

Th1/Th2/Th17-related cytokines in the bronchoalveolar lavage fluid of patients with sarcoidosis: association with smoking

Tomasz Urbankowski¹, Grażyna Hoser², Joanna Domagała-Kulawik³

¹ The Alveolus Student Interest Group, Department of Internal Diseases, Pneumology and Allergology, Medical University of Warsaw, Warszawa, Poland

² Laboratory of Flow Cytometry, Department of Clinical Cytology, Medical Center of Postgraduate Education, Warszawa, Poland

³ Department of Internal Diseases, Pneumology and Allergology, Medical University of Warsaw, Warszawa, Poland

KEY WORDS

bronchoalveolar lavage, cytokines, sarcoidosis, smoking

ABSTRACT

INTRODUCTION Sarcoidosis is a multiorgan granulomatous disease of unknown etiology. The predominance of Th1-related cytokines is observed in the bronchoalveolar fluid (BALF) in pulmonary sarcoidosis. Recently, Th17 cells have been postulated to be involved in the pathogenesis of sarcoidosis. Sarcoidosis is more prevalent in nonsmokers than in smokers. The exact effect of smoking on granulomatous inflammation in this disease remains unclear.

OBJECTIVES The aim of the study was to evaluate the Th1/Th2/Th17-related cytokine concentration in the BALF of patients with pulmonary sarcoidosis in relation to smoking status.

PATIENTS AND METHODS The study included 74 patients with confirmed pulmonary sarcoidosis. Data on smoking status were available for 61 patients (26 ever-smokers, 35 never-smokers; mean 11 ± 9.1 pack-years in smokers). The concentrations of interleukin (IL) 17A (IL-17A), IL-10, IL-6, IL-4, and IL-2 as well as interferon γ (IFN- γ) and tumor necrosis factor α (TNF- α) were measured in BALF supernatants using a flow cytometry method – the Cytometric Bead Array.

RESULTS The median concentration of IL-6, IFN- γ , and IL-17A (2.19 pg/ml, 1.28 pg/ml, and 6.08 pg/ml, respectively) did not differ significantly between smokers and nonsmokers. TNF- α , IL-10, IL-4 and IL-2 levels were below the detection limit in most patients. We observed a significant correlation between IFN- γ concentration and the number of macrophages in BALF ($r = 0.66$, $P < 0.05$) and between IL-17A and IL-6 levels ($r = 0.94$, $P < 0.05$).

CONCLUSIONS We confirmed a significant role of IL-6, IFN- γ , and IL-17A in the local inflammatory response in sarcoidosis. However, the interpretation of the limited number of cytokine measurements should be made with caution. Further studies are needed to explain the effect of smoking on the pathomechanism of inflammation in sarcoidosis.

INTRODUCTION Sarcoidosis is a multiorgan granulomatous disease of unknown etiology. The annual incidence of sarcoidosis in Poland is approximately 10 per 100,000. It most commonly affects people at the age of 20 to 40 years.¹ The characteristic feature of sarcoidosis is formation of noncaseating granulomas in different organs. The most frequent localization of the lesions are lungs and thoracic lymph nodes, which are affected in over 90% of the cases. Other organs that can be affected include peripheral lymph nodes, the heart, liver, skin, eyes, and the nervous and musculoskeletal systems.²

The formation of granulomas is induced by an unknown antigen, which is presented by antigen-presenting cells to naive CD4⁺ T cells (Th0). Th0 cells become activated and differentiate to Th1 cells under the postulated influence of dendritic cells. Th1 cells secrete cytokines (mainly interleukin [IL] 2 [IL-2] and interferon γ [IFN- γ]) and enhance the production of tumor necrosis factor α (TNF- α) by macrophages. Thus, the local immune response is stimulated, including migration and proliferation of T cells and mononuclear cells.^{3,4}

Correspondence to:

