Distribution and Chemotactic Mechanism of CD4+ T Cells in Traumatic Tracheal Stenosis

tingmei feng[1], yan chen[1], jinmei wei[1], sen tan[1], and liu guangnan[1]

[1]The Second Affiliated Hospital of Guangxi Medical University

January 30, 2024

Abstract

Abstract: A systemic and local inflammatory immune imbalance is thought to be the cause of traumatic tracheal stenosis (TS). However, with CD4+ T lymphocytes being the predominant immune cells in TS, the mechanism of action and recruitment has not been described. In our research, using flow cytometry, ELISA, immunofluorescence, and Transwell chamber assays, the expression, distribution, and potential chemotactic function of CD4+ T cells in TS patients were examined before and after treatment. The results showed that the untreated group had significantly more CD4+ T cells and their secreted TGF-β1 than the treated group. Additionally, the untreated group's CD4+ T cells showed a significant rise in CCL22 and CCL1, as well as a larger proportion of CCR4 and CCR8. CD4+ T cells and CD68+ macrophages located in TS also expressed CCL1 and CCL22. In vitro, anti-CCL1 and anti-CCL22 can partially block the chemoattractant effect of TS bronchoalveolar lavage (BAL) on purified CD4+ T cells. The findings of this study indicated that TS contained unbalanced CD4 immune cells that were actively recruited locally by CCR4/CCL22 and CCR8/CCL1. As a result, it is anticipated that CD4 immune rebalancing can serve as a novel treatment for TS.

Distribution and Chemotactic Mechanism of CD4$^+$T Cells in Traumatic Tracheal Stenosis

Feng **TM[1]**, ***Chen*Y[1]**, ***Wei* JM[1]**, ***Tan*S[1]**, ***Liu* GN[1*]**

Department of Respiratory Medicine, The Second Affiliated Hospital of Guangxi Medical University, Nanning, China.

***** Correspondence should be addressed to Guangnan Liu;*gnliu63@hotmail.com*

Abstract: A systemic and local inflammatory immune imbalance is thought to be the cause of traumatic tracheal stenosis (TS). However, with CD4$^+$ T lymphocytes being the predominant immune cells in TS, the mechanism of action and recruitment has not been described. In our research, using flow cytometry, ELISA, immunofluorescence, and Transwell chamber assays, the expression, distribution, and potential chemotactic function of CD4$^+$ T cells in TS patients were examined before and after treatment. The results showed that the untreated group had significantly more CD4$^+$ T cells and their secreted TGF-β1 than the treated group. Additionally, the untreated group's CD4$^+$ T cells showed a significant rise in CCL22 and CCL1, as well as a larger proportion of CCR4 and CCR8. CD4$^+$ T cells and CD68$^+$ macrophages located in TS also expressed CCL1 and CCL22. In vitro, anti-CCL1 and anti-CCL22 can partially block the chemoattractant effect of TS bronchoalveolar lavage (BAL) on purified CD4$^+$ T cells. The findings of this study indicated that TS contained unbalanced CD4 immune cells that were actively recruited locally by CCR4/CCL22 and CCR8/CCL1. As a result, it is anticipated that CD4 immune rebalancing can serve as a novel treatment for TS.

Keywords: Chemokines; CD4$^+$ T; Traumatic tracheal stenosis

Introduction

Traumatic tracheal stenosis (TS) refers to the proliferation of granulation and inflammation and scar tissue in the trachea caused by tracheal intubation, tracheotomy, trauma, and other factors. Patients typically experience a narrowing of the tracheal lumen along with clinical dyspnea, coughing, and wheezing. This lowers their quality of life and, in extreme cases, can result in death from respiratory failure brought on by airway obstruction [1-4]. TS can be treated by surgical and bronchoscopic procedures [5,6], but numerous patients still develop granulation tissue and restenosis after interventions, and the recurrence rate of postoperative TS reaches as high as 40-70% [7].

The cause of TS is complex. Recent findings have shown that immune cell dysregulation is a significant contributor to TS [8-10]. Previous research has reported that B and T cells are involved in mediating granulation tissue formation in laryngotracheal stenosis, while $CD4^+$ T cells act a pivotal part in the fibrosis process [10]. There are various subtypes of $CD4^+$ T lymphocytes. Prior studies have revealed that the Th1/Th2/Th17/Treg lineages can be linked to one another, change into different lineages, and play distinct roles in the progression of fibrosis [11]. Th1 cells are primarily thought to have an anti-fibrotic effect in related studies [12]; nevertheless, they can also be detrimental to bone regeneration [13] and fibrotic disorders [14,15]. There is controversy around the function of regulatory T cells (Tregs) in fibrosis and their connection to a specific disease model [16]. But none of them have been reported in TS.

Transforming growth factor (TGF)-β is a key factor in fibrosis. Studies have revealed that a huge portion of TGF-β accumulates locally in TS, causing excessive proliferation of fibroblasts, increased collagen synthesis, extracellular matrix deposition and fibrosis through the action of TGF-β/Smad2/3, ERK1/2, and other pathways, and eventually leading to airway stenosis [17-19]. Recent findings have also strongly suggested that TGF-β is critical for developing and differentiating 20

Forkhead box protein 3 (FoxP3) Tregs [20]. Tregs are mostly produced in the thymus (tTreg), but they can also be produced extrathymically at peripheral sites (pTreg) or induced in cell culture (iTreg) in the presence of transforming growth factor (TGF-β) [21]. All Treg cell types express FoxP3, especially the thymic population (tTreg), and FoxP3 serves as a spectrum-specific and key transcription factor for maintaining Treg cell phenotype and suppressor function [22-25]. This study hypothesizes the presence of local immune cell infiltration and imbalance in TS and focuses on the secretion, aggregation and relationship with TGF-β1 of $CD4^+$ T cells and its subtype Tregs before and after TS treatment.

According to studies, $CD4^+$ T cells create the environment necessary for monocytes to differentiate into fibroblasts and participate in the negative control of fibrosis by secreting cytokines [26]. Additionally, a variety of inflammatory cytokines and chemokines are produced locally by macrophages, which contribute to the development of the inflammatory response, fibrosis, and abnormal wound healing. When there is an imbalance of Th1/Th2 cells in tissue fibrosis, Th1 cells secrete cytokines such as INF-γ, which is mainly involved in the inhibition of fibrosis [27], while Th2 cells mainly secrete IL-4, which promotes the development of fibrosis [28]. CXCR3 and CCR5 are predominantly expressed on the surface of polarized Th1 cells, whereas CCR4 and CCR8 are mainly distributed in Th2, mast cells and eosinophils. Therefore, different chemokine types can be used to evaluate the Th1/Th2 balance [29]. These chemokine receptors expressed on lymphocytes can be gained and detected in cells obtained by bronchoalveolar lavage (BAL) and tracheal granulation tissue biopsy. According to a larger body of data on pulmonary fibrosis, CXCR3 and its ligands CXCL11 (IFN-inducible T-cell α-chemoattractant, I-TAC) [30] and CXCL10 (IFN-γ-inducible protein, IP-10) [31] play an important role in bleomycin-induced lung fibrosis models in mice [32]. Belperio and his team [33] found that the bleomycin-induced lung fibrosis model in mice was associated with the CCR4 receptor and its agonists CCL17 (thymic and activation-regulated chemokine, TARC) and CCL22 (macrophage-derived chemokine, MDC). In a recent report, it was discovered that the CCR5 ligands CCL3 (macrophage inflammatory protein (MIP)-1) and CCL4 (MIP-1) are both considerably raised in BAL fluid of patients with idiopathic pulmonary fibrosis and have an essential impact in animal models of lung fibrosis [34,35]. Furthermore, Liu et al. confirmed that CCL1 was recruited into the lung through fibroblasts expressing its receptor CCR8 and triggered the AMFR-SPPY1 pathway, thereby promoting the development of pulmonary fibrosis [36]. However, little is known about cellular immune function in TS, and there are no data on the

specific role played by relevant chemokines in TS. This study aims to assess the ratios of $CD4^+$ T cells with the receptors CXCR3, CCR4, CCR5, and CCR8 in BAL fluid and synchronized peripheral blood of TS patients before and after treatment.

Results

General information

The 10 patients with TS were caused by tracheal intubation (n = 6) and tracheotomy (n = 4), and the 10 control subjects had normal functional data. All the patients with TS underwent the comprehensive treatment, during which pulmonary function (FEV1 and FEV1/Pred%) and blood gas analysis (PO2 and PaO2/FiO2) improved, and ESR decreased. Compared to the control subjects, the untreated TS patients showed a decrease in lung function indexes, blood gas analysis indexes, CRP, and ESR, all of which were statistically significant, as shown in Table 1.

Comparison of $CD4^+$ T cells and Tregs expression in the untreated groups, treated groups, and normal healthy groups

This research chose $CD3^+$ and $CD4^+$cells to define $CD4^+$ T cells (Figure 1A) and $CD3^+$, $CD4^+$, and $CD25^+$ to define Tregs (Figure 1C) during flow cytometry. It was found that the untreated patients had a significantly higher expression of $CD4^+$ T cells and Tregs in BAL fluid and Tregs in peripheral blood than the treated patients and the control subjects (for $CD4^+$ T cells and Tregs in BAL fluid, $p = 0.007$ and $p = 0.015$, vs. the treated patients; for $CD4^+$ T cells and Tregs in peripheral blood,$p = 0.522$ and $p = 0.000$, vs. the treated patients,$p = 0.000$ and $p = 0.000$ vs. the control subjects; Figure 1B and 1D).

It was further discovered that the expression of FoxP3 mRNA, a crucial transcription factor of Tregs, was higher in tissue and peripheral blood of the untreated patients than that of the control subjects ($p = 0.018$ and $p = 0.006$, respectively; Figure 1D).

