Assays followed manufacturer instructions: 100 μL of standards, serum, or BALF supernatant was added to wells and incubated on a shaker for 2.5 hours, followed by washing, primary antibody incubation (1 hour), enzyme conjugate addition (45-minute incubation), substrate solution addition (30-minute incubation), and stop solution to terminate the reaction; absorbance was measured at 450 nm using a Thermo Scientific microplate reader, with sample optical density (OD) values calculated via standard curves. IL-6 and IL-17 were selected as targets due to their established role as pro-inflammatory drivers of airway inflammation in *P aeruginosa*–colonized bronchiectasis[2,14,32]; while IL-10 (anti-inflammatory) and IFN-γ (T_H1-type cytokine) were not analyzed here, archived samples are retained

for their quantification in future studies to explore BL-mediated regulation of the pro-inflammatory/anti-inflammatory axis and T_H1/T_H17 responses.

Clinical Symptoms Evaluation

Clinical symptom score (CSS) was assessed to evaluate changes in patients' key symptoms. The score was based on a composite of patient-reported cough frequency and sputum volume, graded on a scale from 0 (none/absent) to 3 (severe/frequent). This tool was developed for this study to capture clinically relevant changes. Clinical symptoms were assessed by recording changes in cough and sputum production before treatment and 3 months after the initiation of treatment. Sputum production was scored as follows: 0 points for no sputum, 1 point for mild (≤50 mL/24 h), 2 points for moderate (51–100 mL/24 h), and 3 points for severe (>100 mL/24 h). Cough was scored as: 0 points for no cough, 1 point for mild (coughing less than 10 times a day without interfering with work, no night symptoms), 2 points for moderate (10–20 times a day, interfering with normal activities, night symptoms present), and 3 points for severe (more than 20 times a day, severely impacting work, unable to sleep at night). The total clinical symptom score was the sum of the sputum and cough scores.

Re-infection Frequency

The frequency of respiratory tract reinfections within 12 months was compared between the treatment and control groups. Acute exacerbations during the study were defined by O'Donnell's criteria[15]: presence of ≥4 symptoms (change in sputum volume or color, worsening dyspnea, increased cough frequency/intensity, persistent fever, severe wheezing, malaise, or reduced exercise capacity, decreased lung function, progressive lung lesions on X-ray/CT, changes in lung auscultation sounds). For baseline PA-colonized patients, re-infection required PA re-isolation with ≥2-fold colony count increase (≥10^7 CFU/mL), confirmed via standard microbiological methods (agar inoculation, 37 °C incubation, biochemical tests, MALDI-TOF MS). Re-infection frequency was total episodes per group divided by patient number, with between-group differences analyzed via chi-square test (p<0.05 significant).

Pulmonary Function

All participants underwent comprehensive pulmonary function testing, which included spirometry to measure forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), and maximal expiratory flow at 25% of FVC (MEF25). The lung diffusion capacity for carbon monoxide (D_{LCO}) was also measured using the single-breath method. These tests were performed by trained personnel using a Vmax 229 pulmonary function instrument, with each parameter measured 3 times, and the best value recorded. All tests were performed in accordance with the American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines.

Statistical Analysis

The sample size was calculated a priori based on the primary outcome (change in FEV_1% predicted). With an assumed mean difference of 8% and a standard deviation of 10%, a sample of 65 participants provides 80% power at a 2-sided alpha of 0.05, accounting for dropouts. Data were analyzed using Statistic Package for Social Science (SPSS) 25.0 (IBM, Armonk, NY, USA). Measurement data were first tested for normality. Distribution normality was determined using the Shapiro Wilks test.[16] Normally distributed data were expressed as mean ± standard deviation ($\bar{x} \pm s$) and analyzed using t tests. Skewed data were expressed as median (P25, P75) and analyzed using rank-sum tests. Categorical data were expressed as n (%) and analyzed using chi-square tests. For the correlation analysis, Pearson correlation coefficient analysis was used when the index met the normal distribution and Spearman rank correlation coefficient analysis when the normal distribution was not met. A p value <0.05 was considered statistically significant.

RESULTS

General Information

A total of 33 patients in the treatment group and 32 patients in the control group were thoroughly analyzed. As shown in Table 1, the treatment group had an average age of 61.4 years, with 39.4% male and 60.6% female participants. Among them, 45.5% were smokers, and 54.5% were non-smokers. In contrast, the control group presented an average age of 61.3 years, comprising 43.7% male and 56.3% female. The smoking status was comparable to that of the treatment group, with 46.9% identified as smokers and 53.1% as non-smokers. The comparative analysis of these baseline characteristics revealed that no any significant difference was observed between the 2 groups (all p>.05), indicating a well-

matched cohort that provides a robust basis for subsequent analyses of treatment effects.

