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Clinical significance of detection of mononuclear phagocyte subsets in blood and bronchoalveolar lavage fluid (BALF) in pulmonary sarcoidosis

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ABSTRACT

This study aimed to investigate the clinical significance of the detection of mononuclear phagocytes subsets in pulmonary sarcoidosis blood and bronchoalveolar lavage fluid (BALF). For this purpose, a total of 52 patients with pulmonary sarcoidosis were selected as the study group, 52 healthy people served as the "NC Group a" (peripheral blood mononuclear cell control group), 47 patients with chronic cough and no pulmonary sarcoidosis who underwent bronchoscopy were used as "control group b" (alveolar lavage fluid macrophage control group). Fasting peripheral blood and BALF were collected, and flow cytometry was used to detect monocytes and macrophage subpopulations. The monocytes and macrophage subpopulations of the study group were compared before and after treatment. The results showed that the proportion of CD14++CD16- subgroup of patients with pulmonary sarcoidosis was lower than that of healthy people (74.21±4.10% vs 84.32±4.76%); The proportion of CD14++CD16+ subgroups of patients with pulmonary sarcoidosis was higher than that of healthy people (7.42±4.08% vs 3.95±1.94%); The proportion of CD14+CD16++ subgroups of patients with pulmonary sarcoidosis was higher than that of healthy people in the control group, but the difference was not significant. After 2 months of treatment, the proportion of CD14++CD16- subgroups in peripheral blood mononuclear cells increased, and the proportion of CD14++CD16+ subgroups decreased. The proportion of M1 in patients with pulmonary sarcoidosis was lower than that in patients with non-pulmonary nodules (24.32±11.36% vs 47.03±13.86%); the proportion of M2 in patients with pulmonary sarcoidosis was higher than the proportion of M2 in patients with non-pulmonary nodules (75.40±10.23% vs 52.53±12.01%). After treatment, the proportion of M1 of BALF macrophages in patients with pulmonary sarcoidosis was increased (P<0.05), and the proportion of M2 was reduced (P<0.05). In general, detection of changes in peripheral blood mononuclear cell subpopulations and BALF macrophage subpopulations in patients with pulmonary sarcoidosis has certain clinical significance for the treatment.

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Introduction

Sarcoidosis is an idiopathic multisystem granulomatous disease involving multiple organ systems, most often involving the lungs and thoracic lymph nodes, but can also affect any organ, including the eyes, skin, central nervous system and heart (1). Pulmonary involvement occurs in almost all patients with sarcoidosis, and approximately 30-50% of patients present with respiratory symptoms such as dry cough and chest tightness, especially in patients with significant pulmonary parenchymal or intrabronchial disease on imaging findings. When extrapulmonary organs are involved, such as skin, the clinical manifestations are pigmentation, rash, erythema nodosum, etc. Involving the eye can show visual impairment, conjunctivitis. The histological characteristics of pulmonary sarcoidosis are the presence of non-caseous necrotizing granulomas mainly distributed along the pleura, interlobular septa and bronchovascular bundles (2). To observe at the early stage of chronic lymphocytic infiltrates pneumonia, the further development of the type will appear the cheese necrotizing granulomatous, reduce the alveolitis, development to the chronic phase, lymphocyte infiltration disappeared, granuloma has collagen and hyaline changes, a large number of fibroblast proliferation and fibrosis. Chronic respiratory diseases such as pulmonary fibrosis, pulmonary hypertension and respiratory failure after pulmonary involvement are the primary causes of death in patients with sarcoidosis, which usually suggest poor prognosis (3, 4).

The relationship between sarcoidosis and genetic and environmental risk factors makes it impossible to

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generalize the epidemiology of sarcoidosis worldwide. The prevalence and incidence of sarcoidosis were correlated with age, sex, race and region. According to the results of national epidemiological studies, the prevalence of sarcoidosis varies from country to country. In Sweden, there are about 160 cases of sarcoidosis per 100,000 people (5), in Greece, there are about 5.89 cases per 100,000 people, and in Hokkaido, Japan, the prevalence is lower, about 3.7 cases per 100,000 people (6). The overall prevalence in the United States was 59-60.1 cases / 100,000, 141 cases / 100,000 in African Americans, and only 49.8 cases / 100,000 in whites (7). The prevalence and incidence of sarcoidosis vary greatly, possibly because most patients have a good prognosis and do not need any treatment, while about 25-40% of sarcoidosis will become chronic persistence or progression or deterioration, which will bring serious medical burden. Studies have shown that the progression of sarcoidosis depends on whether granulomatous inflammation can be alleviated (8).