As shown in Figure 2, the cytokine TGF-β1 was highly expressed in $CD3^+CD4^+$ T cells and Tregs in BAL fluid and Tregs in peripheral blood of the untreated patients compared with the treated patients and the control subjects (for $CD4^+$ T cells and Tregs in BAL fluid, $p = 0.009$ and $p = 0.002$, vs. the treated patients; for $CD4^+$ T cells and Tregs in peripheral blood, $p = 0.913$ and $p = 0.004$, vs. the treated patients, $p = 0.012$ and $p = 0.000$ vs. the control subjects; Figure 2C).

Taking into account all the patients without the comprehensive treatment, the levels of cytokine TGF-β1 had a strong positive correlation with the expression of $CD4^+CD25^+$ BAL cells (r = 0.812,$p = 0.004$) and a moderate positive correlation with $CD4^+$ BAL lymphocytes (r = 0.666, $p = 0.035$; Figure 2C), suggesting that the increased accumulation of Tregs and $CD4^+$ T cells may act an important role in the increased secretion of TGF-β1.

Comparison of the expression of chemokines on $CD4^+$ T cells between the untreated and treated groups

The untreated patients had significantly higher expression of CCR4 and CCR8 on BAL fluid and peripheral $CD4^+$ T cells than the treated patients and the control subjects. A reverse trend was detected in BAL fluid and peripheral blood CD4CCR5 and CD4CXCR3 lymphocytes but without any statistical significance (for CD4CCR4, CD4CCR8, CD4CXCR3, and CD4CCR5 in BAL fluid, $p = 0.004$, $p = 0.038$, $p = 0.235$, and$p = 0.129$ vs. the treated patients; for CD4CCR4, CD4CCR8, CD4CXCR3, and CD4CCR5 in peripheral blood, $p = 0.762$, $p = 0.001$, $p = 0.422$, and $p = 0.237$ vs. the treated patients,$p = 0.005$, $p = 0.000$, $p = 0.056$, and $p = 0.075$ vs. the control subjects; Figure 3A and 3B). The above results suggest that the comprehensive treatment of TS tends to affect the expression of these receptors.

Comparison of the expression of chemokines on $CD4^+$ T cells between BAL fluid and peripheral blood

The expression of CCR8 was significantly higher in BAL fluid than that in peripheral blood $CD3^+CD4^+$

T lymphocytes in the untreated patients, while CCR4, CXCR3, and CCR5 on $CD4^+$ T cells showed an opposite trend without statistically significant differences (CCR8CD4 13.7 ± 1.4% vs. 6.9 ± 0.7%, p = 0.002, CCR4CD4 13.1 ±1.3% vs. 14.7 ±1.5%, $p = 0.496$, CXCR3CD4 2.3 ± 0.4% vs. 2.7 ± 0.4%, $p = 0.367$, CCR5CD4 1.4 ± 0.2% vs. 2.1 ± 0.4%, $p = 0.158$; Figures 4A and 4B).

Chemokine evaluation

Taking into account all the patients without the comprehensive treatment, the levels of CCL1 and CCL22 in BAL fluid (CCR8 and CCR4 ligands, respectively) of the untreated patients were higher than those of the treated groups ($p = 0.050$ and $p = 0.005$, respectively; Figure 5A), while the concentration of CXCL10, one of the CXCR3 ligands, decreased significantly in the untreated groups (p= 0.001; Figure 5A). It was also observed that there were high expression levels of CXCL11 (177.4 ± 19.6 pg/mL vs. 180.4 ± 10.2 pg/mL,p = 0.857), medium expression levels of CXCL9 (32.7 ± 3.1 pg/mL vs. 27.5 ± 1.7 pg/mL, $p = 0.085$), CCL3 (54.0 ± 2.1 pg/mL vs. 51.6 ± 1.7 pg/mL,$p = 0.346$), and CCL4 (61.7 ± 3.7 pg/mL vs. 60.1 ± 2.6 pg/mL,p = 0.671), and low expression levels of CCL17 (5.9 ± 0.5 pg/mL vs. 7.9 ± 0.9 pg/mL, $p = 0.065$; Figure 5A) in BAL fluid of the untreated and treated groups.

As shown in Figure 5B, CCL1 BAL levels of the untreated groups were correlated with the expression of CCR8 on CD4 BAL lymphocytes (r = 0.810, $p = 0.005$), and CCL22 was positively associated with CCR4 expression on CD4 BAL cells (r = 0.721, $p = 0.019$). Additionally, there was a positive correlation between CXCL10 BAL levels and CXCR3 expression on CD4 BAL cells (r = 0.638, $p = 0.047$). No association was found between BAL levels of CCL17, CCL3, CCL4, CXCL9 or CXCL11 and CCR4, CCR5 or CXCR3 expression on CD4 cells.

The previous ELISA analysis showed that CCL1 and CCL22 were highly expressed in BAL fluid and were highly positively correlated with their chemokine receptors. Thus, to visualize the distribution of chemokines in BAL fluid, double immunofluorescence staining was performed on cells isolated from BAL fluid, and it was discovered that CCL1 and CCL22 were expressed in $CD4^+$ T cells and $CD68^+$ macrophages (Figure 6). These data imply that $CD68^+$ macrophages and $CD4^+$ T cells may be the source of CCL1 and CCL22 in BAL fluid.

Chemokines CCL1 and CCL22 may be involved in the active recruitment of $CD4^+$ T cells in TS

The aforementioned data indicated that $CD4^+$ T cells in BAL fluid expressed high levels of CCR4 and CCR8, as well as elevated amounts of CCL1 and CCL22. Considering that the interactions between chemokines and their related chemokine receptors affect lymphocyte infiltration significantly [37], the $CD4^+$ T cells that contribute to the local increase in the mechanism of TS may be the process of their active recruitment to $CD4^+$ T cells. In vitro chemotaxis assays revealed that BAL fluid collected from the TS patients exerted chemotactic effects on peripheral $CD4^+$ T cells through potential chemotactic activity. Anti-CCL1 and anti-CCL22 monoclonal neutralizing antibodies, but not anti-CCL17, inhibited this chemotactic process significantly (Figure 7A and 7B), suggesting that the chemokines CCL1 and CCL22 may be key factors in the recruitment of $CD4^+$ T cells from peripheral blood to infiltrate the diseased trachea in TS.

Discussion

This study mainly focused on the comparison of indicators in the TS patients before and after treatment. Surprisingly, it found that the lung function and blood gas analysis indexes of the TS patients effectively improved via transbronchoscopic intervention therapy and systemic anti-infection treatment, and the inflammation indexes significantly reduced. Currently, transbronchoscopic intervention therapy has increasingly taken over as one of the primary treatments for treating benign airway stenosis [38], and local and systemic anti-infection has also been found to be effective for TS, but most of the data are related to animal models [39-41]. The specific indicators of the clinical patients in this research fully demonstrated that comprehensive treatment, including bronchial intervention and systemic anti-infection, could greatly improve various indicators of TS patients. However, it did not definitively distinguish which specific modality of

treatment is more effective, nor did it refine the specific modality of bronchoscopy intervention. This will lay a foundation for our future detailed research.

In this work, data were presented that supported the involvement of $CD4^+$ T cells and Tregs in TS. The results showed that compared with the treated patients and the healthy control subjects, there was a higher expression of $CD4^+$ T cells and Tregs in BAL fluid of the untreated patients, and simultaneously their secreted TGF-β1 expression was increased. Furthermore, TGF-β1 expression in $CD4^+$ T cells and Tregs in BAL fluid was strongly positively linked with those two cell types' expression in BAL fluid in TS.

The basic features of TS include excessive collagen production during tissue healing, as well as fibroblast differentiation and proliferation [3]. The critical step in the repair process after tissue damage is the infiltration of immune cells [42]. This study's experiment confirmed the hypothesis that there was a greater concentration of $CD4^+$ T lymphocytes in the local area of TS. Studies have revealed that Tregs are a small subset of peripherally circulating $CD4^+$ T cells that govern the immunological balance and are crucial for preserving health [20]. In this study, however, a significant accumulation of Tregs were observed in the untreated TS patients. At the same time, the key cytokine TGF-β1, which causes tissue fibrosis and is secreted by both $CD4^+$ T cells and Tregs, particularly the latter, was abnormally elevated. This research proved a strong correlation between the secretion of TGF-β1 and the number of these two immune cell subtypes, indicating that immune cells may be crucial in the onset and progression of TS through aberrant secretion of TGF-β1 and other cytokines. Previous reports on TS fibrosis have mainly focused on the TGF-β1 pathway [43-45]. Therefore, this paper discussed the new possibility that the imbalance of immune cells and the secretion of cytokines may affect the occurrence and development of TS. At present, studies have constantly explored the potential of immune cells as targeted therapies. In animal models, T-cellular immunotherapy can specifically target pathological mouse heart fiber vitamin cells to slow down the fibrosis process [46], so it is hoped that this indicator will become a targeted therapy for TS. However, this study did not delve into the mechanism of immune cell influence on TGF-β1, which will be investigated in the next step.

Earlier studies have shown that Th1 cells are the primary source of INF-γ, an inflammatory cytokine that can decrease fibroblasts' proliferation and collagen production [27], and thus Th1 cells inhibit fibrosis. Th2 cells that secrete IL-4 have been shown to induce fibroblast aggregation and promote fibroblast activation and proliferation [28]. In this study, Th2-targeted CCR4 and CCR8 chemokine receptors, as well as the chemokine ligands CCL22 and CCL1, were shown to be considerably enhanced in the TS BAL fluid of the untreated group. It has been shown that CCL22 chemotactic $CCR4^+$ macrophages contribute to fibrosis localization by releasing large amounts of cytokines and thus promote fibrosis. In turn, macrophages can further affect the function of $CD4^+$ T cells [47]. Conversely, despite a proportional drop in the Th1-targeted CCR5 and CXCR3 chemokine receptors in the untreated group, no significant difference was identified, likely due to limited specimen size. Additionally, this study discovered that the levels of CXCL10, a chemokine that draws CXCR3-positive cells, were decreased in the untreated patients. According to recent studies, CXCR3 and CXCL10 are strongly associated with the angiogenesis process [30,31,48]. According to Tager et al., receiving bleomycin caused lung fibrosis in CXCL10-deficient mice to worsen [31]. Moreover, recent studies have shown that CXCR3 exerts an angiostatic effect mediated by alternative splicing of the CXCR3 gene (CXCR3-B) [49]. The balance between angiostatic and angiogenic processes may change if CXC chemokines and their receptors become dysregulated during inflammatory events. The untreated TS subjects in this study had low levels of CXCR3, and CXCL10 expression may be beneficial to the local angiogenic activity of TS. Therefore, starting from chemokine receptors, this study discovered a possible local Th1/Th2 subpopulation imbalance in TS, and also found that Th2 cells, which are associated with fibrosis, were elevated, while Th1 cells, which inhibit fibrosis, were decreased.