Tomasz Urbankowski, Katedra i Klinika Chorób Wewnętrznych, Pneumologii i Alergologii, Warszawski Uniwersytet Medyczny, ul. Banacha 1a, 02-097 Warszawa, Poland, phone: +48-22-599-25-62, fax: +48-22-599-15-60, e-mail: tomasz.urbankowski@gmail.com
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The above changes are reflected in local Th1/Th2 imbalance with the shift towards Th1-related cytokine expression. The predominance of Th1-related cytokines (e.g., IL-2, IFN- γ , and TNF- α) over Th2-related cytokines (e.g., IL-4, IL-5, and IL-10) is observed in the bronchoalveolar lavage fluid (BALF) of patients with pulmonary sarcoidosis.⁵

TNF- α , a cytokine derived mainly from macrophages, monocytes, and Th1 cells, plays a pivotal role in granulomatous inflammation.^{4,6} Monoclonal anti-TNF- α antibodies are currently a novel treatment strategy for patients with severe course of sarcoidosis.³ IL-6 is another predominantly macrophage/monocyte-derived cytokine that also plays a role in the pathogenesis of sarcoidosis.⁷

Recently, Th17 cells have been described as the third, distinct from Th1 and Th2, CD4⁺ cell subset.⁸⁻¹⁰ Th17 cells are characterized by the release of IL-17 and other cytokines that have been postulated to participate in the pathogenesis of sarcoidosis, beside the Th1-related response.^{11,12}

It is well established that sarcoidosis is more prevalent in nonsmokers than in smokers.^{2,13-16} However, the exact effect of smoking on granulomatous inflammation in sarcoidosis remains unclear. Smoking contributes to the deterioration of host defense and seems to augment immunosuppression.¹⁷ However, the effect of smoking on immunity interacts with other factors, for example, drugs, infection, and exposure to environmental pollution. Smoking causes migration of alveolar macrophages into the airway lumen. A 2- to 3-fold increase in macrophage number in the BALF of smokers is observed compared with nonsmokers. The function of alveolar macrophages is impaired by cigarette smoking, including antigen-presenting activity and phagocytic capacity. Also, the secretion of proinflammatory cytokines (including IL-6 and TNF- α) by alveolar macrophages was reported to be decreased in smokers.¹⁷

An increased proportion of cytotoxic T cells and a shift in CD4⁺/CD8⁺ ratio towards CD8⁺ cells are observed in the BALF of smokers.¹⁷ The effect of smoking on pulmonary Th1/Th2 balance remains controversial.

The aim of the study was to evaluate the Th1/Th2/Th17-related cytokine concentration in the BALF of patients with pulmonary sarcoidosis in relation to smoking status.

PATIENTS AND METHODS **Patients** The study included 74 patients with confirmed pulmonary sarcoidosis (33 women and 41 men; mean age, 41.9 \pm 12.1 years; range, 25–74 years).

Patients underwent clinical examination as well as laboratory tests and investigations, including chest radiograph, high-resolution computed tomography, spirometry, plethysmography, diffusion lung capacity for carbon monoxide measurement, and bronchoscopy with bronchoalveolar lavage (BAL). Angiotensin converting enzyme activity was measured using the Cushman and Cheung's method¹⁸ as modified by Lieberman.¹⁹ All patients provided written informed

consent before each diagnostic procedure. All patients provided written informed consent before each diagnostic procedure.

Diagnosis of sarcoidosis was established according to the American Thoracic Society / European Respiratory Society / World Association of Sarcoidosis recommendations.² Patients were diagnosed with sarcoidosis either on the basis of the presence of noncaseating granulomas in a transbronchial needle aspiration sample or on the basis of clinicoradiological picture compatible with BAL cytological examination.

Data on smoking status were available for 61 patients – there were 26 ever-smokers and 35 never-smokers. The group of smokers comprised 10 current smokers and 16 ex-smokers. The mean number of pack-years in smokers was 11 \pm 9.1.

Bronchoalveolar lavage All patients underwent flexible bronchoscopy with BAL performed according to recommendations.²⁰ Bronchoscopy was performed under local anesthesia (2% lidocaine) and was preceded by premedication with the use of midazolam and atropine. BAL was performed after macroscopic evaluation of the airways. Bronchoscope was wedged in the segmental or subsegmental bronchus. Then, 200 ml of saline (in aliquots of 20–50 ml) at the temperature of 37°C was administered.

