SARCOIDOSIS VASCULITIS AND DIFFUSE LUNG DISEASES 2015; 32; 305-312

Comparison of the diagnostic value of different lymphocyte subpopulations in bronchoalveolar lavage fluid in patients with biopsy proven sarcoidosis

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Abstract. *Background:* Bronchoalveolar lavage is considered a helpful tool in the diagnosis of diffuse parenchimal lung diseases such as sarcoidosis. CD4/CD8 ratio is higly specific but not sensitive to distinguish sarcoidosis and other intestitial lung diseases. We aimed to compare the diagnostic value of CD4/CD8 ratio and other lmphocyte subpopulations such as CD3+16+56, CD103+, CD4+CD103+, CD8+CD103+ in bronchoalveolar lavage to distinguish sarcoidosis and other nonsarcoidosis interstitial lung diseases. *Methods:* Using the bronchoscopy records from 2006 to 2013, we evaluated 68 patients with biopsy proven sarcoidosis and 72 patients with clinicoradiological and/or biopsy proven diffuse parenchimal lung diseases. Cut off values, sensitivity and specificity were given for aforementioned parameters. *Results:* Bronchoalveolar lavage CD4/CD8 ratio, CD4+ T lymphocyte percentage, CD4+103+, CD3+CD103-, CD8+CD103+/CD103+ ratio were significantly higher in sarcoidosis than other diffuse parenchimal lung diseases whereas CD3+103+, CD3+16+56+, CD8+, CD8+CD103+, CD8+CD103+/CD8+ were significantly lower. Best cut off value of CD4/CD8 was 1.34 with sensitivity and specificity 76.4%, 79.4% respectively. The cut off values of CD4/CD8 of >3.5 and >2.5 had specificity 95.9% and 95.3%, respectively and sensitivity 52%, 41 %, respectively. *Conclusion:* CD4/CD8 ratio is highly specific but not sensitive for sarcoidosis diagnosis. Thus, BAL flow cytometry is not diagnostic alone without appropriate clinicoradiological and/or histopathological findings. *(Sarcoidosis Vasc Diffuse Lung Dis 2015; 32: 305-312)*

Key words: sarcoidosis, bronchoalveolar lavage, CD4/CD8, CD103+

Abbreviations

ATS: American Thoracic Society
AUC: Area under ROC curve
BAL: Bronchoalveolar lavage
COP: Cyrptonized organizing pneumonia
DPLD: Diffuse paranchimal lung disease
DLCO: The diffusing capacity of lung for carbonmonoxide
DLCO/VA: The diffusing capacity divided by the alveolar volume
ERS: European Respiratory Society
FOB: Fibre optic broncoscopy
ILD: Interstitial lung disease
+LR: Positive likelihood ratio
NK: Natural killer
PAP: Pulmonary alveoalar proteinosis
ROC: Receiver operating characteristics
TCR: T-cell receptor
WASOG: World Association for Sarcoidosis and Other Granulomatous Disorders

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Introduction

Sarcoidosis is a multisystem granulomatous disorder of unknown causes (1). Its prevalence varies between 0.03–640 per 100.000 (2,3). The most commonly affected organs are lung and intrathoracic lymph nodes (4). Diagnosis of the sarcoidosis is based on histological evidence of noncaseating epithelioid cell granulomas with appropriate clinical and radiological findings (1). Transbronchial lung biopsy is the recommended procedure in most cases. Its diagnostic yield depends largely on the experience of the operator, ranging from 40% to 90% (5). Analysis of bronchoalveolar lavage (BAL) fluid for total and differential counts of inflammatory cells is considered as a standard procedure in the diagnostic work-up of patients with diffuse paranchimal lung diseases (DPLD) such as sarcoidosis and hypersensitivity pneumonia. BAL fluid was considered helpful in strengthening the diagnosis in patients with sarcoidosis in the absence of biopsy (6). The BAL CD4/CD8 ratio has been considered a useful supplement in the diagnostic workup of sarcoidosis. The BAL fluid shows an increase in lymphocytes in 90% of sarcoidosis patients at the time of diagnosis. An elevated CD4/CD8 ratio (>3.5) in BAL may confirm the diagnosis and obviate the need for confirmation by additional biopsy (7). In patients with sarcoidosis BAL CD4/CD8 ratio was reported in a wide range, median 3.8-6.1 (4, 8-10) and mean 2.92-9.05 (11-20). But CD4/CD8 ratio can invert and in elderly subjects it may normally increase Thus an elevated BAL CD4/CD8 ratio does not fully discriminate sarcoidosis from other DPLD despite its high specificity (4,21) other BAL cellular markers have been searched to help in sarcoidosis differential diagnosis. The integrin CD103 is expressed on CD4+ T-cells in BAL. Due to the high influx of circulating T-cells to the granulomata in sarcoidosis, a reduction in the fraction of BAL CD4+CD103+ T-cells has been suggested as a marker of sarcoidosis, in combination with lymphocytosis and CD4/CD8 ratio. In selected groups of patients, decreases in the fraction of CD4+ CD103+ T-cells are significantly associated with sarcoidosis (15).