Besides, this paper focused on the chemotactic mechanism of $CD4^+$ T cells. At present, it is a poorly understood mechanism for $CD4^+$ T accumulation in TS, and it is speculated that the mechanism of increased $CD4^+$ T content in TS may be related to active recruitment in the TS microenvironment. Chemotactic receptors are essential for T-cell migration, but how they affect $CD4^+$ T-cell migration to local TS remains unclear. Therefore, this study explored whether CCL1 and CCL22 are related to the process of $CD4^+$ T

aggregation in the local area of TS. The findings showed that the untreated groups had considerably higher levels of CCL1 and CCL22 in their BAL fluid than the treated groups, and immunofluorescence suggested that $CD68^+$ macrophages and $CD4^+$ T cells may be the cellular sources of these chemokines. Moreover, the receptors corresponding to CCL1 and CCL22, namely CCR8 and CCR4, were obviously expressed on the surface of both TS and homologous peripheral blood $CD4^+$ T cells. These data suggest that CCL1 and CCL22 in TS may be associated with local accumulation of $CD4^+$ T cells. Subsequent in vitro chemotactic tests further confirmed that BAL in TS could recruit peripheral $CD4^+$ T lymphocytes. In the untreated group, anti-CCL1 or anti-CCL22 monoclonal neutralizing antibodies dramatically inhibited the chemotactic activity of BAL in TS. Thus, the chemotactic effects of CCL1 and CCL22 may mobilize peripheral $CD4^+$ T cells to infiltrate the TS region.

To summarize, our findings showed that a great accumulation of immune cells, including $CD4^+$ T cells and their important subtype Tregs, accumulated in the untreated TS patients, and it was suspected that these cells might be involved in the fibrosis progression through the release of TGF-β1. In this research, it was found that Th1/Th2 cells were locally imbalanced in TS, of which Th2 cells may play a role by actively recruiting TS through CCR4/CCL22 and CCR8/CCL1. However, after comprehensive clinical treatment, the phenotype of these immune cells changed. This study can serve as a breakthrough point for the future study of immune cells in TS fibrosis and lay a foundation for the exploration of TS immunotherapy.

Materials and Methods

Subjects

The study protocol was approved by the Ethics Committee of the Second Affiliated Hospital of Guangxi Medical University (Nanning, China), and written consent was obtained from the subjects. Ten patients with TS were recruited, and they all had tracheal injury due to tracheal intubation or tracheotomy. The proliferative scar tissue was pathologically confirmed, and BAL fluid and blood specimens were collected before and after comprehensive treatment (interventional pulmonology procedures, systemic anti-infection, sputum removal, and rehabilitation exercises).

General detection

Pulmonary function tests, including total spirometry (VC), exertional spirometry (FVC), first second exertional expiratory volume (FEV1), first second exertional expiratory volume as a percentage of predicted value (FEV1/Pred%), and first second exertional expiratory volume as a percentage of exertional spirometry (FEV1/FVC%), were performed for the 10 patients before and one month after the comprehensive treatment. In addition, blood gas analysis and inflammatory indexes, including arterial partial pressure of oxygen (PO2), partial pressure of carbon dioxide oxygen (PCO2), oxygenation index (PaO2/FiO2), C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR), were assessed for all the patients before and after treatment.

Sample collection and processing

BAL samples (60-120 mL) were collected from each patient before and after treatment, and they were processed in accordance with the European Respiratory Society (ERS) guidelines [50]. Simultaneously, 20 mL blood was drawn. BAL samples were immediately placed in ice and then centrifuged at 1,200 × g for 5 min, and the BAL supernatant was collected for subsequent ELISA and chemotactic assays. The cell pellet of BAL fluid and blood was resuspended in phosphate buffered saline (PBS), and mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation (Solarbio, Beijing, China) within 1 h.

Flow cytometry

Dead cells were stained with BD Horizon Fixable Viability Stain 700. Moreover, the expression of markers on T cells from BAL fluid and blood was stained with antibodies, including anti-human CD3-APC/Cy7, CD25-APC, CD4-BB515, CXCR3-APC, CCR4-PE, CCR5-APC, and CCR8-PE (BD Biosciences, Franklin

Lakes, NJ, USA). Cells were acquired with a flow cytometer (Beckman Coulter, Brea, CA, USA), and data were analysed with FlowJo 10.

Enzyme-linked immunosorbent assay (ELISA)

The levels of CCL1, CCL3, CCL4, CXCL9, CXCL10, CXCL11, CCL17, and CCL22 were evaluated in undiluted BAL samples between the untreated and treated patients with commercial immunoassays (Fankew, China) according to the manufacturer's instructions.

Immunofluorescence assay

The cell suspensions were obtained by BAL centrifugation, dehydrated, embedded in paraffin, sectioned in paraffin, dewaxed, rehydrated using an alcohol gradient, antigen repaired, and closed. They were then incubated with CCL1 (Biorbyt, UK), CCL22 (Biorbyt, UK), CD4 (ProteinTech Group, Chicago, IL, USA), and CD68 (ProteinTech Group, Chicago, IL, USA) antibodies at 4degC overnight, followed by incubation with the corresponding fluorescent substance-labeled secondary antibodies for 1 h. After mounting with DAPI (Solarbio, Beijing, China), immunofluorescence images were recorded using an orthofluorescence microscope with phase contrast (Nikon, Japan).

Quantitative real-time polymerase chain reaction (RT-PCR)

RNA was extracted and purified from the 10 TS patients and the 10 control subjects using an RNA isolation kit (Vazyme Biotech, Nanjing, China). As a key Treg cell transcription factor [51,52], the mRNA level of FoxP3 was analysed using quantitative RT-PCR. The cycle threshold (CT) value of FoxP3 was normalized against GAPDH (ΔCT) for all samples. Gene expression was displayed as the relative fold change (2-$\Delta\Delta$CT). All samples were investigated in triplicate.

The primer sequences used in the quantitative polymerase chain reaction (qPCR) were as follows: Gene: FoxP3 (Forward: 5'- AGTTCCTCCACAACATGGACTACT-3'; Reverse: 5'- ATTGAGTGTC-CGCTGCTTCTCT -3'). Gene GAPDH: (Forward: 5'- ACATCGCTCAGACACCATG-3'; Reverse: 5'-TGTAGTTGAGGTCAATGAAGGG-3'). All the reagents were purchased from Takara.

Cell isolation andTranswell chamber assay

$CD4^+$ T cells were isolated by magnetic-activated cell sorting (MACS) based on negative selection using the $CD4^+$ T-cell isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany). The purity of $CD4^+$ T cells was typically $> 96\%$, as detected by flow cytometry.

For chemotaxis assays, a 6.5 mm Transwell with a 5.0 μm pore polycarbonate membrane insert (Corning Costar, Corning, NY, USA) was used. BAL supernatants were collected as described previously. The purified $CD4^+$ T cells were cultured in a Transwell chamber, and BAL supernatants and neutralizing antibodies against the chemokines were cultured in a lower layer culture plate. Grouping was as follows: PBS control group, BAL supernatant group, BAL + anti-CCL1 (Thermo Fisher Scientific, USA) group, BAL + anti-CXCL10 (Thermo Fisher Scientific, USA) group, and BAL + anti-CCL22 (Thermo Fisher Scientific, USA) group. These groups were incubated in an incubator at a constant temperature for 16 h without or with neutralizing antibodies against the chemokines. The cells on the upper surface of the membrane were removed with a cotton swab, and the cells that migrated to the lower side were fixed with methanol and stained with 0.1% crystal violet. The membrane was visualized with an inverted microscope at 200x magnification. Cell counting was performed using the ImageJ software. Four representative visual fields were selected for each membrane, and six separate experiments were performed.

Statistical tests

SPSS V.26.0 (SPSS Inc., Chicago, USA) was employed for the statistical tests. Data are expressed as the mean ± SEM. Within-group comparisons before and after treatment were made using paired t tests or Wilcoxon's signed-rank tests as appropriate. Between-group data were analysed using independent t tests or Mann-Whitney U tests as appropriate. Statistical differences between groups were evaluated using one-way

ANOVA followed by the Newman-Keuls test for normally distributed continuous variables. Correlations were assessed with Spearman's rank tests. $p< 0.05$ was considered statistically significant.

Ethical Approval

This study was approved by the ethics committee of the Second Affiliated Hospital of Guangxi Medical University [Approval number: 2022(KY-0761)].

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was supported in part by grants from the National Natural Science Foundation of China (No. 82060003) and in part by a grant from the Guangxi Clinical Medical Research Center for Respiratory Diseases Program (No. 2022AC04005).

Authors' contributions

L.G.N. and F.T.M. designed the study and drafted the manuscript. F.T.M. and C.Y. were responsible for cell isolation, culture, and Transwell chamber assay. F.T.M. and W.J.M. performed flow cytometry, ELISA, RT-PCR, and immunofluorescence. T.S. recruited the subjects and collected clinical samples. All authors read, critically revised, and approved the final manuscript.

References

1. **Wain, J. C., Jr.,** Postintubation tracheal stenosis.*Semin Thorac Cardiovasc Surg* . 2009. **21** : 284-289.

