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Association between M1 and M2 macrophages in bronchoalveolar lavage fluid and tobacco smoking in patients with sarcoidosis

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KEY WORDS

alveolar macrophages, bronchoalveolar lavage, CD163, CD40, sarcoidosis

ABSTRACT

INTRODUCTION Sarcoidosis is a granulomatous disease, which most often affects the lungs. The role of alveolar macrophages (AMs) in granuloma formation in sarcoidosis has been established. Recently, 2 macrophage populations have been described: M1 and M2. In our styudy, we focused on the effect of tobacco smoking on sarcoidosis. The number of AMs in the lungs of smokers is significantly increased; therefore, it is interesting to study the effect of smoking on AM polarization in sarcoidosis.

OBJECTIVES The aim of the present study was to identify M1 and M2 macrophages in bronchoalveolar lavage (BAL) fluid from patients with sarcoidosis and assess the effect of smoking on these cells.

PATIENTS AND METHODS The study included 36 patients with confirmed sarcoidosis (18 smokers and 18 nonsmokers). Macrophage populations in BAL fluid were assessed by immunocytochemistry using anti-CD40 and anti-CD163 antibodies (for M1 and M2, respectively). The BAL fluid concentration of interleukin 10 (IL-10) was measured using an enzyme-linked immunosorbent assay.

RESULTS We identified 3 populations of macrophages stained with anti-CD40 and anti-CD163 antibodies: small strongly positive cells, large weakly positive cells, and negative cells. The median proportions of these macrophages were 61%, 35%, and 2%, respectively, for CD40, and 55.5%, 35%, and 5%, respectively, for CD163; the proportions did not differ significantly between smokers and nonsmokers. Only the proportion of CD163-negative cells was significantly lower in smokers compared with nonsmokers (3.3% vs. 9.5%, $P < 0.05$). The IL-10 concentration in BAL fluid was below the detection limit.

CONCLUSIONS We did not observe any association between tobacco smoking and macrophage polarization in patients with sarcoidosis. However, our study revealed 2 populations of CD40- and CD163-positive cells, which may indicate that macrophages are involved in granuloma formation and provide direction for future research.

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INTRODUCTION Sarcoidosis is a multiorgan granulomatous disease, which most often affects the lungs. It is estimated that from 1 to 40 per 100,000 inhabitants are affected by sarcoidosis.[1] The disease is observed all over the world irrespective of the race, sex, and age.[2,3] The annual incidence of sarcoidosis in Poland is approximately 10 per 100,000 people.[4] The etiology of sarcoidosis is unknown but, in a recent study, Dubaniewicz et al.[5] highlighted the possible role of macrophages. Sarcoidosis is characterized by the accumulation of T lymphocytes, mononuclear phagocytes, and granulomas in affected tissues.[6]

Alveolar macrophages (AMs) are the main lung cell population that serves as the first line of cellular defense against pollutants owing to their antigen presenting function and phagocytic properties.[7] Granulomas are closed, centrally organized collections of macrophages and epithelial cells surrounded by lymphocytes. Macrophages differentiate into epithelioid cells (after chronic cytokine stimulation), lose some phagocytic capacity,

and form multinucleated giant cells.[3] Thus, AMs play an important role in the pathogenesis of sarcoidosis. Macrophages form the core of granuloma in sarcoidosis and are involved in macrophagic alveolitis.[8] Activated macrophages produce tumor necrosis factor α (TNF-α) and other cytokines (e.g., interleukin [IL] 6) that promote granuloma formation.[9,10]

Macrophages are morphologically similar and, until recently, they have been considered as a uniform cell population but are now divided into 2 phenotypes, M1 and M2, which differ phenotypically and, almost certainly, functionally.[11] M2 macrophages have a suppressive and immunoregulatory function and promote angiogenesis, wound healing, and IL-10 and IL-1ra release.[12] M1 macrophages are activated by lipopolysaccharide and interferon γ (INF-γ), while M2, by IL-4, IL-13, IL-10, and immune complexes.[13,14] The macrophage phenotype is characterized by high capacity to produce cytokines and present surface antigens. This different polarization of macrophages is also detected by their phenotyping using immunocytochemistry staining with anti-CD40 and anti-CD163 antibodies for M1 and anti-CD206 antibodies for M2 macrophages.[15,16]

There are some data on the role of IL-10 in the regulation of immune reaction. IL-10 is an anti-immflamatory cytokine capable of limiting inflammatory responses and promoting the development of regulatory T cells.[17] Moreover, elevated IL-10 levels play an important role in macrophage polarization.[18,19] We aimed to analyze the involvement of IL-10 in macrophage polarization in patients with sarcoidosis and compare its concentrations between smokers and nonsmokers.