Filtration of BALF and measurement of its volume were followed by centrifugation for 10 minutes (400 \times g). The supernatants were collected, frozen, and stored at –70°C. The Bürker chamber was used to measure total cell count. Differential cell count was determined on May-Grunwald-Giemsa-stained slides with the use of light microscopy.

Measurement of cytokine concentrations IL-17A, IFN- γ , TNF- α , IL-10, IL-6, IL-4, and IL-2 levels were measured by flow cytometry in previously stored BALF supernatants using Cytometric Bead Array (BD Biosciences, United States). In this method, the range of antibody-coated beads reacts with the investigated material (e.g., serum, BALF). Beads are coated with antibodies against different substances. Each substance-specific bead population has a distinct infrared fluorescence intensity. After being conjugated with material sample, beads are mixed with phycoerythrin-conjugated antibodies against different cytokines. Then, in a quantitative analysis, fluorescence intensity of beads is compared with standard patterns. Cytokine concentration is calculated on the basis of fluorescence intensity measurement with the use of a software designed for the CBA analysis.

Statistical analysis The Statistica 10.0 software (StatSoft, United States) was used for statistical analysis. Differences between groups were analyzed using the Mann-Whitney test. Correlations were analyzed with the Spearman rank correlation coefficient. A $P < 0.05$ was considered statistically significant.

TABLE 1 Demographic data, pulmonary function tests, and laboratory tests in nonsmokers and smokers with sarcoidosis

	Nonsmokers	Smokers
subjects, n	35	26
sex, % of women	51.4	23.1
mean age, y	42.2 ± 11.3	37.8 ± 11.6
FVC, l	4.4 ± 1.5	4.8 ± 1.0
FVC, % predicted	117.1 ± 27.8 ^a	98.1 ± 15.9 ^a
FEV ₁ , l	3.5 ± 1.3	3.7 ± 0.8
FEV ₁ , % predicted	103.9 ± 21.9 ^a	90.5 ± 16.4 ^a
TLC, % predicted	106.0 ± 15.3	102.0 ± 7.0
DL _{CO} , ml/min/mmHg	26.1 ± 7.5	26.5 ± 7.5
Ca, mmol/l	2.1 ± 0.4	2.2 ± 0.4

^a $P < 0.05$

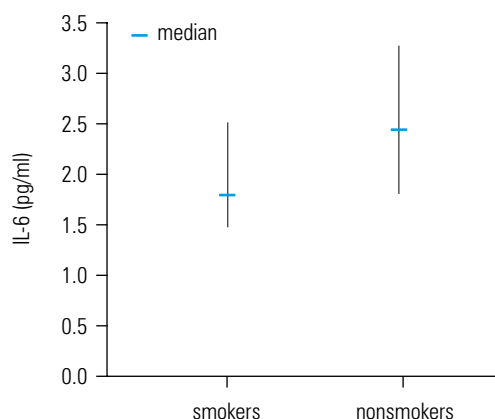
Abbreviations: Ca – calcium, DL_{CO} – diffusing lung capacity for carbon monoxide, FEV₁ – forced expiratory volume in 1 second, FVC – forced vital capacity, TLC – total lung capacity

TABLE 2 Bronchoalveolar lavage fluid cellular profile in nonsmokers and smokers with sarcoidosis

	Nonsmokers	Smokers
total cell count, × 10 ⁶	14.4 ± 7.7	21.1 ± 14.7
macrophages, %	52.0 ± 17.0 ^a	64.0 ± 23.0 ^a
macrophages, × 10 ⁶	7.7 ± 4.7 ^a	12.5 ± 10.8 ^a
lymphocytes, %	39.2 ± 18.5 ^a	25.6 ± 19.5 ^a
lymphocytes, × 10 ⁶	6.2 ± 7.0	5.8 ± 4.3
neutrophils, %	6.8 ± 3.9	6.8 ± 9.0
neutrophils, × 10 ⁶	1.0 ± 0.6	1.8 ± 3.2
eosinophils, %	2.3 ± 1.9	3.5 ± 3.8
eosinophils, × 10 ⁶	0.3 ± 0.3	0.7 ± 0.9

^a $P < 0.05$

FIGURE 1 Interleukin 6 levels in nonsmokers and smokers: median and interquartile range



RESULTS Demographic data and the results of pulmonary function tests and laboratory tests in smoking and nonsmoking patients with sarcoidosis are presented in **TABLE 1**. Smokers had a significantly lower predicted forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV₁) than nonsmokers (98 ± 16.0% vs. 117.1 ± 27.8% and 90.5 ± 16.4% vs. 103.9 ± 21.9%, respectively).