2. **Murgu, S. D., Egressy, K., Laxmanan, B., Doblare, G., Ortiz-Comino, R. and Hogarth, D. K.,** Central Airway Obstruction: Benign Strictures, Tracheobronchomalacia, and Malignancy-related Obstruction.*Chest* . 2016. **150** : 426-441.

3. **Gelbard, A., Francis, D. O., Sandulache, V. C., Simmons, J. C., Donovan, D. T. and Ongkasuwan, J.,** Causes and consequences of adult laryngotracheal stenosis. *Laryngoscope* . 2015. **125** : 1137-1143.

4. **Farzanegan, R., Feizabadi, M., Ghorbani, F., Movassaghi, M., Vaziri, E., Zangi, M., Lajevardi, S. andShadmehr, M. B.,** An Overview of Tracheal Stenosis Research Trends and Hot Topics. *Arch Iran Med* . 2017. **20** : 598-607.

5. **Wang, T., Zhang, J., Qiu, X. J., Wang, J., Pei, Y. H. and Wang, Y. L.,** Scarring Airway Stenosis in Chinese Adults: Characteristics and Interventional Bronchoscopy Treatment. *Chin Med J (Engl)* . 2018. **131** : 276-281.

6. **Ferreirinha, J., Caviezel, C., Weder, W., Opitz, I. and Inci, I.,** Postoperative outcome of tracheal resection in benign and malignant tracheal stenosis. *Swiss Med Wkly* . 2020. **150** : w20383.

7. **Huuskonen, A., Mäkitie, A. and Sihvo, E.,** [Treatment of adult tracheal stenosis]. *Duodecim* . 2015. **131** : 1793-1801.

8. **Motz, K. M., Yin, L. X., Samad, I., Ding, D., Murphy, M. K., Duvvuri, M. and Hillel, A. T.,** Quantification of Inflammatory Markers in Laryngotracheal Stenosis. *Otolaryngol Head Neck Surg* . 2017.**157** : 466-472.

9. **Hillel, A. T., Ding, D., Samad, I., Murphy, M. K. and Motz, K.,** T-Helper 2 Lymphocyte Immunophenotype Is Associated With Iatrogenic Laryngotracheal Stenosis. *Laryngoscope* . 2019. **129** : 177-186.

10. **Ghosh, A., Malaisrie, N., Leahy, K. P., Singhal, S., Einhorn, E., Howlett, P., Cohen, N. A. andMirza, N.,** Cellular adaptive inflammation mediates airway granulation in a murine model of subglottic stenosis. *Otolaryngol Head Neck Surg* . 2011. **144** : 927-933.

11. **Saito, S., Nakashima, A., Shima, T. and Ito, M.,**Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy. *Am J Reprod Immunol* . 2010. **63** : 601-610.

12. **Wynn, T. A.,** Fibrotic disease and the T(H)1/T(H)2 paradigm.*Nat Rev Immunol* . 2004. **4** : 583-594.

13. **Liu, Y., Wang, L., Kikuiri, T., Akiyama, K., Chen, C., Xu, X., Yang, R., Chen, W., Wang, S. andShi, S.,** Mesenchymal stem cell-based tissue regeneration is governed by recipient T lymphocytes via IFN-γ and TNF-α. *Nat Med* . 2011. **17** : 1594-1601.

14. **Fielding, C. A., Jones, G. W., McLoughlin, R. M., McLeod, L., Hammond, V. J., Uceda, J., Williams, A. S., Lambie, M., Foster, T. L., Liao, C. T., Rice, C. M., Greenhill, C. J., Colmont, C. S., Hams, E., Coles, B., Kift-Morgan, A., Newton, Z., Craig, K. J., Williams, J. D., Williams, G. T., Davies, S. J., Humphreys, I. R., O'Donnell, V. B., Taylor, P. R., Jenkins, B. J., Topley, N. andJones, S. A.,**Interleukin-6 signaling drives fibrosis in unresolved inflammation.*Immunity* . 2014. **40** : 40-50.

15. **Nevers, T., Salvador, A. M., Velazquez, F., Ngwenyama, N., Carrillo-Salinas, F. J., Aronovitz, M., Blanton, R. M. andAlcaide, P.,**Th1 effector T cells selectively orchestrate cardiac fibrosis in nonischemic heart failure. *J Exp Med* . 2017. **214** : 3311-3329.

16. **Pesenacker, A. M., Broady, R. and Levings, M. K.,** Control of tissue-localized immune responses by human regulatory T cells.*Eur J Immunol* . 2015. **45** : 333-343.

17. **Wu, Y., Li, Y., Bai, Y., Jiang, J., Wang, X., Chen, Y., Wang, X., Huang, G., Gan, Y., Li, Y. andGuo, S.,** Clinical significance of serum transforming growth factor-β1 and procollagen type I N-propeptide in post-tuberculosis tracheobronchial stenosis. *Exp Ther Med* . 2021. **21** : 570.

18. **Cai, Z., Li, H., Zhang, H., Han, S., An, R. and Yan, X.,**Novel insights into the role of hypoxia-inducible factor-1 in the pathogenesis of human post-intubation tracheal stenosis. *Mol Med Rep* . 2013. **8** : 903-908.

19. **Zhang, G., Xue, C. and Zeng, Y.,** β-elemene alleviates airway stenosis via the ILK/Akt pathway modulated by MIR143HG sponging miR-1275. *Cell Mol Biol Lett* . 2021. **26** : 28.

20. **Pillai, V., Ortega, S. B., Wang, C. K. and Karandikar, N. J.,** Transient regulatory T-cells: a state attained by all activated human T-cells. *Clin Immunol* . 2007. **123** : 18-29.

21. **Shevach, E. M. and Thornton, A. M.,** tTregs, pTregs, and iTregs: similarities and differences. *Immunol Rev* . 2014.**259** : 88-102.

22. **Shevach, E. M.,** Regulatory T cells in autoimmmunity*.*Annu Rev Immunol* . 2000. **18** : 423-449.

23. **Takahashi, T., Tagami, T., Yamazaki, S., Uede, T., Shimizu, J., Sakaguchi, N., Mak, T. W. andSakaguchi, S.,** Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4.*J Exp Med* . 2000. **192** : 303-310.

24. **Hori, S., Nomura, T. and Sakaguchi, S.,** Control of regulatory T cell development by the transcription factor Foxp3.*Science* . 2003. **299** : 1057-1061.

25. **Fontenot, J. D., Gavin, M. A. and Rudensky, A. Y.,** Foxp3 programs the development and function of CD4+CD25+ regulatory T cells.*Nat Immunol* . 2003. **4** : 330-336.

26. !!! INVALID CITATION !!! [26].

27. **Motz, K., Samad, I., Yin, L. X., Murphy, M. K., Duvvuri, M., Ding, D. and Hillel, A. T.,** Interferon-γ Treatment of Human Laryngotracheal Stenosis-Derived Fibroblasts. *JAMA Otolaryngol Head Neck Surg* . 2017. **143** : 1134-1140.

28. **Gieseck, R. L., 3rd, Wilson, M. S. and Wynn, T. A.,** Type 2 immunity in tissue repair and fibrosis. *Nat Rev Immunol* . 2018.**18** : 62-76.

29. **Colantonio, L., Recalde, H., Sinigaglia, F. and D'Ambrosio, D.,** Modulation of chemokine receptor expression and chemotactic responsiveness during differentiation of human naive T cells into Th1 or Th2 cells. *Eur J Immunol* . 2002. **32** : 1264-1273.

30. **Burdick, M. D., Murray, L. A., Keane, M. P., Xue, Y. Y., Zisman, D. A., Belperio, J. A. and Strieter, R. M.,** CXCL11 attenuates bleomycin-induced pulmonary fibrosis via inhibition of vascular remodeling. *Am J Respir Crit Care Med* . 2005. **171** : 261-268.

31. **Tager, A. M., Kradin, R. L., LaCamera, P., Bercury, S. D., Campanella, G. S., Leary, C. P., Polosukhin, V., Zhao, L. H., Sakamoto, H., Blackwell, T. S. andLuster, A. D.,** Inhibition of pulmonary fibrosis by the chemokine IP-10/CXCL10. *Am J Respir Cell Mol Biol* . 2004.**31** : 395-404.

32. **Jiang, D., Liang, J., Hodge, J., Lu, B., Zhu, Z., Yu, S., Fan, J., Gao, Y., Yin, Z., Homer, R., Gerard, C. andNoble, P. W.,**Regulation of pulmonary fibrosis by chemokine receptor CXCR3. *J Clin Invest* . 2004. **114** : 291-299.

33. **Belperio, J. A., Dy, M., Murray, L., Burdick, M. D., Xue, Y. Y., Strieter, R. M. and Keane, M. P.,** The role of the Th2 CC chemokine ligand CCL17 in pulmonary fibrosis. *J Immunol* . 2004.**173** : 4692-4698.

34. **Capelli, A., Di Stefano, A., Gnemmi, I. and Donner, C. F.,**CCR5 expression and CC chemokine levels in idiopathic pulmonary fibrosis. *Eur Respir J* . 2005. **25** : 701-707.

35. **Ishida, Y., Kimura, A., Kondo, T., Hayashi, T., Ueno, M., Takakura, N., Matsushima, K. andMukaida, N.,** Essential roles of the CC chemokine ligand 3-CC chemokine receptor 5 axis in bleomycin-induced pulmonary fibrosis through regulation of macrophage and fibrocyte infiltration. *Am J Pathol* . 2007. **170** : 843-854.

36. **Liu, S. S., Liu, C., Lv, X. X., Cui, B., Yan, J., Li, Y. X., Li, K., Hua, F., Zhang, X. W., Yu, J. J., Yu, J. M., Wang, F., Shang, S., Li, P. P., Zhou, Z. G., Xiao, Y. andHu, Z. W.,** The chemokine CCL1 triggers an AMFR-SPRY1 pathway that promotes differentiation of lung fibroblasts into myofibroblasts and drives pulmonary fibrosis.*Immunity* . 2021. **54** : 2042-2056.e2048.