Treatment Outcomes

Table 2 presents a comprehensive evaluation of pulmonary function, clinical symptom scores (CSS), and re-infection rates (RIR) before and after treatment in both the treatment and control groups. Initially, no significant disparities were observed between the groups in terms of pulmonary function indices (FEV_1, FVC, MEF 25%, MEF 50%, MEF 75%), residual volume, diffusion capacity, CSS, or RIR, with all *p* values greater than .05. After treatment, the treatment group exhibited statistically significant improvements in several key areas: CSS significantly decreased, and pulmonary function showed marked enhancement in FEV_1, FVC, MEF25%, MEF50%, and MEF75%, along with improvements in residual volume and diffusion capacity, all with *p* values less than .001. Notably, the RIR significantly declined in the treatment group (Figure 2). In contrast, the control group demonstrated significant improvements in clinical symptoms and pulmonary function but did not achieve a corresponding significant reduction in RIR. When comparing the 2 groups post-treatment, the treatment group had significantly lower CSS and RIR than the control group ($p<0.05$). While both groups exhibited improvements in pulmonary function, the treatment group showed more substantial enhancements, particularly in overall pulmonary function and small airway function.

Effects of Combined BLs and Alveolar Lavage on Airway Microenvironment and Immune Inflammation

Our study delved into the impact of BLs in conjunction with alveolar lavage on immune markers in patients with bronchiectasis colonized by PA. Table 3 illustrated that, prior to treatment, no significant variances were observed between the 2 groups concerning peripheral blood lymphocytes, $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, and the $CD4^+/CD8^+$ ratio, all showing *p* values above .05. Following treatment, the control group, which received only alveolar lavage, did not exhibit notable changes in these immune markers. Conversely, the treatment group, which was administered BLs alongside alveolar lavage, demonstrated a significant elevation in the activity levels of peripheral blood lymphocytes, $CD4^+$ T lymphocytes, and $CD8^+$ T lymphocytes, with *p* values falling below 0.05 when compared to their pre-treatment levels (Figure 3). Additionally, while initial expression levels of the pro-inflammatory cytokines IL-17 and IL-6 were comparable between the groups ($p>0.05$), the control group maintained consistent levels post-treatment. In stark contrast, the treatment group showed a substantial decrease in the levels of both IL-17 and IL-6 after receiving the combined treatment (all $p<0.001$, Figure 4). Moreover, when compared to the control group post-treatment, the treatment group's levels of IL-17 and IL-6 were significantly lower, underscoring the potential therapeutic efficacy of the combined BLs and alveolar lavage in modulating the airway microenvironment and reducing immune inflammation.

Table 1. Baseline characteristics of subjects in the treatment and control groups ($\bar{x} \pm s/M_{25}–M_{75}$)

Variables	Treatment group (n, %)	Control group (n, %)	*p*
Total samples	33	32	
Age	61.4 ± 2.76	61.3 ± 2.32	.982
Sex			.722
Male	13 (39.4)	14 (43.7)	
Female	20 (60.6)	18 (56.3)	
Smoke			.909
Yes	15 (45.5)	15 (46.9)	
No	18 (54.5)	17 (53.1)	

Table 2. Comparative analysis of pulmonary functions and clinical outcomes in treatment and control groups pre- and post-intervention.

Variables	Treatment group		p^a	Control group		p^a	p^b	p^c
	Before treatment	After treatment		Before treatment	After treatment			
FEV_1, L	1.40 ± 0.57	2.04 ± 0.66	<0.001	1.38 ± 0.51	1.90 ± 0.53	<0.001	.860	.355
FVC, L	2.11 ± 0.9	2.82 ± 0.83	<0.001	2.09 ± 0.92	2.69 ± 0.79	<0.001	.932	.530
FEV_1/FVC (%)	69.79 ± 15.7	73.61 ± 17.02	0.160	69.3 ± 16.09	72.1 ± 14.31	0.219	.901	.700
MEF75%	1.11 ± 0.46	1.78 ± 0.53	<0.001	1.1 ± 0.4	1.69 ± 0.36	<0.001	.910	.398
MEF50%	0.85 ± 0.39	1.49 ± 0.7	<0.001	0.79 ± 0.3	1.28 ± 0.36	<0.001	.481	.140
MEF25%	0.47 ± 0.21	0.89 ± 0.23	<0.001	0.51 ± 0.32	0.92 ± 0.3	<0.001	.549	.652
Residual volume	2.99 ± 1.09	2.26 ± 0.86	<0.001	2.93 ± 1.09	2.19 ± 0.64	0.001	.840	.735
Diffusion capacity	4.61 ± 1.29	6.27 ± 1.39	<0.001	5.23 ± 1.55	6.42 ± 2.01	0.001	.083	.729
Clinical symptom scores	4.67 ± 1.16	2.48 ± 0.83	<0.001	4.41 ± 1.24	3.06 ± 1.16	<0.001	.386	.025
Re-infection rates	4.00 ± 1.48	2.45 ± 1.58	<0.001	3.94 ± 1.48	3.56 ± 1.85	0.216	.865	.012

[a]The comparison of intragroup differences before and after treatment (after treatment - before treatment).

[b]The pre-treatment comparison between groups.