In this retrospective study we aimed to determine the diagnostic value of different lymphocyte subpopulations in BAL fluid in patients with biopsy proven sarcoidosis and other DPLDs.

Methods

Between January 1st 2006 and December 31st 2013 patients who underwent fibre optic bronchoscopy (FOB) and BAL with a suspicious of DPLD were eligible for study. FOB records were investigated and only the patients with biopsy proven sarcoidosis and the patients with a final diagnosis of DPLD which was based on clinicoradiological and/or biopsy results, were enrolled the study. All patients were new diagnosed and none of them was received steroid treatment before BAL performed. FOB and BAL were performed as described below: The subjects were premedicated topically with lidocaine that was delivered via an atomizer. The bronchoscope was inserted usually transnasally and wedged to segmental or subsegmental bronchi. BAL was performed in the right middle lobe or lingula in the presence of diffuse involvement, or in the area of greatest radiological abnormality. Sterile isotonic saline was instilled in at least three 40 mL aliquots. Each aliquot was retrieved with gentle manual aspiration. Only the second aliquot was used for BAL analysis. Sarcoidosis was diagnosed as described below: A clinical and radiological pattern consistent with sarcoidosis and presence of noncaseating granulomas on endobronchial mucosa (13 patients), transbronchial lung (24 patients), transbronchial needle aspiration (3 patients), endoscopic ultrasound-guided fine needle aspiration of mediastinal or hilar nodes (7 patients), mediastinal lymph node biopsy by mediastinoscopy or video assisted thoracic surgery (11 patients), open lung biopsy (3 patients) peripheral lymph node dissection (3 patients), skin biopsy (4 patients) and exclusion of other known causes of granulomatous disease. Nonsarcoidosis DPLD patients were classified as CTD-ILD (20 patients), pneumoconiosis (14 patients, 3 slicosis, 11 coal worker's pneumoconiosis), IPF (12 patients), infection (5 patients), other ILDs (a total of 16 patients, 2 PAP, 1 COP, 2 NSIP, 1 drug related ILD, 10 unclassified) and malignancy (4 lymphoma, 1 kaposi sarcoma) (Figure 1). Patients were grouped as sarcoidosis and non sarcoidosis DPLD and variables and results of BAL fluid analysis were compared between two groups.