37. **Moser, B. and Loetscher, P.,** Lymphocyte traffic control by chemokines. *Nat Immunol* . 2001. **2** : 123-128.

38. **Zhang, J., Wang, J., Wang, T., Xu, M., Dang, B. W., Pei, Y. H. and Zhang, C. Y.,** [A pilot study on interventional bronchoscopy in the management of airway stenosis with benign hyperplasia].*Zhonghua Jie He He Hu Xi Za Zhi* . 2011. **34** : 334-338.

39. **Huang, Z., Wei, P., Gan, L., Li, W., Zeng, T., Qin, C., Chen, Z. andLiu, G.,** Protective effects of different anti-inflammatory drugs on tracheal stenosis following injury and potential mechanisms.*Mol Med Rep* . 2021. **23** .

40. **Huang, Z., Wei, P., Gan, L., Zeng, T., Qin, C. and Liu, G.,**Role of Erythromycin-Regulated Histone Deacetylase-2 in Benign Tracheal Stenosis. *Can Respir J* . 2020. **2020** : 4213807.

41. **Huang, Z., Wei, P., Gan, L., Li, W., Zeng, T., Qin, C., Chen, Z. andLiu, G.,** Expression of histone deacetylase 2 in tracheal stenosis models and its relationship with tracheal granulation tissue proliferation. *Exp Ther Med* . 2021. **21** : 444.

42. **Gurtner, G. C., Werner, S., Barrandon, Y. and Longaker, M. T.,** Wound repair and regeneration. *Nature* . 2008. **453** : 314-321.

43. **Shi, W., Fang, Y., Jiang, Y., Jiang, S., Li, Y., Li, W., Xu, M., Aschner, M. andLiu, G.,** Plumbagin attenuates traumatic tracheal stenosis in rats and inhibits lung fibroblast proliferation and differentiation via TGF-β1/Smad and Akt/mTOR pathways.*Bioengineered* . 2021. **12** : 4475-4488.

44. **Xiao, Y., Zhou, L., Zhang, T., Qin, C., Wei, P., Luo, L., Luo, L., Huang, G., Chen, A. andLiu, G.,** Anti-fibrosis activity of quercetin attenuates rabbit tracheal stenosis via the TGF-β/AKT/mTOR signaling pathway. *Life Sci* . 2020. **250** : 117552.

45. **Zhou, L., Li, Y., Gan, L. M., Qin, E. Y., Meng, X. Y., Gan, J. H., Li, W. T., Qin, C. C. andLiu, G. N.,** [Effects of different drugs on bronchial stenosis by TGF-β/mTOR signaling pathway in rabbit model]. *Zhonghua Yi Xue Za Zhi* . 2019. **99** : 1898-1903.

46. **Aghajanian, H., Kimura, T., Rurik, J. G., Hancock, A. S., Leibowitz, M. S., Li, L., Scholler, J., Monslow, J., Lo, A., Han, W., Wang, T., Bedi, K., Morley, M. P., Linares Saldana, R. A., Bolar, N. A., McDaid, K., Assenmacher, C. A., Smith, C. L., Wirth, D., June, C. H., Margulies, K. B., Jain, R., Puré, E., Albelda, S. M. andEpstein, J. A.,**Targeting cardiac fibrosis with engineered T cells. *Nature* . 2019.**573** : 430-433.

47. **Yogo, Y., Fujishima, S., Inoue, T., Saito, F., Shiomi, T., Yamaguchi, K. and Ishizaka, A.,** Macrophage derived chemokine (CCL22), thymus and activation-regulated chemokine (CCL17), and CCR4 in idiopathic pulmonary fibrosis. *Respir Res* . 2009. **10** : 80.

48. **Jiang, D., Liang, J., Campanella, G. S., Guo, R., Yu, S., Xie, T., Liu, N., Jung, Y., Homer, R., Meltzer, E. B., Li, Y., Tager, A. M., Goetinck, P. F., Luster, A. D. andNoble, P. W.,** Inhibition of pulmonary fibrosis in mice by CXCL10 requires glycosaminoglycan binding and syndecan-4. *J Clin Invest* . 2010. **120** : 2049-2057.

49. **Lasagni, L., Francalanci, M., Annunziato, F., Lazzeri, E., Giannini, S., Cosmi, L., Sagrinati, C., Mazzinghi, B., Orlando, C., Maggi, E., Marra, F., Romagnani, S., Serio, M. andRomagnani, P.,** An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4. *J Exp Med* . 2003.**197** : 1537-1549.

50. Technical recommendations and guidelines for bronchoalveolar lavage (BAL). Report of the European Society of Pneumology Task Group.*Eur Respir J* . 1989. **2** : 561-585.

51. **Sakaguchi, S., Yamaguchi, T., Nomura, T. and Ono, M.,**Regulatory T cells and immune tolerance. *Cell* . 2008.**133** : 775-787.

52. **Sakaguchi, S.,** Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses.*Annu Rev Immunol* . 2004. **22** : 531-562.

Table and Figure

Table 1. Characteristics of the patients enrolled

	Pre-T (n=10)	Post-T (n=10)	CTRL (n=10)
Age, yr	37.5 ± 4.5	37.5 ± 4.5	30.3 ± 1.8
Sex, M/F	7/3	7/3	6/4
VC, mL	2.6 ± 0.2^{+}	2.7 ± 0.2	3.6 ± 0.1
FVC, mL	2.6 ± 0.2^{+}	2.7 ± 0.2	3.6 ± 0.1
FEV1, mL	1.8 ± 0.1^{*+}	2.0 ± 0.1	3.2 ± 0.1
FEV1/Pred, %	55.5 ± 5.9^{*+}	59.5 ± 5.9	91.3 ± 0.6
FEV1/FVC, %	70.3 ± 3.7^{+}	76.3 ± 4.1	89.4 ± 1.1
PO2, mmHg	78.6 ± 3.0^{*+}	91.2 ± 1.9	97.2 ± 0.5
PCO2, mmHg	39.1 ± 1.2	39.7 ± 1.2	37.5 ± 0.6
PaO2/FiO2, mmHg	354.8 ± 21.6^{*+}	434.2 ± 9.0	462.9 ± 2.3
CRP, mg/L	18.9 ± 5.0^{+}	7.5 ± 1.8	2.8 ± 0.3
ESR, mm/h	65.3 ± 8.6^{*+}	15.8 ± 1.8	7.2 ± 1.3

Definition of abbreviations: Pre-T = Pre-treatment; Post-T = Post-treatment; CTRL = healthy control subjects; data are shown as mean ± SEM. * $p < 0.05$ versus Post-T; +$p < 0.05$ versus CTRL.

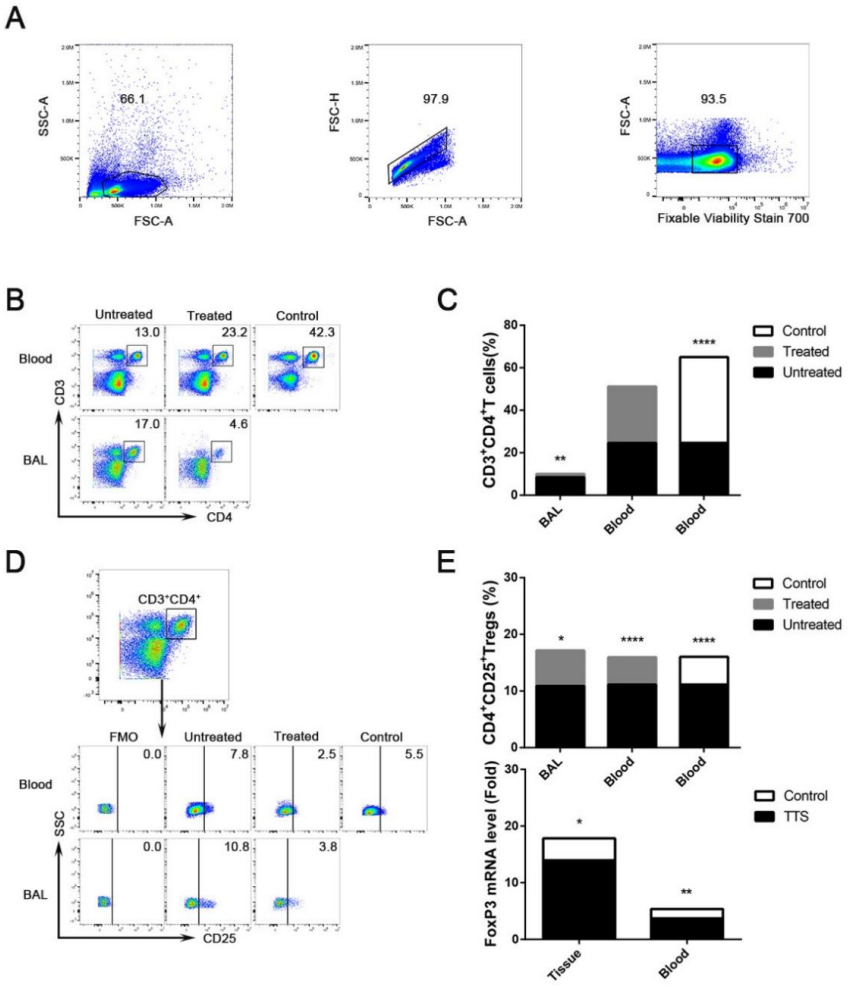


Figure 1. The expression of $CD3^+CD4^+$ T cells and Tregs in BAL fluid and peripheral blood of the untreated groups, treated groups, and control subjects. (A) (C) Representative flow cytometric dot plots of $CD3^+CD4^+$ T cells and Tregs in BAL fluid, blood, and control. (B) (D) Comparisons of percentages of $CD3^+CD4^+$ T cells and Tregs in BAL fluid, blood, and control (n = 10). (E) qPCR analysis of FoxP3 in the tissue and peripheral blood of the patients and control subjects. Within-group comparisons were made using paired t tests or Wilcoxon's signed-rank tests. Between-group data were analysed using independent t tests or Mann-Whitney U tests. The bar graph shows the mean. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.000$.