The etiology of sarcoidosis is unknown, but studies have suggested that it is caused by interaction with environmental antigens or genetic susceptibility to antigens. In addition, microbial and environmental antigens (organic and inorganic) have also been reported to be associated with sarcoidosis etiology, but no clear link has been established (9, 10). A range of different genetic factors may be involved in different cases of sarcoidosis. And each factor or a group of factors may be associated with susceptibility of sarcoidosis patients with different groups, such as in Germany, the crowd found the genes associated with sarcoidosis BTNL2, later in the United States has also been found in white is associated with sarcoidosis, whereas in African-American found genetic susceptibility may also be associated with the different clinical manifestations of sarcoidosis (11).

By definition, the diagnosis of sarcoidosis requires the exclusion of other similar or granulomatous diseases. There is no specific diagnostic method for sarcoidosis, and its diagnosis depends on the compatibility of histological and imaging features under the condition of consistent clinical manifestations, as well as the exclusion of other causes (12). Bilateral symmetric lymphadenopathy is an important basis for the diagnosis of pulmonary sarcoidosis, which is different from malignant tumor and tuberculosis (13). For pulmonary sarcoidosis, the diagnostic rate of endobronchial ultrasound-guided aspiration via bronchial needle (ebus-tbna) is close to 75% (14, 15). Higher diagnostic rates can be achieved by combining Ebus-TBNA with endobronchial mucosal biopsy and transbronchial lung biopsy (16). The routine biomarker of pulmonary sarcoidosis is the serum angiotensin-converting enzyme level, which has poor diagnostic sensitivity, only about 41% (17). Bargagli E and Chen E S showed that serum amyloid A (SAA), as A potential biomarker, was elevated in patients' serum, but with low specificity (18, 19).

Studies on biomarkers in bronchoalveolar lavage fluid (BAL) and serum have also been reported for exploring diagnostic markers for pulmonary sarcoidosis. Bronchoalveolar lavage fluid (BALF) can obtain the alveolar surface lining fluid, so the examination of its contents is helpful to the diagnosis, treatment, prognosis and pathogenesis of pulmonary diseases. BALF is designed to inject normal saline into the bronchoalveoli through bronchoscopy and aspirate the fluid on the surface of the bronchoalveoli to diagnose pulmonary diseases, evaluate efficacy and prognosis by examining cellular components, soluble substances and infectious pathogens. It is suitable for the diagnosis of lung infection, especially the pathogen diagnosis of opportunistic lung infection in immune-impaired patients, and the diagnosis of unknown lung shadow, suspected lung infection or identification with other diseases. In this study, mononuclear phagocyte subsets in blood and bronchoalveolar lavage fluid of patients with pulmonary sarcoidosis were detected and their clinical significance was discussed.

Materials and methods

Research Objects

Research objects were collected from September 2019 to January 2021 hospitalized patients with pulmonary sarcoidosis, a total of 52 cases as a study group, and the 52 healthy cases as “control group a” (peripheral blood mononuclear cells in the control group), 47 cases of collecting line bronchoscopy chronic cough without pulmonary nodules patients as “control group b” control group).

Inclusion criteria of patients in study group: 1) Diagnostic criteria of pulmonary nodules (2); 2) Clinical symptoms: cough, sputum and blood in

sputum; 3) Chest radiographs showed a few half-films in the upper lobe of one or both lungs; 4) No treatment before admission; 5) Good communication skills, high cooperation and compliance; 6) Voluntarily participate in the study and sign the informed consent. This study has been approved by the medical Ethics Committee of our hospital. Exclusion criteria: 1) Recent cold, lung infection and other diseases; 2) Cannot tolerate bronchoscopy; 3) Obese patients (obesity affects monocyte differentiation); 4) Pregnant and lactating women; 5) Mental illness and other cognitive and communication disorders.

Control group A inclusion criteria: 1) Healthy, no smoking history, a normal chest X-ray and electrocardiogram; 2) Voluntarily participate in the study and sign the informed consent. Exclusion criteria: 1) Recent cold, lung infection and other diseases; 2) Obese patients (obesity affects monocyte differentiation); 3) Mental illness and other cognitive and communication disorders.