BALF cellular profile in smokers and nonsmokers with sarcoidosis is presented in **TABLE 2**. Smokers had a significantly higher number and percentage of macrophages than nonsmokers (12.5 ± 10.8 × 10⁶ vs. 7.7 ± 4.7 × 10⁶ and 64.0 ± 23.0% vs. 52.0 ± 17.0%, respectively). Furthermore, the percentage of lymphocytes was significantly lower in smokers than in nonsmokers (25.6 ± 19.5% vs. 39.2 ± 18.5%).

The median concentration of IL-6 in the BALF of patients with sarcoidosis was 2.19 pg/ml (interquartile range [IQR], 1.50–3.07) and did not differ significantly between smokers and nonsmokers. **FIGURE 1** shows the comparison of IL-6 concentrations between smokers and nonsmokers. The median concentration of IFN-γ was 1.28 pg/ml (IQR, 1.16–1.29), and there was no significant difference between smokers and nonsmokers. The median concentration of IL-17A was 6.08 pg/ml (IQR, 4.11–7.16). The concentrations of TNF-α, IL-10, IL-4, and IL-2 were below the detection limit in the majority of cases. There were no significant differences in cytokine concentrations between patients in stages I, II, and III of sarcoidosis.

We observed a significant correlation between IFN-γ concentration and the number of macrophages in BALF ($r = 0.66$, $P < 0.05$). There was also a significant correlation between IL-6 concentration and the number of neutrophils ($r = 0.28$, $P < 0.05$; **FIGURE 2**). Furthermore, there was a significant correlation between IL-17A and IL-6 concentrations ($r = 0.94$, $P < 0.05$). There were no significant correlations between the level of any cytokine and serum angiotensin-converting enzyme activity.

In smokers, the concentration of IL-6 correlated significantly with the number and percentage of eosinophils ($r = 0.51$, $P < 0.05$ and $r = 0.44$, $P < 0.05$, respectively). Moreover, there was a negative correlation between IL-6 concentrations and the percentage of predicted FVC and FEV₁ in smokers ($r = -0.69$, $P < 0.05$ and $r = -0.56$, $P < 0.05$, respectively).

DISCUSSION In our study, we focused on the measurement of cytokine concentrations in the BALF of patients with sarcoidosis. We aimed to find a correlation between cytokine profile (Th1/Th2 balance) and smoking status. The central concept of our study was based on the state-of-the-art description of Th1-mediated response and its predominance in the pathogenesis of sarcoidosis.^{11,12} Previously, we observed that cigarette smoking significantly affected the cellular composition of BALF. However, in that study, the clinical picture and results of the pulmonary function test did not differ significantly between smokers and nonsmokers.²¹ A possible association between smoking status and the etiology and pathogenesis of sarcoidosis has not been fully investigated yet. The analysis of this mechanism may help recognize the elements of immune response to environmental agents in the pathogenesis of sarcoidosis.