[c]The post-treatment comparison between groups.

[d]FEV_1: forced expiratory volume in the first second; FVC: forced vital capacity; MEF: maximal expiratory flow.

Table 3. Changes in IL-17, IL-6, and immune cells in BAL fluid and peripheral blood of treatment and control groups.

Variables	Treatment group		p^a	Control group		p^a	p^b	p^c
	Before treatment	After treatment		Before treatment	After treatment			
Lymphocyte	1.35 ± 0.78	2.11 ± 0.85	<.001	1.47 ± 0.56	1.5 ± 0.61	.496	.492	.002
$CD4^+$ cells	235.85 ± 93.77	266.94 ± 86.03	.018	239.03 ± 132.56	233.59 ± 127.17	.112	.912	.222
$CD8^+$ cells	212.48 ± 86.48	294.94 ± 81.03	<.001	214.22 ± 91.96	221.81 ± 90.33	.107	.938	.001
$CD4^+$/ $CD8^+$	1.28 ± 0.72	0.94 ± 0.31	.002	1.21 ± 0.67	1.12 ± 0.58	.001	.667	.121
IL-6, ng/L, BALF	79.33 ± 76.25	27.44 ± 33.1	<.001	82.29 ± 80.7	76.99 ± 76.01	.397	.880	.002
IL-17, ng/L, BALF	83.34 ± 79.34	26.48 ± 25.41	<.001	89.12 ± 85.54	79.26 ± 76.45	.071	.779	.001
IL-6, ng/L, Blood	22.9 ± 16.02	9.23 ± 6.31	<.001	23.2 ± 16.08	20.97 ± 11.07	.375	.941	<.001
IL-17, ng/L, Blood	26.61 ± 18.56	10.97 ± 6.33	<.001	22.43 ± 16.75	21.11 ± 12.71	.6262	.344	<.001

[a]The comparison of intragroup differences before and after treatment (after treatment - before treatment).

[b]The pre-treatment comparison between groups.

[c]The post-treatment comparison between groups.

[d]BALF: bronchoalveolar lavage fluid; IL: interleukin.

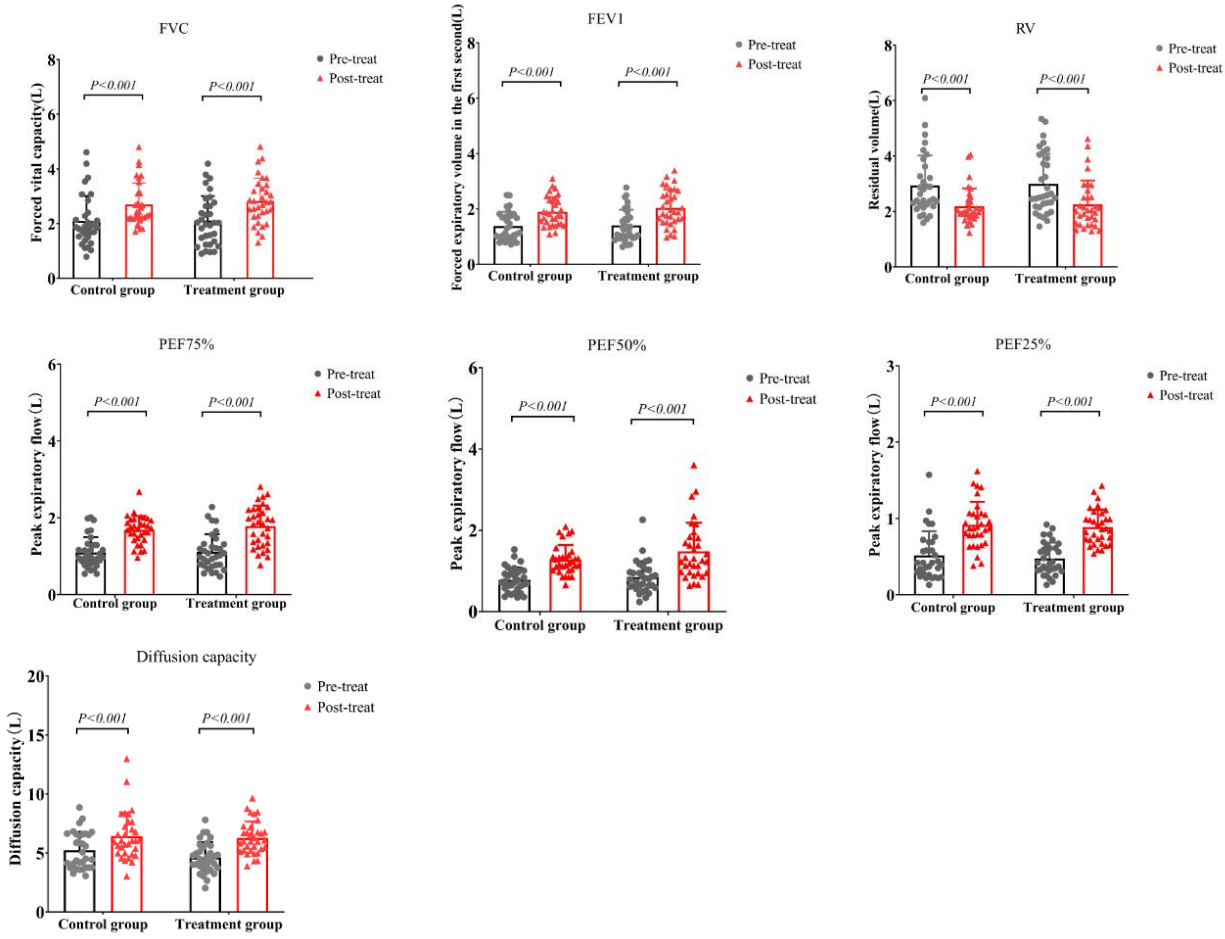
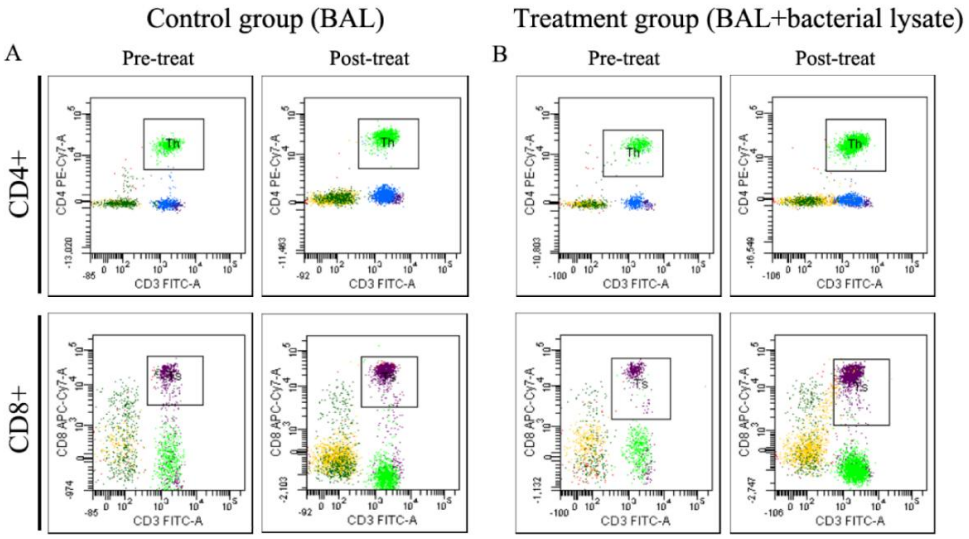