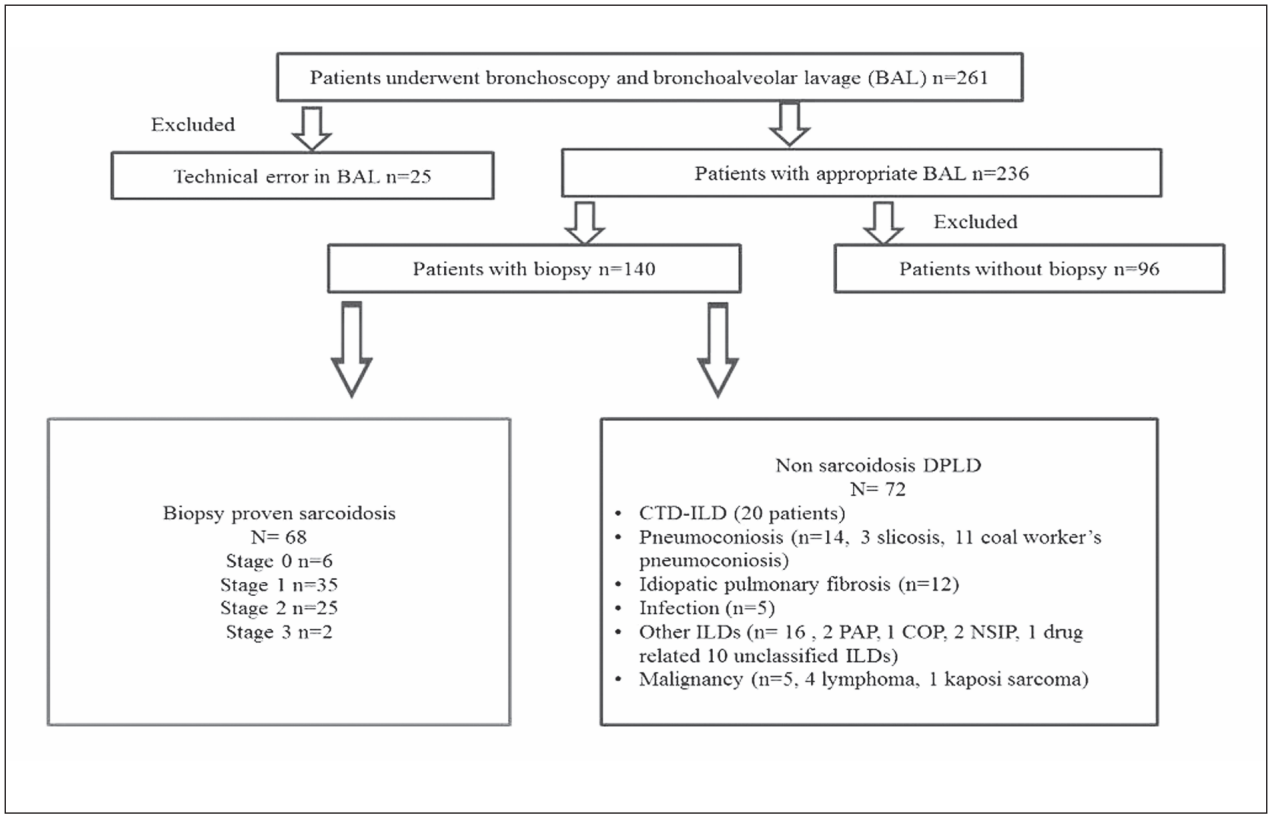

Fig. 1. Sarcoidosis was classified as: Stage 0: Chest radiography is normal (Diagnosis based on skin or lmphy node biopsy), Stage I: Bilateral hilar lymph node, Stage II: Bilateral hilar lymph node and parenchymal involvement and stage III: Only parenchymal involvement. CTD-ILD was diagnosed based on appropriate clinicoradiological findings suggested CTD and positive serology and/or biopsy, pneumoconiosis was diagnosed based on history of exposure to occupational dust, appropriate radiological findings and/or biopsy, unclassified ILDs were diagnosed if clinicoradiological findings suggested ILD and biopsy results consistent with interstitial fibrozis and/or inflammation

Flow Cytometry

BAL fluid cells were washed in phosphate-buffered saline (PBS) and resuspended at 1×10^6 cells/ml in 0.1% bovine serum albumin (BSA) in PBS and stained with the indicated antibodies: CD14-PE, CD45-FITC, CD4- PC5, CD8-PE, CD3-FITC, CD3-PE, CD103-FITC, CD19 –PE, TCR γ ò-FITC, CD16+56 PE. Isotype-matched antibodies were used as negative controls. All antibodies purchased from Beckman Coulter Company (Fullerton, CA, US). Following staining and incubation at room temperature, samples were immediately analyzed by flow cytometry (Beckman Coulter Company, FC500, Fullerton, CA, US). Data from a minimum of 10,000 cells were recorded for each tube. Standardized gating was performed without any clinical information about the individual patients.