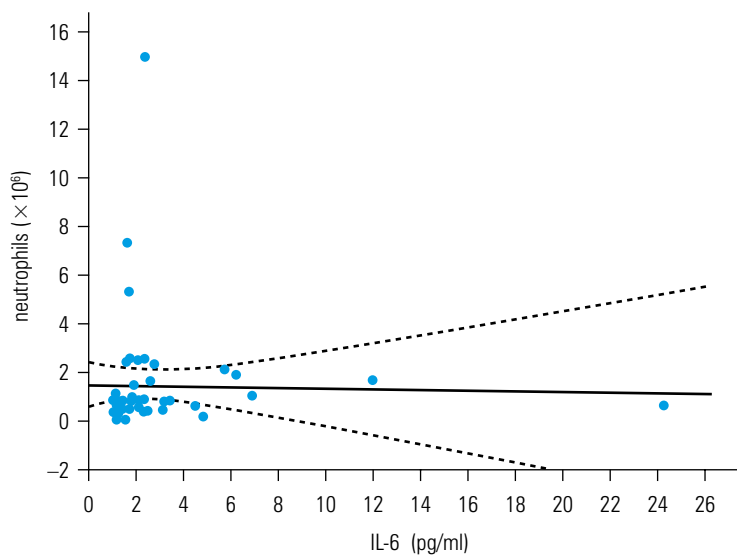


FIGURE 2 Scatter plot showing a correlation between the number of neutrophils and interleukin 6 levels in the bronchoalveolar lavage fluid

Unfortunately, we did not achieve the expected results. However, we confirmed the role of IL-6, IFN- γ , and IL-17 in the local inflammatory response in the lungs of patients with sarcoidosis.

We investigated lung environment using BAL, which is a well-established method in the diagnosis and evaluation of sarcoidosis.^{5,22} We applied a novel technology for the measurement of cytokine concentration in BALF – the Cytometric Bead Array. This method is still rarely used and has not been well established for the common measurement of IL concentration in the BALF. In our study, the measurements were conducted for the first time and we used the samples of BALF supernatants which were nonconcentrated. Therefore, the limitation of our study is the lack of representative results in many cases. We measured the concentration of a wide range of cytokines; however, only IL-6, IFN- γ , and IL-17A levels could be detected with the use of our method. Nevertheless, those cytokines are important in the pathogenesis of sarcoidosis. In our study, concentrations of cytokines were similar to those reported by other investigators,^{23–25} who used standardized enzyme-linked immunosorbent assay in their studies.

The effect of smoking on the immune system is pleiotropic but significant changes are observed in smokers with the smoking history of more than 20 pack-years (Chądryński R.; personal communication). Therefore, a small number of current smokers in our study group (16% of the patients) and a short smoking history may explain the lack of significant differences between smokers and nonsmokers. However, it should be noted that we carefully selected 2 groups of patients, ever- and never-smokers, and our study group is among the largest reported in the related literature. We observed the effect of smoking on the cellular composition of BALF, and a significant predominance of macrophages was noted in smokers. In our patients, IFN- γ concentrations in BALF could be detected, and we observed a correlation between the number of

macrophages and IFN- γ . IFN- γ is a Th1-related cytokine, which is known to participate in granuloma formation.^{2,7,26} A shift in Th1/Th2 balance toward Th1-related response seems to be favorable in sarcoidosis, while Th2-related immune response is associated with the occurrence of lung fibrosis.⁴ We hypothesize that smoking, by stimulating macrophages and favoring Th1-related response, protects from the development of structural abnormalities in the lungs, but this needs to be confirmed in further studies.

IL-6 is a pluripotent proinflammatory cytokine, which was reported to play a role in the local immune response in sarcoidosis.^{2,7} As previously stated, IL-6 concentrations in our study were comparable with the results reported by other authors.^{22,23} Interestingly, we found a correlation between IL-6 concentration and the number of eosinophils among smokers. The role of eosinophil influx into the lung milieu in smokers was reported.¹⁷ On the other hand, an increased percentage of eosinophils in the BALF of patients with sarcoidosis was observed (Domagała-Kulawik J.; unpublished data). We can speculate that eosinophil influx and IL-6 expression in smokers is rather a nonspecific reaction.

In our study, the results of pulmonary function tests were within the normal range with slightly lower FEV₁ in smokers. We observed that increased IL-6 concentration correlated with the deterioration of lung function in this group of patients.

Our results confirm a number of important interactions of cytokines. IL-6 is known to promote differentiation of Th cell to Th17 cells.^{27,28} We observed a correlation between IL-17 and IL-6 concentrations, which is consistent with well-known pathways.²⁸ Recently, Facco et al.¹¹ reported increased pulmonary infiltration by Th17 cells and concluded that the predominance of this subpopulation promotes progression of the disease. Many studies confirmed the role of regulatory cells, which have been postulated to interact with Th17 cells, in autoimmune diseases, including sarcoidosis.^{11,28,29} Although our results are consistent with the current knowledge, a limited number of cytokine measurements should be interpreted with caution.