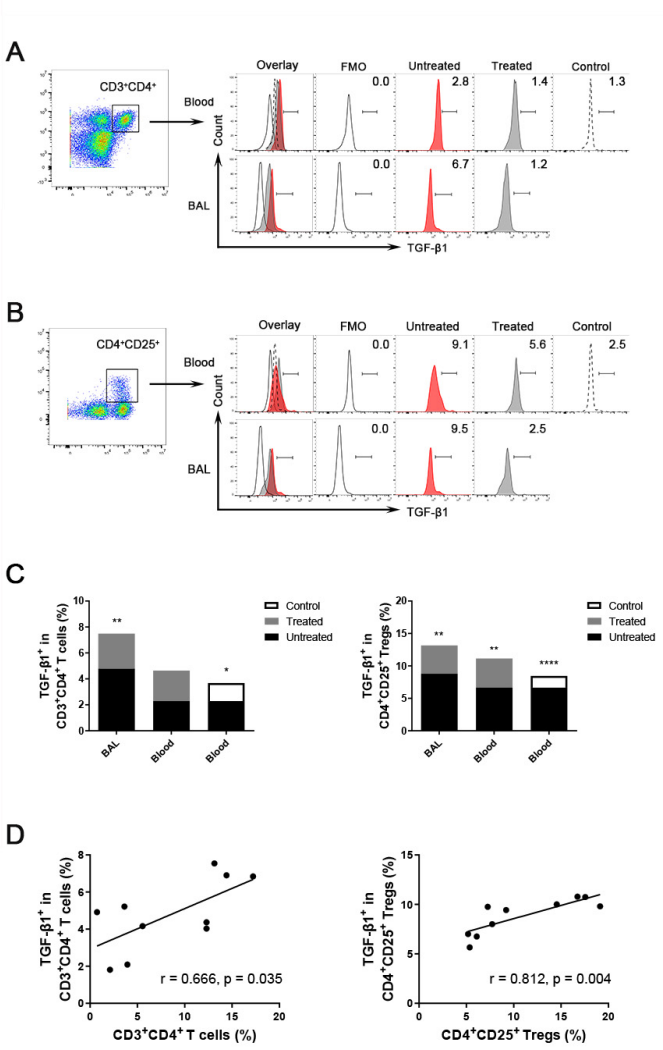


Figure 2. Τηε εξπρεσσιον οφ ΤΓΦ-β1^{+}"Δ4^{+} Τ ςελλς ανδ ΤΓΦ-β1^{+} Τρεγς ιν ΒΑΛ φλυιδ ανδ περιπηεραλ βλοοδ οφ τηε υντρεατεδ γρουπς, τρεατεδ γρουπς, ανδ ςοντρολ συβθε-ςτς. (A) (B) Representative flow cytometric dot plots of TGF-β1^{+} CD4^{+} T cells and TGF-β1^{+} Tregs in BAL fluid, blood, and control. (C) Comparisons of the percentages of TGF-β1^{+} CD4^{+} T cells and TGF-β1^{+} Tregs in BAL fluid, blood, and control (n = 10). (D) Correlation between TGF-β1^{+} CD4^{+} T cells and CD3^{+}CD4^{+} T cells and correlation between TGF-β1^{+} Tregs and Tregs in BAL fluid of the untreated groups. Within-group comparisons were made using paired t tests or Wilcoxon's signed-rank tests. Between-group data were analysed using independent t tests or Mann-Whitney U tests. Correlations were determined by Spearman's rank correlation coefficients. The bar graph shows the mean. *p< 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.000.

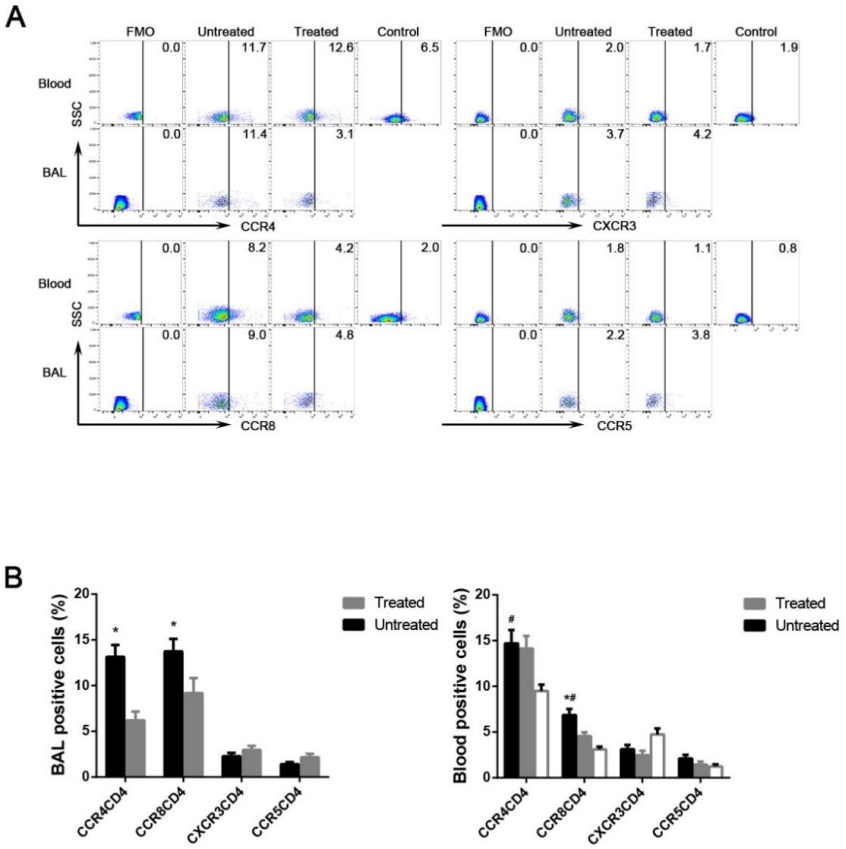


Figure 3. The expression of different chemokines on the CD4 surface in BAL fluid and peripheral blood of the untreated groups, treated groups, and control subjects. (A) Representative flow cytometric dot plots of CCR4, CCR8, CCR5, and CXCR3 on $CD4^+$ T cells in BAL fluid, blood, and control. (B) Comparisons of percentages of CCR4, CCR8, CCR5 and CXCR3 on $CD4^+$ T cells in BAL fluid and blood of the untreated groups, treated groups, and control subjects (n = 10). Data are expressed as the mean ± SEM. Within-group comparisons before and after treatment were made using paired t tests or Wilcoxon's signed-rank tests, and between-group comparisons were analysed using one-way ANOVA followed by Newman-Keuls tests. * $p < 0.05$ compared with the treated groups, and # $p < 0.05$ compared with the control groups.

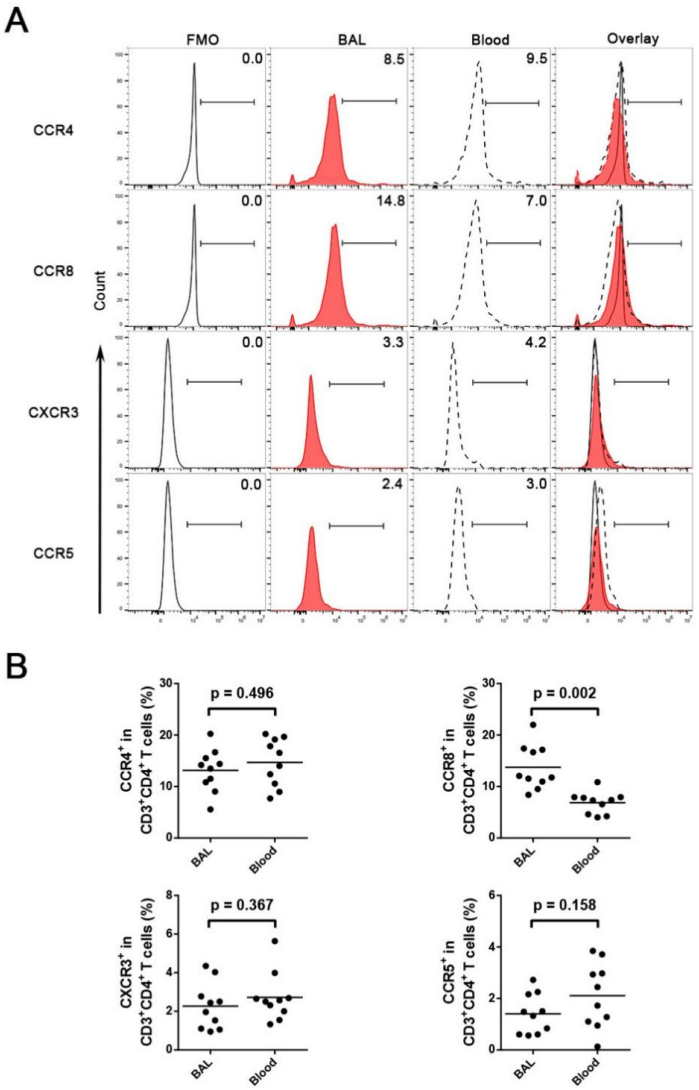


Figure 4. The percentages of CCR4, CCR8, CXCR3, and CCR5 on CD4$^+$ T cells in BAL fluid and homologous peripheral blood. (A) Representative flow cytometric dot plots showing the expression of CCR4, CCR8, CXCR3, and CCR5 on CD4$^+$ T cells in BAL fluid and blood. (B) Comparisons of percentages of CCR4, CCR8, CXCR3, and CCR5 on CD4$^+$ T cells in BAL fluid and blood (n = 10). Horizontal bars indicate means. Comparisons were made using paired t tests or Wilcoxon signed-rank tests.