Control group B inclusion criteria: 1) healthy, no smoking history, cough of unknown cause for more than 8 weeks, chest X-ray and ecg examination normal; 2) No abnormality was found under bronchoscopy; 3) Normal lung function examination; 4) Voluntarily participate in the study and sign the informed consent. Exclusion criteria: 1) Recent cold, lung infection and other diseases; 2) Cannot tolerate bronchoscopy; 3) Mental illness and other cognitive and communication disorders.

Collection and treatment of peripheral blood samples

After admission, 5mL of fasting peripheral venous blood was collected, and heparin was anticoagulated and sent to the laboratory.

BALF collection and processing

BALF was collected through an organ cannula. The lung segment affected by the lesion was selected as the lavage site, and the operation was carried out according to the Clinical Application Guide (Plan) of fiberoptic bronchoscopy. Via tracheal biopsy hole will be 150 ml sterile physiological salt water temperature of 37℃ 3 times by bronchoscopy mirror insert shipped bronchoalveolar lavage catheter soft inside, and then with a syringe or pressure suction slowly recovery, after treated with silicone oil in the container, the container with ice around surrounded, BALF after double sterile gauze filter, in 4℃ under the condition of cooling for 10 min, Send it to the lab.

After each recovery, the injected liquid is mixed together for the test. Considering that the first recovered liquid is often mixed with endobronchial components, in order to prevent interference, the first recovered liquid can be separated from other recovered liquids for inspection, and the recovery rate exceeds 40% is considered as successful recovery.

Flow cytometry detection of peripheral blood mononuclear cell subsets

Flow cytometry detection of peripheral blood mononuclear cell subsets were to take 600 μL EP tube, add 2 μL CD45-FITC, 3 μL CD14-PE, 2 μL CD16-PE-CY5 monoclonal antibody to detection tube, add 2 μL CD45-FITC, 3 μL CD14-PE, 2 μL MGG G1-PE-CY5 monoclonal antibody to each tube, add 30 μL intravenous whole blood to each tube, mix well with vortex meter, and incubate at room temperature under dark conditions for 15 min. The erythrocytes were dissolved, and 600 μL of erythrocyte lysate was added to flow test tube. The mixture was mixed by a vortex analyzer, and the mixture was placed at room temperature for 10 min under dark conditions. Flow-count fluorescent microspheres were taken out and mixed by vortex for 10 s. 30 μL fluorescent microspheres were added into each sample tube by reverse sampling method and mixed by vortex meter.

Cytomics FC500 flow cytometer was used for monocyte phenotype analysis and FlowJo software was used for analysis.

Flow cytometry was used to detect BALF macrophage subsets

10 mL of collected BALF was filtered through a 300-mesh metal mesh to remove impurities and sputum, centrifuged at 300 g for 5 min, and the supernatant was discarded. Wash with 0.5% FBS phosphate buffer twice, take 200μL BALF, add 20μL fluorescent antibody, incubate at room temperature with dark for 20 min, centrifuge at 300 g for 5 min, discard the supernatant. 2 mL 10% hemolysin was added and incubated at room temperature in dark for 10 min, centrifuged at 200 g for 5 min, the supernatant

was discarded, phosphate buffer was added at 300 g for 5 min, the supernatant was discarded, and cells were suspended with 0.5 mL phosphate buffer.

Cytomics FC500 flow cytometer was used for phenotype analysis of macrophages and FlowJo software was used for analysis.

Treatment methods

The patients with pulmonary sarcoidosis were treated in accordance with the relevant standards in the 2018 edition of Chinese Expert Consensus on the Diagnosis and Treatment of Pulmonary Nodules. Two months after treatment, peripheral blood samples and BALF have collected again, and monocyte subsets and macrophages were detected respectively.

Statistical Methods

SPSS 22.0 statistical software was used. The dose data were expressed as X ± S. T-test was used for comparison between groups, and P < 0.05 was considered statistically significant.

Results and discussion

General data of peripheral blood mononuclear cells research subjects

Comparison of gender, age, body mass index (BMI) and other general data between patients in the study group and healthy people in peripheral blood monocyte control group A was shown in Table 1, with no statistical significance (P > 0.05), indicating comparability.