Statistical analyse

We used SPSS ver. 19.0 software for statistical analyses. Descriptive statistics of categorical variables were given as frequency and percentage, continues variables were given as mean (±standart deviation) or median (minimum–maximum). The intergroup comparisons of frequencies and percentages were made by using the chi-square test and Fisher's exact test. Student's *t*-test or the nonparametric Mann–Whitney *U*-test was used to compare the means of variables, where appropriate. A receiver operator characteristic (ROC) curve analysis was used to evaluate the predictive performance of different BAL parameters for sarcoidosis diagnosis in order to provide the best cut-off value and the area under the ROC curve (AUC) values for sarcoidosis as well as sensitivity and specificity. The significance level was set at $p < 0.05$ for interpretations.

All authors participated in the design, conduct and analysis stages of the study. Authors had full access to data.

Results

A total of 140 patients, 68 biopsy proven sarcoidosis and 72 nonsarcoidosis DPLD, were enrolled the study. Demographic data and characteristics of patients were given in Table 1. Mean age was significantly higher in nonsarcoidosis group than sarcoidosis group. Proportion of female sex was significantly higher in sarcoidosis than nonsarcoidosis 69% vs 45%, respectively.

Median BAL CD4/CD8 ratio and lymphocyte percentage were significantly higher in sarcoidosis group than nonsarcoidosis DPLD, 2.76 vs 0.77 p= 0.000, respectively and 18.5% vs 4.85%, p=0.000, respectively. Median BAL neutrophil percentage was found significantly lower in sarcoidosis than nonsarcoidosis 9.2% vs 19.6%, p= 0.000 (Table 2).

The median percentage of BAL neutrophil, CD3+103+, CD3+16+56+, CD8+, CD8+CD103+, CD8+CD103+/CD8+ were significantly lower in sarcoidosis group than nonsarcoidosis group whereas the median percentage of BAL lymphocyte, CD4+, CD4+103+, CD3+CD103-, CD8+CD103+/CD103+ ratio were significantly higher in sarcoidosis group than nonsarcoidosis group. TCR γ delta

Table 1. Descriptive statistics of sarcoidosis and nonsarcoidosis patients

	Sarcoidosis	Nonsarcoidosis	p
Age, mean±SD	45.1±12.7	56.1±12.2	.000
Female, n (%)	47 (69)	33 (45)	0.007
Smoking status, n (%)			
Current	12 (17.6)	25 (34.7)	0.025
Never	39 (57.3)	25 (34.7)	0.003
Former	17 (25.1)	22 (31.6)	0.08
Elevated Serum ACE, n (%)	17 (25)	3(4)	0.001
Elevated liver enzym, n (%)	7 (10.2)	0	N/A
Hypercalcemia, n (%)	1 (1.4)	0	N/A
FEV1 %pred., mean±sd	91.6±21.5	88.5±19.6	0.434
FVC %pred., mean±sd	91.1±26.3	84.8±24.4	0.207
DLCO %pred., mean±sd	72.8±22.2	62.2±22.6	0.027

Data presented as mean ± standart deviation,
ACE: serum angiotensin converting enzyme, DLCO: The diffusing capacity of lung for carbonmonoxide, FEV1: Forced expiratory volume in 1 second, FVC: forced vital capacity

Table 2. Comparing BAL lymphocyte subpopulations of patients with biopsy proven sarcoidosis and nonsarcoidosis DPLD