Figure 2. Lung function metrics in the control and treatment groups before and after treatment. Significant improvements were observed in FVC, FEV_1, RV, PEF75%, PEF 50% and PEF25%, as well as DC after treatment (all $p<0.05$). FEV_1 indicates forced expiratory volume in the first second; FVC, forced vital capacity; RV, residual volume; PEF, peak expiratory flow; DC, diffusion capacity.



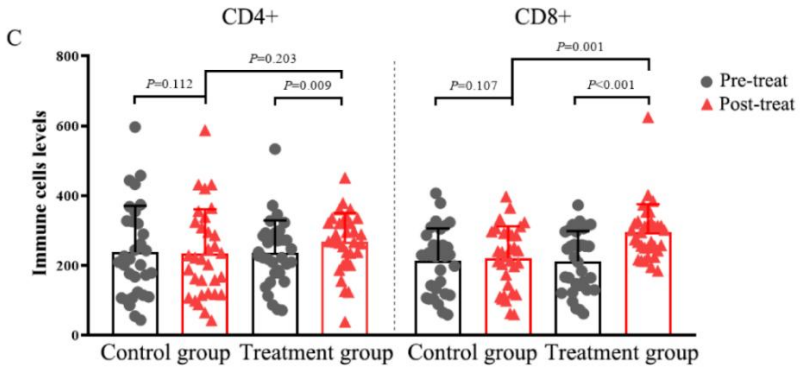


Figure 3. Flow cytometric results of immune cells populations in the control and treatment groups. (A–B) Displaying flow cytometry plots of $CD4^+$ and $CD8^+$ cells in the treatment group (A) and control group (B) before and after treatment. (C) Presenting the levels of $CD4^+$ and $CD8^+$ T in both the treatment and control groups prior to and following treatment. In the control group, no significant differences were found in $CD4^+$ and $CD8^+$ T levels before and after treatment (all $p>0.05$). Conversely, in the treatment group, $CD4^+$ and $CD8^+$ T lymphocyte levels significantly increased post-treatment (all $p<0.05$), with $CD8^+$ T levels in the treatment group being significantly higher than those in the control group after treatment ($p<0.05$).