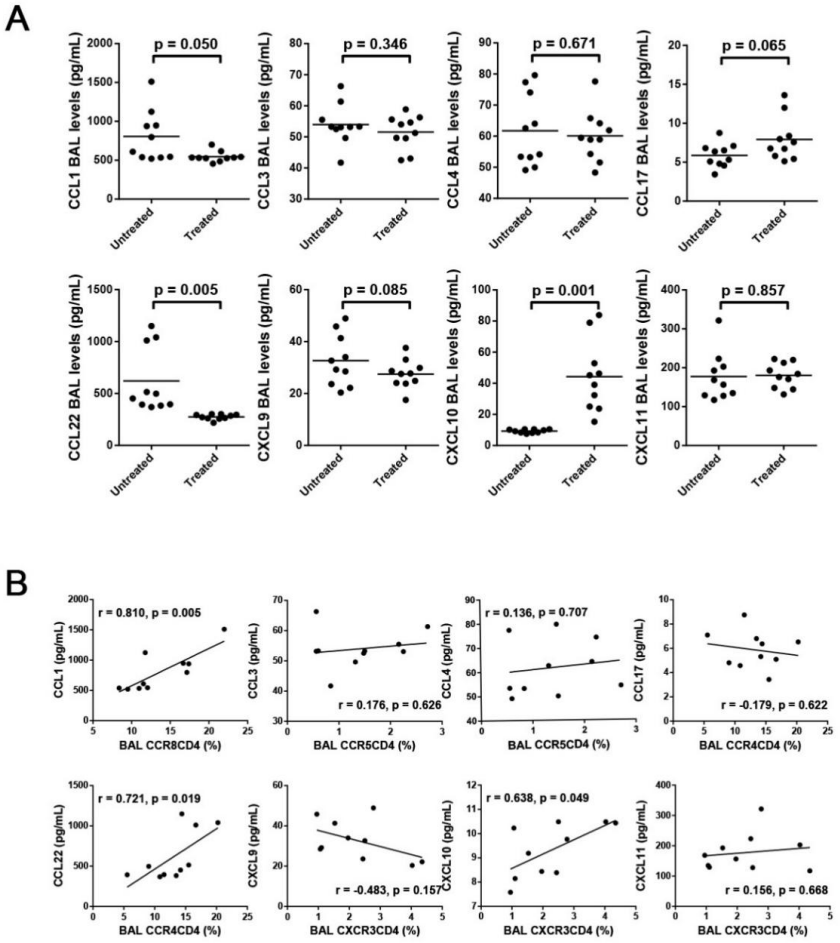


Figure 5. The expression levels of chemokines in BAL fluid and correlation analysis with chemokine receptors. (A) The statistical graph shows the concentration contrast of chemokines CCL1, CCL3, CCL4, CCL17, CCL22, CXCL9, CXCL10 and CXCL11 between the untreated and treated groups (n = 10). (B) Correlation analysis between chemokines and paired chemokine receptors. Horizontal bars indicate means. Comparisons were made using paired t tests or Wilcoxon signed-rank tests, and correlations were determined by Spearman's rank correlation coefficients.

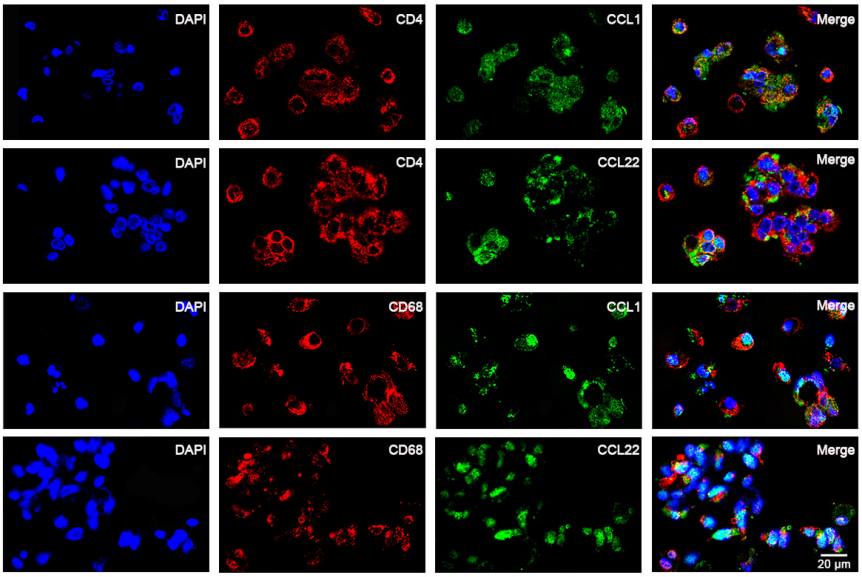


Figure 6. CCL1 and CCL22 were expressed in CD4$^+$ T cells and CD68$^+$ macrophages in TS BAL fluid. Macrophages and CD4$^+$ T cells were tagged with anti-CD68 and anti-CD4 murine monoclonal antibodies, followed by rhodamine-labeled goat anti-mouse serum staining (red). The expression levels of CCL1 and CCL22 were labeled with rabbit anti-human polyclonal antibodies and then labeled with FITC in goat anti-rabbit IgG (green). Two-colour immunofluorescence staining showed that some CD68$^+$ macrophages and some CD4$^+$ T cells expressed CCL1 and CCL22. Scale bar = 20 μm.