	Sarcoidosis	Nonsarcoidosis	p
CD4/CD8	2.76 (0.46-16.14)	0.77 (0,1-8.2)	.000
Lymphocyte%	18.1 (1.1-80.2)	4.85 (0,2-66)	.000
Neutrophil %	9.2 (1.7-46)	19.6 (0.3-79)	.000
CD3+16+56+(%)	4.8 (0.1-18.1)	7.3 (0.3-42,6)	.004
CD4(%)	59.2 (18.3-91.9)	29.1 (1.8-82.9)	.000
CD8(%)	23.4 (7.1-69.0)	40.5 (8.5-84)	.000
CD16+56+(%)	3.3 (0.2-32)	4.35 (0.4-29)	0.621
TCR γ delta(%)	2.9 (0.1-39)	3.45 (0.2-15.2)	0.146
CD103+	30.1 (5.6-54.6)	38.5 (12.5-90.2)	0.059
CD3+103+(%)	22.3 (5.5-46.4)	34.9 (12.7-63.3)	0.024
CD4+CD103+(%)	11.9 (1.9-45)	6.4 (0.8-31)	0.019
CD8+CD103+(%)	10.6 (0.6-35.1)	27.8 (5.7-69.2)	.000
CD3+CD103-(%)	64.3 (29.1-89.2)	36.0 (20.7-61.0)	.000
CD4+CD103+/CD4	0.19 (0.03-0.85)	0.23 (0.04-0.8)	0.424
CD8+CD103+/CD8	0.51 (0.08-0.98)	0.71 (0.13-0.95)	0.002
CD4+CD103+/CD8+CD103+	1.11 (0.46-17.3)	0.51 (0.18-1.51)	0.046
CD4+CD103+/CD103+	0.62 (0.05-0.92)	0.14 (0.04-0.39)	0.017
CD8+CD103+/CD103+	0.23 (0.27-0.99)	0.46 (0.08-0.6)	0.005

Data presented as median (min- max)

Table 3. Cut-off values of different lymphocyte subpopulation for sarcoidosis diagnosis that were obtained from ROC curve

	Cutoff	Sensitivity%	Specificity%	AUC	+LR	P
CD4/CD8	1.34	76.4	79.4	0.844	1.34	0.0001
CD4%	39	80	80	0.851	1.25	0.0001
Lymphocyte%	9.4	85	72	0.776	1.36	0.001
CD4+CD103+%	6.75	70	59	0.69	1.21	0.018
CD3+16+56+(%)	7.6	78.9	48.5	0.648	1.54	0.0025
CD8+CD103+/CD103+	0.45	88.4	53	0.707	1.91	0.0004
CD8+CD103+/CD8	0.67	83.3	60.8	0.728	2.13	0.0002

+LR: Positive likelihood ratio, AUC: area under receiver operating characteristic curve

natural killer, CD103+, CD4+CD103+/CD4 and CD16+56+ percentages were lower in sarcoidosis but difference was not significant.

ROC curve analysis was performed and the best cut off value of CD4+/CD8+ for the sarcoidosis diagnosis was found as 1.34 with the sensitivity, specificity and AUC 76.4%,79.4% and 0.85,respectively (Table 3). We also performed ROC curve analysis for other parameters which were significantly different between groups (Figure 2), such as CD8+CD103+/CD8+, CD8+CD103+/CD103 and CD3+16+56+ for the diagnosis of sarcoidosis and best cut off values were 0.45, 0.67 and 7.6%, respectively, sensitivity 88.6%, 83.3%, 78.9% specificities 53.7%, 60.8% 48.5% and AUCs were 0.72, 0.70, 0.64, respectively (Table 3). Different cut-off values of CD4/CD8 that were obtained from ROC curves for sarcoidosis diagnosis is presented in table 4. While the cut off value of CD4/CD8 ratio increased, spesificity raised but sensitiviy decreased. A cut off value of 3.5 for CD4/CD8 was highly specific (95.9%) but not sensitive (41%) for diagnosis of sarcoidosis. We found that a cut off value of 2.5 for BAL CD4/CD8 had same specificity when compared to cut off value of 3.5 (for both 95.9%) and higher sensitivity (52%, 41% respectively) for the diagnosis of sarcoidosis. We found that the CD4/CD8 ratio was >3.5 in 42.7%, 2.5–3.5 in 8.9%, 1.0–2.5 in 32.3% and <1 in 16.1% of sarcoidosis patients (Table 5).