It is well known that sarcoidosis is more prevalent among nonsmokers.[20,21] However, the postulated protective role of smoking on inflammation in sarcoidosis remains unclear. Smoking contributes to the deterioration of host defense and might lead to increased immunosuppression. Moreover, it causes migration of AMs into the airway lumen and an increase in the total number of cells in bronchoalveolar lavage (BAL) fluid.[7] This is due to a significant increase in the number of macrophages in BAL fluid in smokers, which is approximately 2- to 3-fold higher compared with that in nonsmokers.[22] The function of AMs is impaired by cigarette smoking, including antigen-presenting activity and phagocytic capacity.[7]

The aim of the present study was to identify M1 and M2 populations of macrophages in BAL fluid from patients with sarcoidosis in relation to the smoking status.

PATIENTS AND METHODS

Patients The study included 36 patients with confirmed pulmonary sarcoidosis (25 men and 11 women; mean age, 37.9 ±9.9 years; range, 26–74 years). Data on the smoking status were available for all patients. There were 18 ever-smokers (5 current smokers and 13 ex-smokers; mean pack/years, 8.3; range, 1–23) and 18 never-smokers. There were patients with stages I, II, and III of sarcoidosis. All patients underwent clinical examination and bronchoscopy with bronchoalveolar lavage and laboratory tests. All patients provided written informed consent before each diagnostic procedure. Diagnosis of sarcoidosis was established according to the recommendations of the American Thoracic Society / European Respiratory Society / World Association of Sarcoidosis,[23] either on the basis of the presence of noncaseating granulomas in a transbronchial needle aspiration sample or bronchial biopsy or on the basis of clinical and radiological manifestations compatible with cytological examination of the BAL fluid.[24]

Bronchoalveolar lavage Bronchoscopy was performed under local anesthesia (lidocaine, 2%) and was preceded by premedication with midazolam and atropine. The bronchoscope was wedged in the segmental or subsegmental bronchus, and 200 ml of saline (in the aliquots of 20–50 ml) at a temperature of 37°C were administered. Next, BAL fluid was processed according to the recommendations.[22] The fluid was filtered through nylon gauze, the volume was measured, and the fluid was centrifuged for 10 min (400 × *g*). The Bürker chamber was used to measure the total cell count. Cell pellets were suspended in 300 ml of phosphate buffered saline and gently spread on slides. For each sample, 6 slides with BAL fluid were prepared. Light microscopy was used to determine differential cell count on 2 slides using May–Grünwald–Giemsa staining. Four slides were air-dried, frozen immediately, and stored at a temperature of 20°C.

Immunocytochemical staining and analysis M1 and M2 macrophage subpopulations were analyzed using targeted anti-CD40 and anti-CD163 antibodies (AbD Serotec, Raleigh, North Carolina, United States), respectively. Previously frozen slides of the BAL fluid were thawed and dried for 1 h at room temperature, and then fixed in aceton at a temperature of –20°C for 10 min and dried. The endogenous peroxidase activity was blocked by incubation with hydrogen peroxide (3%) for 10 min. To reduce unspecific binding of the antibody, the slides were incubated for 30 min with normal horse blocking serum (ImmPRESS™, 2.5%; Vector Laboratories Inc, Burlingame, California, United States). Then, mouse primary antibodies were applied, CD40 on one slide and CD163 on the other for each patient, diluted 1:75 in normal horse serum (ImmPRESS™, 2.5%; Vector). The incubation time for primary antibodies was 60 min. Detection was done with horse antimouse ImmPRESS™ Detection Kit (Vector) and 3.3'-diaminobenzidine as a chromogen (Dako, Glostrup, Denmark). All slides were counterstained with Mayer's hematoxylin, dehydrated, and mounted.