Table 1. General data of peripheral blood mononuclear research subjects

	Control group B (n=47)	Study group (n=52)	P
Gender			＞0.05
Male	23（44.23%）	21（40.38%）	
Female	29（55.77%）	31（59.62%）	
Age	37.65±7.26	37.24±6.19	＞0.05
BMI/kg/m^2	22.36±3.59	22.05±2.86	＞0.05

The proportion of monocyte subsets in pulmonary sarcoidosis patients and healthy people

Monocyte CD14 and CD16 expression levels were divided into three subpopulations: CD14++CD16-, CD14++CD16+, and CD14+CD16++. As shown in Figure 1, the proportion of CD14++CD16- subgroup in the study group was significantly lower than that in control group A (74.21± 4.10% vs 84.32± 4.76%, P < 0.05). The proportion of CD14++CD16+ subgroup in the study group was significantly higher than that in control group A (7.42±4.08 % vs 3.95±1.94 %, P < 0.05). The proportion of CD14+CD16+ subgroup in the study group was higher than that in the control group, but the difference was not statistically significant (7.84±1.59% vs 8.43±1.66%, P > 0.05).

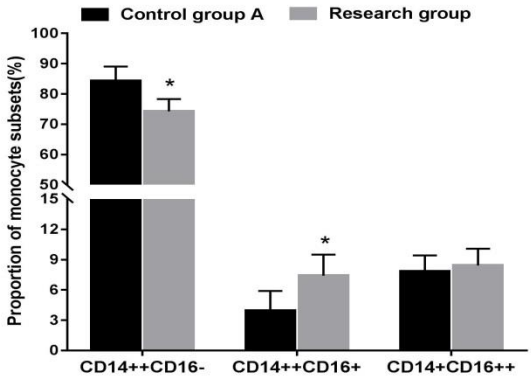

Figure 1. Proportional results of monocyte subsets. Note: Compared with control group A, *P < 0.05

General information of BALF macrophage research subjects

Comparison of gender, age, body mass index and other general data of non-pulmonary sarcoidosis patients between the study group and BALF macrophage control group B was shown in Table 2, with no statistically significant differences (P > 0.05), indicating comparability.

Table 2. General data of BALF macrophages research subjects

	Control group B (n=47)	Study group (n=52)	P
Gender			＞0.05
Male	20（42.55%）	21（40.38%）	
Female	27（57.45%）	31（59.62%）	
Age	37.43±8.15	37.24±6.19	＞0.05
BMI/kg/m^2	21.89±3.64	22.05±2.86	＞0.05

Proportion of macrophage subsets in patients with pulmonary sarcoidosis and patients without pulmonary nodules

The proportion of BALF macrophage subsets in patients in the study group and control group B was shown in Figure 2. The proportion of M1 in patients

in the study group was significantly lower than that in control group B without pulmonary nodules (24.32± 11.36% vs 47.03± 13.86%, P < 0.05). The proportion of M2 in the study group was significantly higher than that in control group B (75.40±10.23 % vs 52.53±12.01 %, P < 0.05).

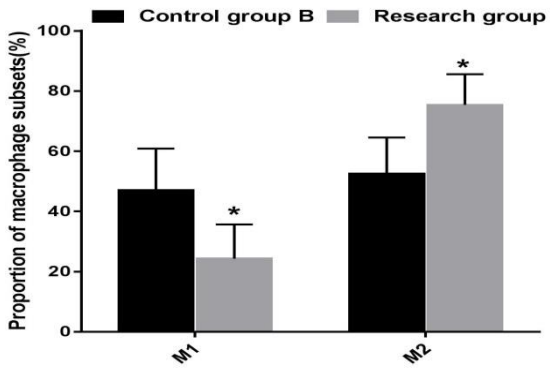

Figure 2. Proportional results of macrophage subsets. Note: Compared with control group B, *P < 0.05

The proportion of monocyte subsets in peripheral blood of pulmonary sarcoidosis patients before and after treatment

The proportion of peripheral blood monocyte subsets in patients with pulmonary sarcoidosis before and after 2-month treatment is shown in Figure 3. Compared with before treatment, the proportion of CD14++CD16- subsets in patients with pulmonary sarcoidosis after treatment is significantly increased (P < 0.05), and the proportion of CD14++CD16+ subsets is significantly decreased (P < 0.05). The proportion of the CD14+CD16+ subgroup decreased, and the difference was not statistically significant (P > 0.05).