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Cytokiny o profilu Th1/Th2/Th17 w płynie z płukania oskrzelowo-pęcherzykowego u chorych na sarkoidozę – związek z paleniem papierosów

Tomasz Urbankowski¹, Grażyna Hoser², Joanna Domagała-Kulawik³

1 Studenckie Koło Naukowe „Alveolus”, Katedra i Klinika Chorób Wewnętrznych, Pneumonologii i Alergologii, Warszawski Uniwersytet Medyczny, Warszawa

2 Pracownia Cytometrii Przepływowej, Centrum Medyczne Kształcenia Podyplomowego, Warszawa

3 Katedra i Klinika Chorób Wewnętrznych, Pneumonologii i Alergologii, Warszawski Uniwersytet Medyczny, Warszawa

SŁOWA KLUCZOWE

cytokiny, palenie tytoniu, płukanie oskrzelowo-pęcherzykowe, sarkoidoza

STRESZCZENIE

WPROWADZENIE Sarkoidoza jest wielonarządową chorobą ziarniniakową o nieznanej etiologii. W płucnej postaci sarkoidozy obserwuje się przewagę cytokin pochodzących od limfocytów Th1 w płynie z płukania oskrzelowo-pęcherzykowego (*bronchoalveolar lavage fluid* – BALF). Od niedawna postuluje się udział limfocytów Th17 w patogenezie sarkoidozy. Sarkoidoza występuje częściej wśród osób niepalących niż wśród palaczy. Dokładny mechanizm wpływu palenia na zapalenie ziarniniakowe w tej chorobie pozostaje niejasny.

CELE Celem badania była ocena stężeń cytokin związanych z różnicowaniem limfocytów Th1, Th2 i Th17 w BALF uzyskanym od pacjentów chorych na sarkoidozę oraz ocena zależności stężeń od palenia tytoniu podanego w wywiadzie.

PACJENCI I METODY Badaniem objęto 74 pacjentów z potwierdzoną postacią płucną sarkoidozy. Dane dotyczące palenia były znane u 61 chorych (26 kiedykolwiek palących, 35 niepalących; średnia 11 ± 9.1 paczolat w grupie palących). Stężenia interleukiny (IL) 17A (IL-17A), IL-10, IL-6, IL-4 i IL-2 oraz interferonu γ (IFN- γ) i czynnika martwicy guza α (*tumor necrosis factor- α* – TNF- α) mierzono w supernatantach z BALF przy użyciu cytometrii przepływowej – metodą Cytometric Bead Array.

WYNIKI Mediany stężeń IL-6, IFN- γ i IL-17A (odpowiednio 2,19 pg/ml, 1,28 pg/ml i 6,08 pg/ml) nie różniły się znacząco między grupą palących i niepalących. Stężenia TNF- α , IL-10, IL-4 i IL-2 były u większości chorych poniżej progu wykrywalności. Stwierdziliśmy istotną korelację między stężeniem IFN- γ i liczbą makrofagów w BALF ($r = 0,66$; $p < 0,05$) oraz między stężeniem IL-17A i IL-6 ($r = 0,94$; $p < 0,05$).

WNIOSKI Potwierdziliśmy istotną rolę IL-6, IFN- γ oraz IL-17A w miejscowej odpowiedzi zapalnej w sarkoidozie. Jednakże interpretacja ograniczonej liczby pomiarów stężeń cytokin powinna być ostrożna. Wpływ palenia tytoniu na patomechanizm reakcji zapalnej w sarkoidozie wymaga dalszych badań.

Adres do korespondencji:
Tomasz Urbankowski, Katedra i Klinika Chorób Wewnętrznych, Pneumonologii i Alergologii, Warszawski Uniwersytet Medyczny, ul. Banacha 1a, 02-097 Warszawa, tel.: 22-599-25-62, faks: 22-599-15-60, Praca wpłynęła: 01.04.2012. Przyjęta do druku: 02.04.2012. Nie zgłoszono sprzeczności interesów.
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