Light microscopy was used to count 300 macrophages on each slide under high magnification (×1000), and the proportions of CD40- and CD163-positive cells were calculated. To avoid

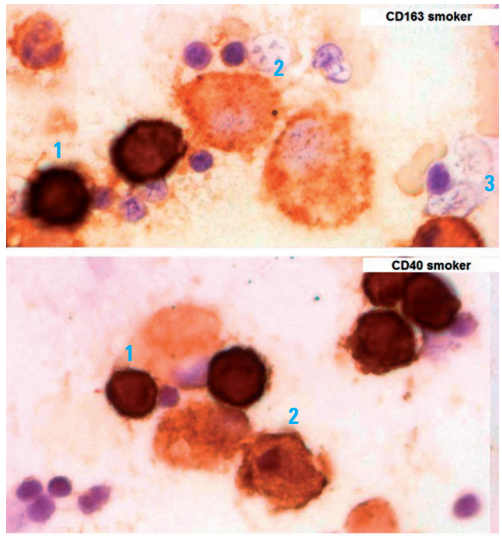

FIGURE 1 Three populations of macrophages stained with CD40 and CD163: 1, small strongly positive cells (+++); 2, large weakly positive cells (+); and 3, negative cells (–)

TABLE 1 Proportions of macrophages stained for CD40 and CD163 in smokers and nonsmokers with sarcoidosis

	Variable	Smokers	Nonsmokers
CD40	(+++), %	61.0 (57.0–68.0)	61.0 (51.0–71.0)
	(+), %	35.0 (30.0–40.0)	34.5 (25.0–42.0)
	(–), %	2.0 (1.0–3.0)	3.0 (1.0–5.0)
CD163	(+++), %	56.5 (49.0–43.0)	55.0 (43.0–66.0)
	(+), %	40.5 (33.0–47.0)	31.0 (24.0–40.0)
	(–), %	3.3 (2.0–4.0)	9.5 (6.0–11.4)[a]

Data are presented as median (interquartile range).

a $P < 0.05$

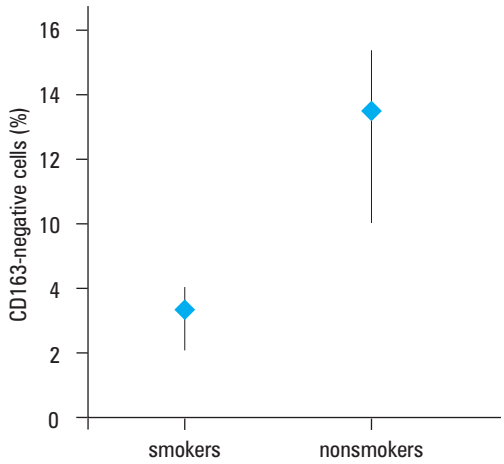

FIGURE 2 Median levels of CD163-negative cells in smokers and nonsmokers

observer bias, the slides were analyzed by 2 independent pathologists.

Enzyme-linked immunosorbent assay The IL-10 concentration in BAL fluid was measured using the commercially available kit, Quantikine ELISA Human IL-10 Immunoassay (R&D System, Minneapolis, Minnesota, United States) according to the manufacturer's protocol. The absorbance was measured at 450 nm using a Microplate reader (model StatFox-2100; Awarness Technology Inc, Palm City, Florida, United States). The lower limit of detection for IL-10 was 3.9 pg/ml.

Statistical analysis The Statistica 10.0 software (StatSoft, Poland) was used for statistical analysis. A P value of less than 0.05 was considered statistically significant. Differences between the groups were analyzed using the Mann–Whitney test. Relations between quantitative variables were analyzed using Spearman correlation coefficients.

RESULTS We found 3 populations of macrophages stained with anti-CD40 and CD163 antibodies: small strongly positive cells (+++), large weakly positive cells (+), and negative cells (–) (FIGURE 1). The median proportions of CD40-positive macrophages in the whole group were as follows: (+++), 61% (interquartile range, 54.5–69.0); (+), 35% (27.5–40.5); and (–), 2% (1.0–4.0). The median proportions of CD163 macrophages in BAL fluid were as follows: (+++), 55.5% (48.0–63.0); (+), 35% (27.0–47.0); and (–), 5% (3.0–10.0).