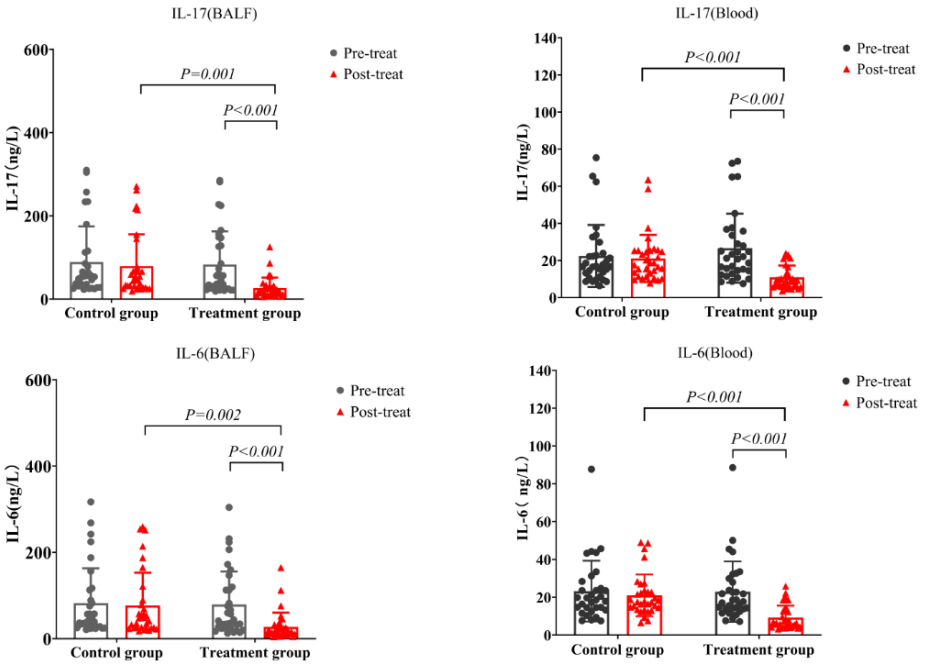


Figure 4. Inflammatory factors in the control and treatment groups before and after treatment. In the treatment group, IL-6 and IL-17 levels in blood and BALF decreased after treatment ($p<.05$). No significant changes were observed in IL-6 and IL-17 levels in blood and BALF in the control group before and after treatment ($p>.05$). IL indicates inflammatory factor; BALF, bronchoalveolar lavage fluid.

Correlation Analysis Between Immune Biomarkers and Clinical Effects

The relationship between changes in immunological biomarkers and clinical outcomes were further assessed. As shown in Figure 5, in the control group, higher IL-6 levels in BALF were inversely correlated with FEV_1 and diffusion capacity, while elevated IL-17 levels in BALF negatively correlated with FEV_1, MFE 50%, MEF 25%, and diffusion capacity, indicating reduced lung function. In the treatment group, lymphocyte and $CD8^+$ cell

counts were positively correlated with key lung function metrics (eg, FEV_1, MEF 75%, MEF 50%, MEF 25%, and diffusion capacity) and inversely correlated with residual volume and CSS, suggesting improved pulmonary function and symptom relief. Additionally, IL-6 and IL-17 levels in both BALF and blood exhibited significant negative correlations with lung function measures (such as FEV_1, FVC, MEF 75%, MEF 50%, and MEF 25%) and positive associations with CSS and RIR, indicating a potential link between these biomarkers and clinical deterioration. In the combined analysis, these trends persisted. Lymphocyte counts showed positive correlations with FEV_1, FVC, MEF 75%, MEF 50%, MEF 25%, and diffusion capacity, and negative correlations with residual volume. Similarly, $CD8^+$ cell counts were positively correlated with FEV_1, FVC, MEF 75%, MEF 50%, MEF 25%, and diffusion capacity, and negatively associated with residual volume and RIR. IL-6 and IL-17 levels in both BALF and blood consistently showed inverse correlations with lung function (eg, FEV_1, FVC, MEF 75%, MEF 50%, MEF 25%) and positive correlations with CSS and RIR. These results suggest that lymphocyte and $CD8^+$ cell counts may be indicative of improved lung function, while elevated IL-6 and IL-17 levels correlate with worsened symptoms and higher re-infection risk, supporting their potential role as biomarkers for monitoring treatment response.

Adverse Reactions

Over the 3-month study, patients in both BLs+BAL and BAL alone groups received monthly safety monitoring: clinical checks for allergic reactions (rash, pruritus, dyspnea) and lab tests for liver (ALT, AST) and kidney (Scr, BUN) function (Roche Diagnostics kits).