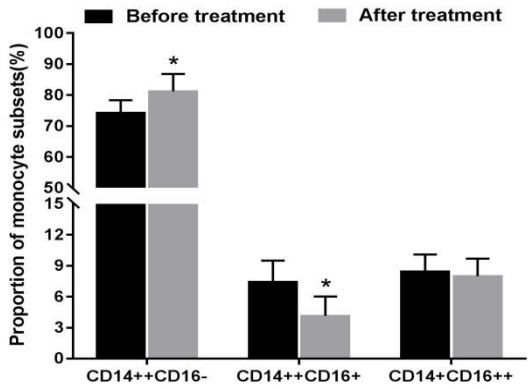

Figure 3. Proportional results of monocyte subsets before and after treatment. Note: compared with before treatment, *P < 0.05

The proportion of BALF macrophage subsets in pulmonary sarcoidosis patients before and after treatment

Figure 4 shows the proportion of BALF macrophage subsets in patients with pulmonary sarcoidosis before and after 2-month treatment. Compared with before treatment, the proportion of M1 was significantly increased (P < 0.05) and M2 was significantly decreased (P < 0.05) in patients with pulmonary sarcoidosis.

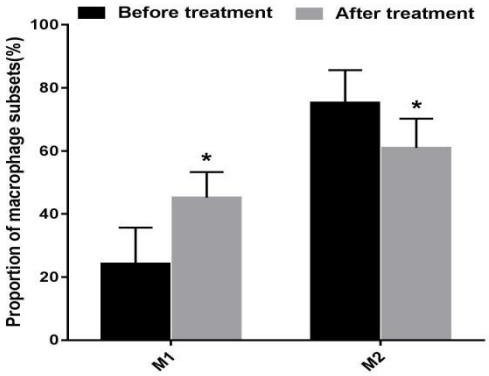

Figure 4. The proportion of macrophage subsets before and after treatment. Note: compared with before treatment, *P < 0.05

Pulmonary sarcoidosis is mainly found in European and American countries, with a relatively low incidence in China. However, pulmonary nodules may damage either one or multiple organs, causing serious multiple injuries to patients' health. At the early stage of the disease, there will be a large number of alveolitis infiltrated by macrophages and monocytes, which depletes the alveoli (20, 21).

Bronchoalveolar lavage (BALF) is a useful diagnostic adjunctive, and a 15% to 25% increase in BALF lymphocyte count supports granulomatous disease (including sarcoidosis, but also seen in accelerated silicosis), whereas a count of > 50% is more suggestive of hypersensitive pneumonic cell nonspecific interstitial pneumonia (22). Alveolar macrophages, resident mononuclear phagocytes of the lung, are currently considered to play a key role in the pathogenesis of diseases by inducing replication and functional activation of lung T lymphocytes and stimulating lung fibroblasts to produce collagen (23). Mononuclear phagocytes originate from myeloid cells

derived from bone marrow and then circulate in the blood as monocytes during a steady-state and inflammation and fill tissue as macrophages (24). The mononuclear phagocyte system consists of circulating monocytes and tissue-resident macrophages that play a role in acute lung injury and fibrosis. Macrophages can be divided into alveolar macrophages and interstitial macrophages according to the anatomical position of the lung. Among them, alveolar macrophages have the function of clearing particles and microorganisms in the alveolar cavity, and interstitial macrophages have the function of inhibiting inflammatory response, fibrosis and antigen presentation, and play an important role in the innate immune response of the lung (25, 26). Activation states of macrophages include M1 polarization and M2 polarization, M1 polarization is related to Th1 immune response, and M2 polarization is related to Th2 immune response. M1-like macrophages respond first after lung tissue injury and then are replaced by M2-like macrophages that contribute to tissue repair and fibrosis (27).