We observed the highest proportion of small cells staining positively (+++) for CD40 in all patients with sarcoidosis. The median proportions of large cells staining positively (+) for CD40 and CD163 did not differ significantly between smokers and nonsmokers (TABLE 1). The median proportion of CD163-negative cells was significantly lower in smokers compared with nonsmokers (3.3 vs. 9.5, $P = 0.0002$; FIGURE 2). We observed a significantly higher number of CD40- and CD163-positive macrophages in BAL fluid from smokers compared with that from nonsmokers (FIGURE 3).

The IL-10 concentration in BAL fluid was below the detection limit. We observed a significant positive correlation between the proportion of CD40- and CD163-negative cells in nonsmokers and no correlation in smokers: the Spearman correlation coefficients were 0.66 ($P < 0.003$) and 0.33 ($P < 0.19$), respectively (FIGURE 4). Moreover, a negative correlation was observed between small cells (+++) and large cells (+) staining positively for CD163 both in smokers and nonsmokers ($r = -0.96$ and $r = -0.77$, respectively; $P < 0.05$). A similar correlation was obseved between small cells (+++) and large cells (+) staining positively for CD40 ($r = -0.86$ and $r = -0.91$, respectively; $P < 0.05$). We observed a negative correlation between CD163- and CD40-negative macrophages and pack/years in smokers ($r = -0.59$ and $r = -0.55$, respectively; $P < 0.05$).

DISCUSSION In our study, we focused on the analysis of macrophage populations in BAL fluid from patients with sarcoidosis. We aimed to find a correlation between macrophage subpopulations (M1 and M2) and the smoking status. The most striking finding of our study was that staining with anti-CD40 and anti-CD163 antibodies allowed to identify 3 different populations of AMs in BAL fluid. Our results confirmed the presence

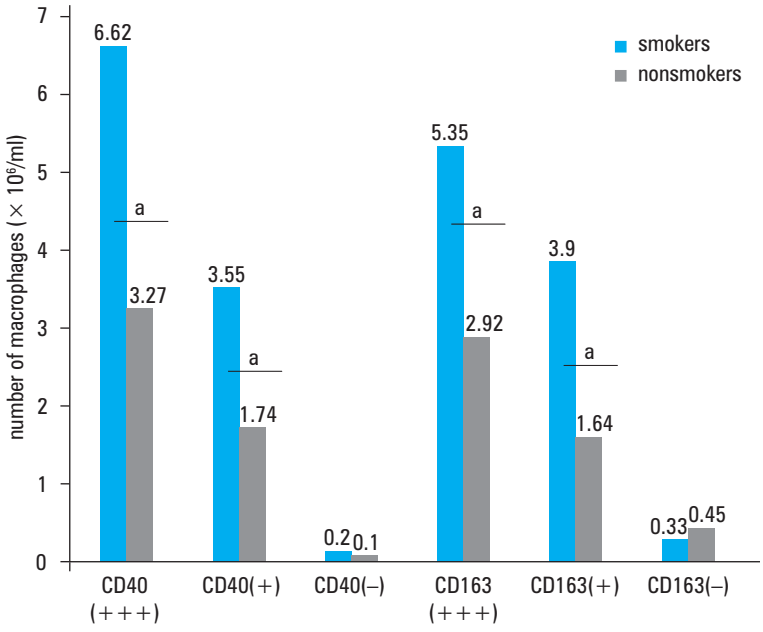

FIGURE 3 Number of CD40- and CD163--positive and negative macrophages in bronchoalveolar lavage fluid from smokers and nonsmokers with sarcoidosis
a $P < 0.05$
For explanation of (+++), (+), and (–), see **FIGURE 1**

of double-stained macrophages with complex expression of CD40 and CD163.

The limitation of our study was that we only characterized the phenotype and not the function of M1/M2 cells.

Previously, we observed that cigarette smoking significantly affected the cellular composition of the BAL fluid. Smokers had a higher number of macrophages and a lower percentage of lymphocytes compared with nonsmokers.[7,25] In this study, we also confirmed that smokers had a higher total number of macrophages compared with nonsmokers (15.2 and 8.7 × 10^6/ml, respectively; $P = 0.03$).