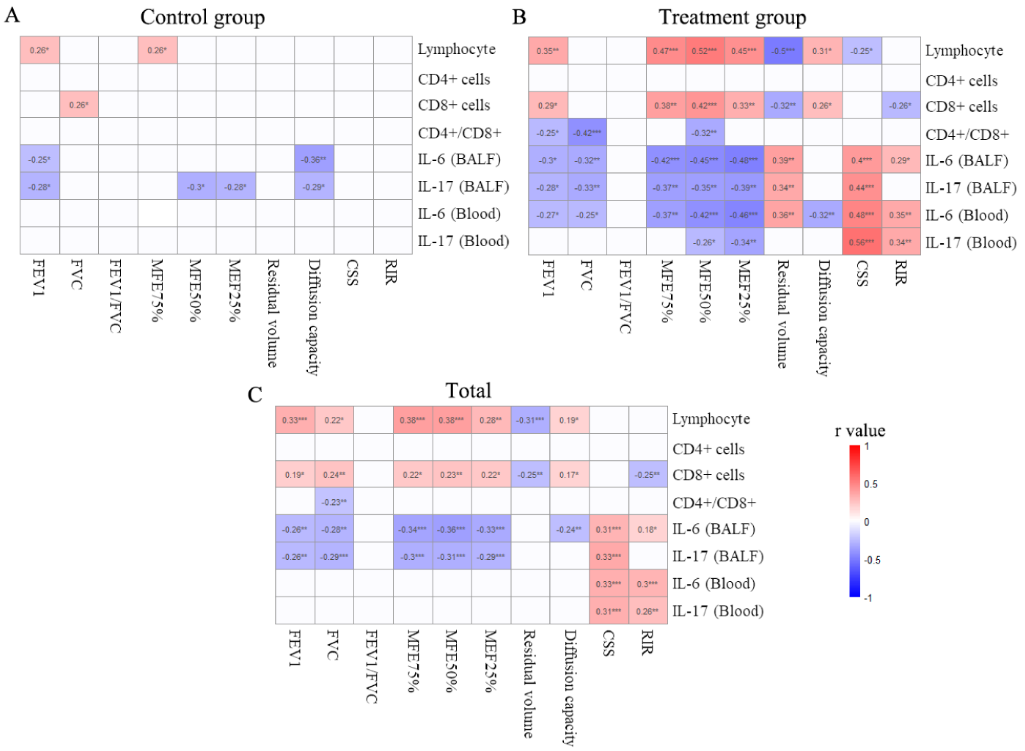


Figure 5. Heatmaps of correlation analysis between immune biomarkers and clinical outcomes. The color intensity of each cell in the heatmap corresponds to the strength of the correlation. (A) Displaying results for the control group, with IL-6 and IL-17 levels in BALF negatively correlated with lung function metrics (FEV_1, diffusion capacity). (B) Showing the treatment group, where lymphocyte and $CD8^+$ cell counts are positively correlated with lung function and negatively correlated with CSS. (C) Presenting combined analysis, highlighting that elevated IL-6 and IL-17 levels correlate with worse symptoms and higher RIR. *p<0.05, **p<0.01, ***p<0.001.

DISCUSSION

For this study, we summarize the core findings: In patients with PA-colonized bronchiectasis, combined BLs and BAL therapy-compared to BAL alone-yielded 4 key outcomes. First, it significantly reduced pro-inflammatory cytokines (IL-6, IL-17) in both BALF and serum. Second, it markedly increased peripheral blood $CD4^+$ and $CD8^+$ T-cell counts. Third, it improved lung function (notably small airway function, as reflected by elevated MEF25, $p<0.001$) and clinical symptom scores. Fourth, it lowered respiratory reinfection rates. Correlation analyses further confirmed that higher $CD4^+/CD8^+$ T-cell counts correlated with better lung function, while elevated IL-6/IL-17 were associated with worse symptoms and higher RIR-directly linking the therapy's immunomodulatory effects to clinical benefits.

Bronchiectasis is defined by abnormal bronchial dilation driven by persistent infections and immune dysregulation, with PA colonization exacerbating disease via biofilm formation, immune evasion, and antimicrobial resistance. This dilation results from the disruption of the muscular and elastic tissue structures within the bronchial walls. Pathogen infections play a crucial role in the development and progression of bronchiectasis. However, the role of immune dysregulation should not be overlooked, as both hyperactive and weakened immune responses can impair the body's ability to combat pathogenic infections, leading to recurrent infections and subsequent bronchial damage.[17]

The pathophysiological mechanisms of bronchiectasis include persistent bacterial infections, immune response dysregulation, impaired mucociliary clearance, and airway obstruction.[18] As the disease progresses and acute exacerbations recur, patients experience more severe clinical symptoms, affecting their quality of life and lung function.[19] Studies have found that 70.2% of patients with bronchiectasis have a decline in lung function, with 41.1% exhibiting airway obstruction, impaired diffusing capacity for carbon monoxide (D_{LCO}) (55.7%), airflow obstruction (41.1%), hyperinflation (15.7%), and restriction (8.0%).[20] In bronchiectasis patients, the annual rate of FEV_1 decline was −31.6 mL/year, with a faster decline observed in older patients and those with chronic bronchial infection by PA, an increased number of previous severe exacerbations, and a higher baseline FEV_1 value.[21]