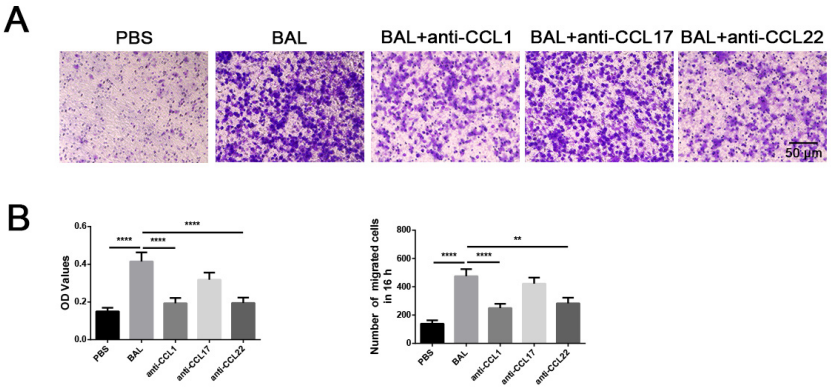
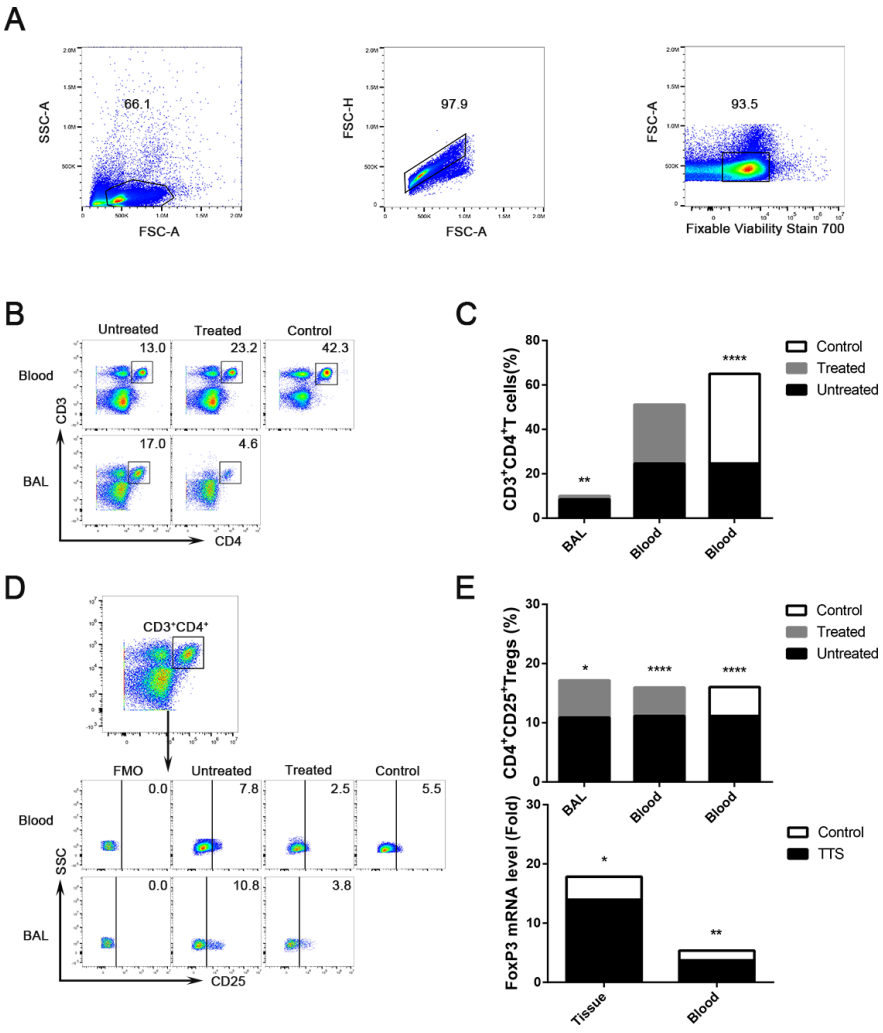
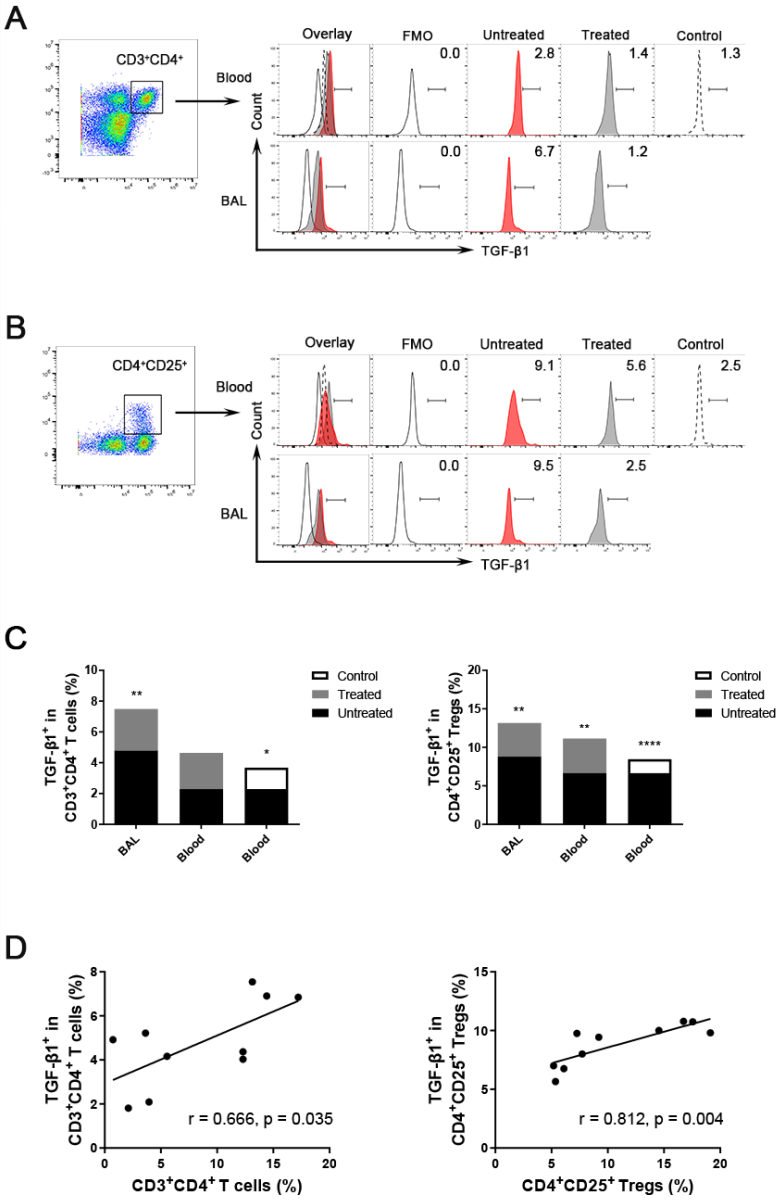
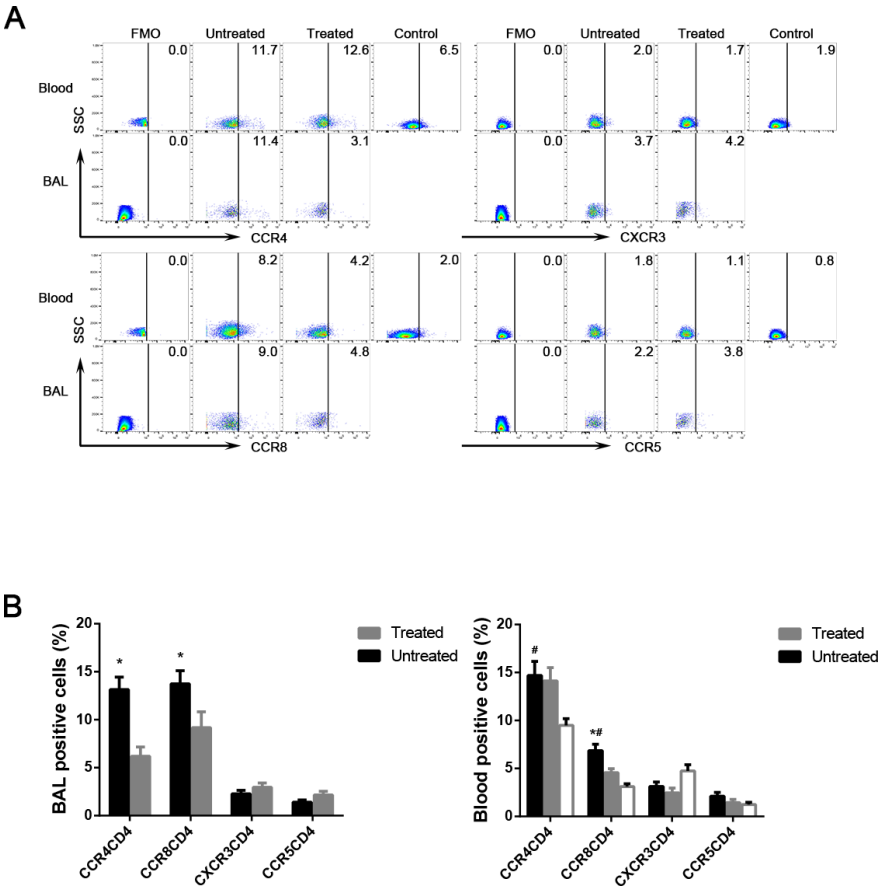


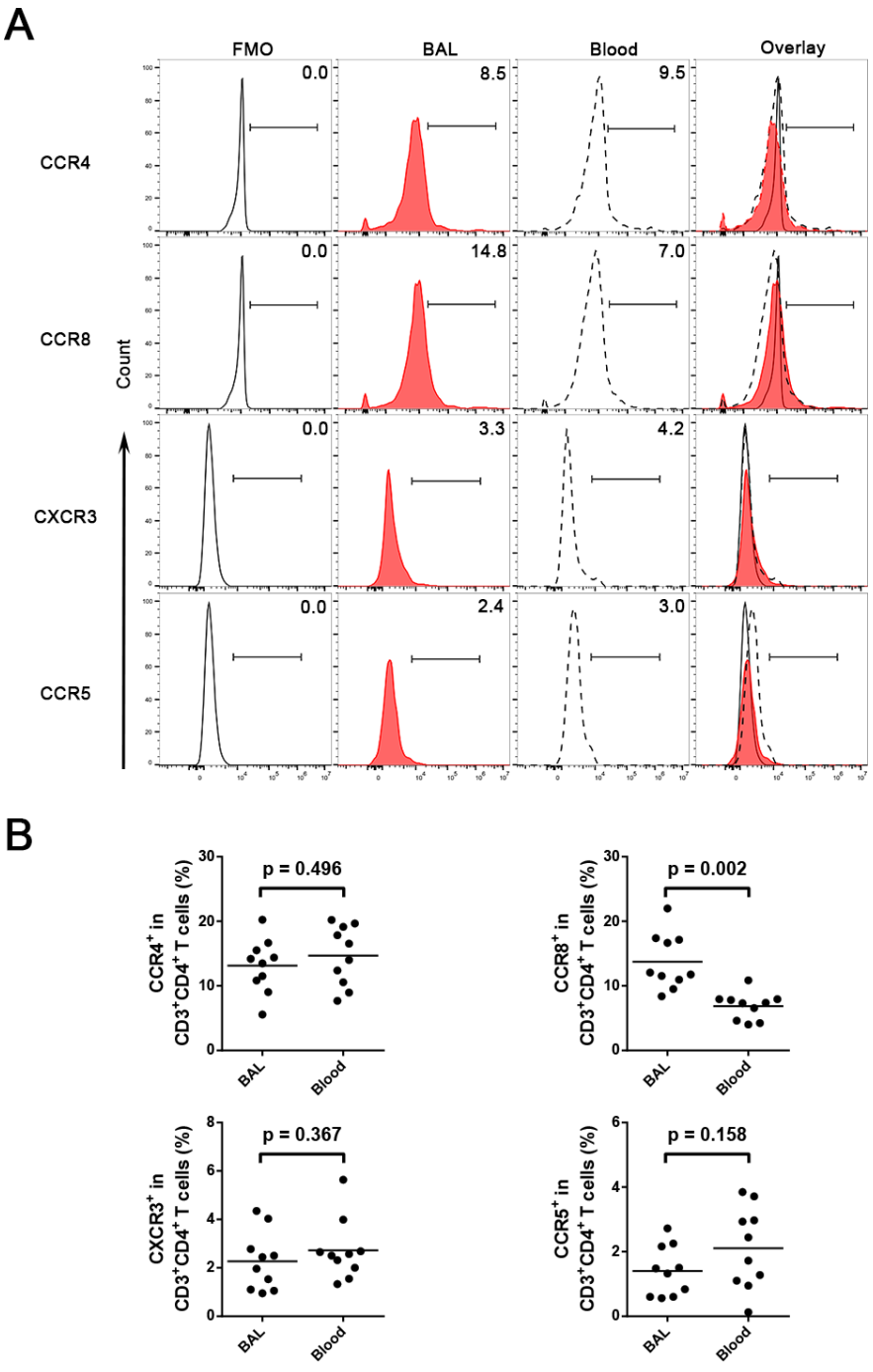
Figure 7. CCL1 and CCL22 in BAL of TS showed chemotactic activity against CD4$^+$ T cells. The Transwell method was adopted for migration assay. Purified CD4$^+$ T cells (1×10^5) were plated into the upper chamber of a 6.5 mm Transwell (5 μm pore size). Three neutralizing antibodies (anti-CCL1 0.1 μg/mL; anti-CCL17 1 μg/mL; and anti-CCL22 1 μg/mL) were added to the lower chamber. PBS was used as a negative control, and pure BAL was used as a positive control. After 16 h, CD4$^+$ T cells on the upper side were removed, and cells in the lower chamber were fixed with methanol and stained with 0.1% crystal violet. (A) Representative microscopy pictures from six independent experiments, scale bar = 50 μm. (B) Chemotactic assay statistics analysed by direct counting (number of migrated cells in 16 h) and indirect counting (optical density values). Data are expressed as the mean ± SEM. Between-group comparisons were

analysed using one-way ANOVA followed by Newman-Keuls tests. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.000$.









Hosted file

FIGURE 5.tif available at https://authorea.com/users/723361/articles/708045-distribution-and-chemotactic-mechanism-of-cd4-t-cells-in-traumatic-tracheal-stenosis