A possible association between the smoking status and the course of sarcoidosis has not been fully clarified so far. A number of studies indicated that sarcoidosis is more common in nonsmokers.[20,21,26,27] Tobacco smoke significantly affects the local and systemic immune status and should be considered when investigating the mechanisms of lung diseases. Cigarette smoking increases the number of macrophages that are functionally impaired, have reduced phagocytic activity,[28] produce lower levels of proinflammatory cytokines,[29,30] and their metabolic activity is weak.[31] In our previous studies, we investigated the hypothesis that cigarette smoking suppressed granuloma formation in sarcoidosis.[25,32,33]

In this study, we investigated the lung environment using BAL, which is a well-established method in the diagnosis and evaluation of sarcoidosis.[24,34] We applied immunocytochemistry staining to assess AM populations in BAL fluid, which is still not a well-established method for the determination of M1 and M2 macrophages in BAL fluid. While CD163 and CD206 are well-known markers of M2 macrophages,[12,15] it is quite challenging to find a specific marker for the M1 subpopulation. In this study, we used an anti-CD40 antibody, macrophage surface receptor involved in proinflammatory pathways, to detect the M1 population.[35]

Currently, little is known about the effects of smoking on macrophage polarization. Kunz et al.[16] evaluated the effect of the smoking status on CD163-positive macrophages in BAL fluid and induced sputum from patients with chronic obstructive pulmonary disease. They showed that the percentage of CD163-positive macrophages in BAL fluid was similar to that in our study and was higher compared with that in induced sputum.

The profile of macrophages in sarcoidosis is unknown. Wikén et al.[36] observed no differences in M1 and M2 polarization of AMs between patients with sarcoidosis and healthy controls. However, they assessed only the expression of cytokines without estimating M1/M2 markers by immunocytochemistry. Prokop et al.[37] examined whether the polarization of macrophages contributed to myofibrosis in neuromuscular sarcoidosis. Their results confirmed the important role of M2 cell with the expression of CD206, CD301, and arginase 1 in fibrosis, but they focused on tissue rather than alveolar macrophages.[37] Our results showed a significantly higher number of all subtypes of macrophages in smokers with the predominance of CD40-positive cells. However, we noticed a significantly lower proportion of macrophages without the expression of CD163 in BAL fluid from smokers and correlation of this percentage with the number of pack/years. CD163 is a transmembrane protein with an anti--inflammatory effect.[38] It is downregulated by proinflammatory cytokines of confirmed activity in sarcoidosis (such as TNF-α and INF-γ). The alteration of AM polarization toward a higher expression of CD163 in smokers is in line with our previous finding of the possible suppressive action of tobacco smoke on inflammation in sarcoidosis.[33] Interestingly, we found a significant correlation of CD40-negative cells with CD163--negative cells only in nonsmokers. The lack of such a correlation in smoking patients seems to reflect some degree of disruption of homeostasis.

In our study, we found 2 different positive populations and 1 negative population of both CD40 and CD163 cells on all slides. To our knowledge, such findings have not been reported before. Moreover, our calculation indicated double staining and the presence of CD40- and CD163-positive AMs. This finding possibly reflects the different stages of macrophage polarization and shift from the M1 to M2 subtype. The precise assessment of the cells should be confirmed by an accurate method, for example, confocal microscopy. In our preliminary study, we confirmed the specificity of CD40 for M1 and CD163 for M2 by cultured macrophage immunostaining (data not shown). We noticed that anti-CD40 antibodies were strongly specific for the M1 subpopulation and consisted of large cells with weak positive staining. The M2 subpopulation consisted of small cells with strong positive staining with