Our study found that 65 patients with PA colonization in bronchiectasis exhibited a significant decline in lung function, characterized by small airway disease, increased residual volume, and reduced diffusion capacity. The combination of BLs and alveolar lavage therapy significantly improved the patients' FEV_1, FVC, small airway function, and diffusion capacity. Improvement in MEF25 (Peak expiratory flow at 25% of lung volume) is indicative of enhanced small airway function, as MEF25 specifically measures airflow in the distal, smaller airways. Improved MEF25 suggests a reduction in airway obstruction, better mucus clearance, and less inflammation in these airways, which are critical for the recovery process. This is supported by literature indicating that small airways play a pivotal role in bronchiectasis management and recovery.[22] As small airways become less obstructed, overall lung function improves, facilitating better gas exchange and reducing the risk of recurrent infections.[23] Our study demonstrated that MEF25 was negatively associated with clinical cough scores and positively associated with FEV_1, indicating that improved small airway function correlates with enhanced overall lung function, increased sputum clearance, decreased airway mucus, and improved cough symptoms. Furthermore, MEF25 was negatively correlated with alveolar lavage fluid IL-6 and IL-17, suggesting that the decrease in airway inflammation is closely linked to reduced small airway obstruction. Reduced airway inflammation and improved small airway obstruction are essential in mitigating impaired airflow and reducing the risk of recurrent infections, thus playing a crucial role in the management and recovery of bronchiectasis.[24] These findings underscore the importance of targeting small airway inflammation and obstruction in therapeutic strategies for bronchiectasis patients.

Recent research has discovered a significant infiltration of $CD4^+$ and $CD8^+$ T lymphocytes in the bronchial pathology of patients with bronchiectasis.[25] T cells can be divided into 2 main types: $CD4^+$ T cells, known as helper T cells, which play a crucial role in recognizing antigens and aiding in the production of antibodies[26]; and $CD8^+$ T cells, known as cytotoxic T cells, whose increase is detrimental to antibody production and inflammation control.[27] In our study, we observed that patients with PA colonization in bronchiectasis had impaired T lymphocyte function, with the most significant decline observed in $CD4^+$ T cells. The counts of T lymphocytes, $CD4^+$ T

lymphocytes, and $CD8^+$ T lymphocytes were all reduced in these patients. The decrease in immune function increases the likelihood of opportunistic infections, which may be a factor in the recurrent acute exacerbations observed in patients with bronchiectasis. Our findings indicate that bronchiectasis patients with PA colonization have significantly lower T lymphocyte counts, $CD4^+$, and $CD8^+$ T lymphocytes compared to normal values, with $CD4^+$ T lymphocytes being the most affected. This suggests that patients with PA colonization have a more severe reduction in immune function.[28] The various subtypes of T lymphocytes are in a state of mutual antagonism, maintaining the body's immune homeostasis. Any decrease or increase in $CD4^+$ or $CD8^+$ T lymphocytes can lead to immune dysregulation and a significantly increased probability of opportunistic pathogen infections. Studies have found that bronchiectasis patients with PA infection have reduced immune function.[29] In many clinical trials, oral BLs have been proven to minimize the risk of recurrent respiratory infections in children and adults, reduce the need for antibiotics, and significantly improve patient prognosis.[30] Our study found that BLs can enhance T lymphocyte, $CD4^+$ T cell, and $CD8^+$ T cell counts in bronchiectasis patients, effectively improving cellular and humoral immunity. The immune-enhancing effects of BLs are intriguing; however, our cohort study excluded chronic progressive diseases. In conditions like AIDS, where there is a significant decline in both the quantity and function of $CD4^+$ T cells, the effectiveness of BLs may be limited. In the context of severe immune deficiency, the use of BLs should be approached with caution, as they may stimulate immune responses, leading to adverse effects or increased risk of infections. However, it is worth contemplating whether early intervention with BLs could potentially increase the quantity and activity of $CD4^+$ T cells, thereby enhancing immune function.