Flow cytometry has been widely used to detect BALF at home and abroad in order to understand the characteristics and activity of early lung lesions and diagnose lung diseases. In this study, flow cytometry was used to detect peripheral blood monocyte subsets and BALF macrophage subsets in patients with pulmonary sarcoidosis. The results of peripheral blood monocyte subsets in healthy people were used as control group A, and the results of BALF macrophage subsets in patients with non-pulmonary sarcoidosis cough were used as control group B. The proportion of CD14++CD16- subgroup in patients with pulmonary sarcoidosis was significantly lower than that in healthy subjects (78.21±7.1 % vs 84.32±4.76 %, $P < 0.05$). The proportion of CD14++CD16+ subgroup in patients with pulmonary sarcoidosis was significantly higher than that in healthy subjects (7.42±4.08 % vs 3.95±1.94 %, $P < 0.05$). The proportion of CD14+CD16+ subgroup in pulmonary sarcoidosis patients was higher than that in the control group, but the difference was not statistically significant ($P > 0.05$), indicating that the proportion of CD14++CD16- subgroup and CD14++CD16+ subgroup in peripheral blood of pulmonary sarcoidosis patients were significantly increased. After 2 months of treatment, the proportion of CD14++CD16- subgroup in peripheral blood monocytes of patients with pulmonary sarcoidosis was significantly increased ($P < 0.05$), the proportion of CD14++CD16+ subgroup was significantly decreased ($P < 0.05$), and the proportion of CD14+CD16+ subgroup was decreased. However, the difference was not statistically significant ($P > 0.05$), and the proportion of monocyte subsets gradually tended to change in the proportion of healthy people, indicating that treatment intervention changed the proportion of CD14++CD16- and CD14++CD16+ in peripheral blood of patients with pulmonary sarcoidosis. Studies have shown that CD16+ monocytes show an inflammatory phenotype and can cause the secretion of pro-inflammatory factors such as TGF-α, and CD16+ monocytes increase in peripheral blood when the body is inflamed (28, 29). Pulmonary sarcoidosis is a chronic disease characterized by monocyte alveolitis, granuloma, and interstitial fibrosis in some patients (30). Data from this study confirmed that CD16+ monocytes increased in peripheral blood of patients with pulmonary sarcoidosis and decreased after treatment, indicating that treatment effectively reduced inflammatory response in patients with pulmonary sarcoidosis. BALF macrophage detection results showed that the proportion of M1 in patients with pulmonary sarcoidosis was significantly lower than that in patients without pulmonary nodules (24.32± 11.36% vs 47.03± 13.86%, $P < 0.05$). The proportion of M2 in patients with pulmonary sarcoidosis was significantly higher than that in patients without pulmonary nodules (75.40± 10.23% vs 52.53±12.01 %, $P < 0.05$), indicating that M1 was significantly decreased and M2 was significantly increased in BALF of patients with pulmonary sarcoidosis. After treatment, the proportion of M1 in BALF macrophages in patients with pulmonary sarcoidosis was significantly increased ($P < 0.05$) and M2 was significantly decreased ($P < 0.05$), indicating that treatment intervention also changed the proportion of M1 and M2 in BALF macrophages in patients with pulmonary sarcoidosis. It is currently believed that M1 macrophages are the first to respond in the early stage of inflammation, playing an antibacterial and anti-inflammatory role, and M2 macrophages gradually increase in the late stage of inflammation. The data from this study showed an inflammatory response in patients with pulmonary

sarcoidosis, which was consistent with monocyte test results. This is consistent with the results of Misharin et al. (26), that M2 increases in alveoli after acute lung injury because acute lung injury can induce rapid expansion and infiltration of Ly6Chi monocytes in lung tissues (31).

Lepzien et al. (32) conducted an in-depth phenotypic, functional and transcriptomic analysis of MNP subsets in blood and bronchoalveolar lavage fluid (BALF) from 108 patients with pulmonary sarcoidosis and 30 healthy controls. Patients were followed for clinical progression and assessed the function of the MNP subsets at diagnosis and their association with 2-year disease outcome. Results showed an increase in monocytes/monocyte-derived cells in the blood and BALF of patients with sarcoidosis compared with healthy controls. Longitudinal follow-up was also conducted. In patients with longitudinal follow-up, in the absence of exogenous stimulation, the frequency of TNF production by monocytes/monocyte-derived cells in BALF at diagnosis was increased. BALF monocytes/monocyte-derived cells that produce TNF were most frequently present in patients with sarcoidosis compared with macrophages at diagnosis, indicating that pulmonary monocytes/monocyte-derived cells are highly inflammatory and can be used as predictors of disease outcome in patients with sarcoidosis.

In summary, this study investigated the changes in the proportion of peripheral monocyte subsets and BALF macrophage subsets in patients with pulmonary sarcoidosis and compared the proportion of peripheral monocyte subsets and BALF macrophage subsets before and after treatment. It was found that changes in peripheral blood mononuclear cell subsets and BALF macrophage subsets have certain clinical significance for the treatment of pulmonary sarcoidosis, but the sample size of this study is small, and further observation of large sample clinical studies is still needed.

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Interest conflict

The authors declare no conflict of interest.

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