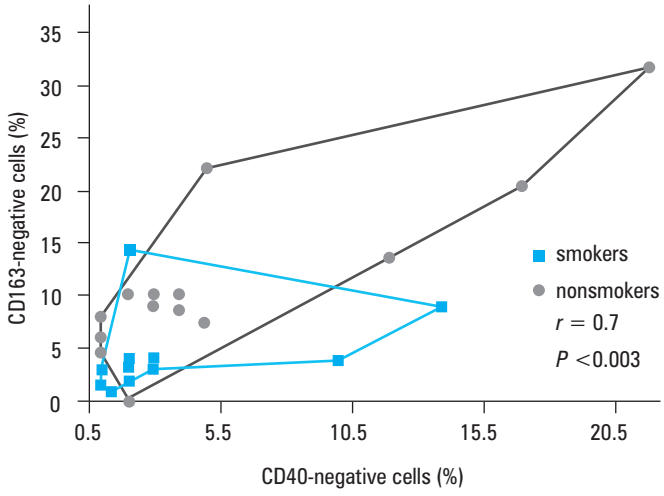

FIGURE 4 Correlations between the proportion of CD163- and CD40--negative macrophages in smokers and nonsmokers

anti-CD163 antibodies. However, further research is needed to confirm those preliminary results.

In conclusion, we observed no evidence for macrophage polarization among patients with sarcoidosis in relation to the smoking status. However, our study revealed 2 populations of CD40- and CD163-positive macrophages in BAL fluid from patients with sarcoidosis with a high proportion of small cells and strong double staining of M1 and M2 markers on macrophages. This may indicate the involvement of these cells in granuloma formation and provides direction for future research. Further studies are needed to explain the effect of smoking and macrophage polarization on the pathomechanism of inflammation in sarcoidosis using the most accurate method.

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ARTYKUŁ ORYGINALNY – KONKURS STUDENCKI 2014*

Makrofagi M1 i M2 w popłuczynach oskrzelowo-pęcherzykowych u chorych na sarkoidozę a palenie tytoniu

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SŁOWA KLUCZOWE

CD40, CD163, makrofagi pęcherzyków płucnych, płukanie oskrzelowo-pęcherzykowe, sarkoidoza

STRESZCZENIE

WPROWADZENIE Sarkoidoza jest chorobą ziarniniakową o najczęstszej lokalizacji w płucach. Znany jest udział makrofagów pęcherzyków płucnych (*alveolar macrophages* – AMs) w tworzeniu ziarniniaków w sarkoidozie. Ostatnio opisano dwie populacje makrofagów: M1 i M2. W naszym badaniu skupiliśmy się na ocenie wpływu palenia tytoniu na sarkoidozę. Liczba AMs w płucach palaczy jest znacznie większa, interesujące jest więc zbadanie wpływu palenia na polaryzację AM w sarkoidozie.

CELE Celem badania była identyfikacja makrofagów M1 i M2 w popłuczynach oskrzelowo-pęcherzykowych (*bronchoalveolar lavage fluid* – BALF) chorych na sarkoidozę i ocena wpływu palenia tytoniu na te komórki.

PACJENCI I METODY Badaniem objęto 36 chorych z potwierdzoną sarkoidozą (18 palących i 18 niepalących). Oceniono populacje makrofagów M1 i M2 w BALF z wykorzystaniem techniki immunocytochemicznej z przeciwciałami przeciwko CD40 i CD163 (odpowiednio dla M1 i M2). Stężenie interleukiny 10 (IL-10) w BALF badano za pomocą testu ELISA.

WYNIKI Stwierdzono występowanie trzech populacji makrofagów wykorzystując znakowanie przeciwciałami przeciwko CD40 i CD163: małe komórki silnie barwiące się, duże komórki słabo barwiące się i komórki niewybarwione. Mediany odsetka populacji tych makrofagów wynosiły odpowiednio: dla CD40 61%, 35%, 2%; dla CD163: 55,5%, 35%, 5% oraz nie różniły się istotnie między pacjentami palącymi i niepalącymi. Jedynie odsetek CD163(–) był istotnie mniejszy w grupie palących w porównaniu z grupą niepalących (3.3% *vs* 9.5%, p <0,05). Stężenie IL-10 w BALF było poniżej granicy detekcji.

WNIOSKI Nie zaobserwowano związku między paleniem papierosów a polaryzacją makrofagów u chorych na sarkoidozę. Jednak badanie ujawniło występowanie dwóch populacji komórek pozytywnie barwiących się pod wpływem CD40 i CD163, co może wskazywać na aktywność makrofagów w powstawaniu ziarniaków i stanowić kierunek dla przyszłych badań.

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