Currently, bronchoscopy with alveolar lavage is often chosen for treatment. The electronic bronchoscope can be inserted directly into the bronchial lesions, using physiological saline to lavage the lesion site repeatedly. This process aspirates the secretions and sputum that block the airways, dilutes inflammatory substances, effectively controls the inflammatory response, and promotes symptom relief.[30] Alleviation of the inflammatory response can also reduce the expression of remodeling factors, unblocking the airways and improving lung function.[31] However, while bronchoscopy with alveolar lavage can alleviate pulmonary blockage symptoms, it is difficult to completely clear the pathogens at the lesion site, leading to potential recurrence of the disease.[31] IL-6 and IL-17 are important cytokines involved in inflammatory responses, reflecting the control of infection and inflammatory responses. There is a close association between bronchiectasis with pulmonary infection and inflammatory response damage. Our previous research indicated that IL-6 and IL-17 are important indicators for predicting infection and judging the severity of infection.[14] The IL-6 signaling pathway operates through 2 distinct modes: classical signaling via the membrane-bound IL-6 receptor (IL-6R) and trans-signaling through the soluble IL-6 receptor (sIL-6R).[32] The trans-signaling pathway primarily mediates IL-6's pro-inflammatory activities, while classical signaling generally elicits anti-inflammatory and protective responses. Elevated levels of IL-6 in patients with bronchiectasis, particularly those colonized by PA, align with our findings.[33] During infection or inflammation, IL-6 produced by the activated innate immune system can inhibit the generation of regulatory T cells (Treg) induced by TGF-β, thus promoting a pro-inflammatory T cell response characterized predominantly by T_H17 cells.[34] The administration of BLs, particularly in conjunction with BAL, likely activates the innate immune system in a manner that significantly reduces reinfection rates in bronchiectasis patients. BLs, comprising fragments of inactivated bacteria, engage pattern recognition receptors (PRRs) on immune cells, including Toll-like receptors (TLRs) and NOD-like receptors (NLRs).[11] These receptors are critical components of the innate immune response, recognizing microbial components such as lipopolysaccharides (LPS), peptidoglycans, and flagellins. Upon oral administration, BLs are absorbed in the intestinal mucosa and preferentially activate key signaling pathways, including the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinases (MAPK) in monocyte-derived dendritic cells. Dendritic cells and peripheral blood mononuclear cells (PBMCs) subsequently regulate the release of cytokines that activate B cells and T cells, enhancing the activity of $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells.[30] This cascade of events alleviates the T_H17 cell-dominated pro-inflammatory response, leading to reduced levels of IL-17 and overall improved immune regulation.

Our data demonstrate that the combination therapy significantly improved lung function, particularly in the small airways as measured by MEF25. Crucially, the improvement in MEF25 was negatively correlated with levels of IL-6 in BALF, suggesting that the functional recovery is intrinsically linked to the resolution of underlying inflammation. This interplay between mechanics and immunology can be explained by a shift in the T_H17/Treg balance. The elevated IL-6 in PA-colonized patients likely promotes a pro-inflammatory T_H17 response, perpetuating tissue damage and impairing host defense. Our intervention, by significantly reducing IL-6, may help to reverse this imbalance, allowing for a more regulated immune environment. This immune reprogramming facilitates mucosal healing and enhances mucus clearance, ultimately manifesting as the observed improvement in small airway function and reduction in exacerbation risk.

This study found a statistically significant difference in overall treatment effectiveness between the 2 groups. Importantly, baseline characteristics including smoking status, key comorbidities (COPD, asthma), and use of medications such as macrolides and ICS were comparable between the groups, supporting the validity of the observed treatment effects. The treatment group showed significant improvements in cough and sputum, RIR, lung function, CSS, and T lymphocyte counts, while the control group showed no significant changes. The treatment group had significantly lower RIR compared to the control group. Broncho-Vaxom is absorbed in the intestinal mucosa after oral administration. This localized immune activation helps "prime" the immune system, leading to a more robust and quicker response to actual infections. Moreover, the enhanced recognition of pathogens by innate immune cells may improve the clearance of bacteria from the lungs, reducing the bacterial load and, enhancing lung function. BLs represent a promising therapeutic strategy for managing bronchiectasis, especially in patients colonized with PA. Their potential to improve lung function, enhance immune responses, and reduce reinfection rates highlights their role in the multifaceted approach required to effectively treat this complex disease. Further studies are warranted to explore the long-term effects and mechanisms underlying the immune-modulatory effects of BLs, which could lead to more tailored and effective treatment strategies for bronchiectasis.

This study has limitations that should be acknowledged. First, it uses a single-center design with a small sample size (N=65), which may limit generalizability to diverse PA-colonized cohorts (eg, patients with comorbid COPD or non-mucoid PA strains). A multicenter RCT with larger enrollment is needed to validate findings. Second, the open-label design (though laboratory analysts and CSS scorers were blinded) may introduce bias in patient-reported symptoms (eg, cough frequency) or adherence, which could influence CSS and RIR assessments. Third, we lacked long-term follow-up (only 3 months); longer assessments (6–12 months) are required to determine if BL+BAL sustains lung function improvements or slows FEV_1 decline-a key measure of bronchiectasis progression. Fourth, mechanistic exploration was limited: we did not assess T_H1/T_H17/Treg subset ratios or signaling pathways (eg, NF-κB, MAPK) underlying BL-induced immunomodulation, nor did we measure PA biofilm clearance or antibiotic resistance changes-data that would clarify how BLs interact with PA to reduce infections.

In summary, alveolar lavage therapy helps to alleviate the clinical symptoms of bronchiectasis patients and enhance lung function. The combination of BLs and alveolar lavage in bronchiectasis patients can significantly reduce cough and sputum symptoms, improve lung function, reduce the number of re-infections, and improve the patient's immune system function. It has few adverse reactions and is a new preventive and therapeutic method for bronchiectasis.

STATEMENT OF ETHICS

Ethics approval and consent to participate: The study was approved by Dongguan Binhaiwan Central Hospital Ethics Committee (Project ethics approval number: L2022001). All participants provided informed consent and signed written consent forms.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

All data generated or analyzed during this study is provided within the manuscript.

AI ASSISTANCE DISCLOSURE

Not applicable.

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