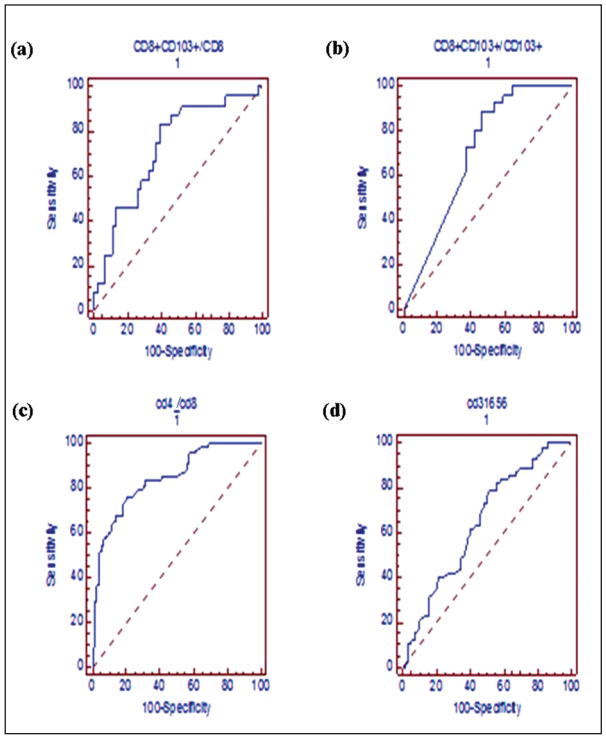

Fig. 2. AUC, sensitivity and specificity of, CD8+103+/CD8(a), CD8+CD103+/CD103 (b), CD4/CD8 (c) and CD3+16+56 (d) for sarcoidosis diagnosis that were obtained from ROC curve analysis

Table 4. Different cut-off values, sensitivity and specificity of BAL CD4/CD8 ratio for sarcoidosis diagnosis

	Cutoff	Sensitivity %	Specificity %	+LR
CD4/CD8	1,34*	76.4	79.4	1.34
	>2	61.7	89.1	5.6
	>2,5	51.4	95.9	12.5
	>3,5	41	95.9	10.2
	>4	36.7	97.2	13.4

*: best cut off value that was obtained from ROC curves, +LR: Positive likelihood ratio

Table 5. Distribution of BAL CD4/CD8 ratios of patients with biopsy-proven sarcoidosis and non sarcoidosis DPLD

CD+4/CD8	Sarcoidosis	Nonsarcoidosis
0-1	11 (16.1)	46 (63.8)
>1-2.5	22 (32.3)	23 (31.9)
>2.5-3.5	6 (8.9)	0
>3.5	29 (42.7)	3 (4.3)

Discussion

We found that BAL CD4/CD8 ratio remains the best parameter for the diagnosis of sarcoidosis with the highest specificity and sensitivity among the other BAL parameters such as CD4+, CD8+, CD103+ CD8+CD103+/CD8+, CD8+CD103+/CD103, CD3+16+56+ and lymphocyte percentage. The best cut- off value of CD4/CD8 ratio for sarcoidosis diagnosis was 1.34 with both high sensitivity and specificity. But the cut off values of CD+/CD8 ratio of 2.5 and 3.5 both had the highest specificity but low sensitivity for sarcoidosis diagnosis.

In BAL fluid, an increased number of lymphocytes, predominantly activated Th cells, can be found in 90% of sarcoidosis patients at the time of diagnosis. CD4+ Th lymphocytes usually regulate immune responses in the lungs as effector T lymphocytes (helper or inducer) (22). The infiltrate of CD4 activated T cells represents the immunological hallmark of sarcoidosis.

The CD4/CD8 ratio is increased in >50 to 60% of patients with sarcoidosis. The diagnostic value of this ratio has been debated recently because of the high variability in sarcoidosis. In about 4–10% of cases, sarcoidosis presents with a T8 lymphocytic alveolitis and a low T4/T8 ratio (23). A lymphocytosis is quite sensitive but less specific, whereas an increased BAL fluid CD4/CD8 ratio is highly specific but not sensitive for sarcoidosis (22). CD4 lymphocytosis may also be observed in other types of lung diseases. Thus the sensitivity of an elevated CD4/CD8 ratio for sarcoidosis is seldom more than 50% in the studies available in literature (21). In the present study we found that the sensitivity and specificity of BAL CD4/CD8 with a cut –off value of 1.34 is 76.4% and 79.4%, respectively.

In the literature a BAL CD4/CD8 ratio of >3.5 has a sensitivity of 52–59% and a specificity of 94–96% (24). In the present study, we found that the CD4/CD8 ratio of >3.5 was highly specific (95.9%) but not sensitive (41%) for sarcoidosis diagnosis. Also we found that the specifity of CD4/CD8 ratio for a cut off value of >2.5 had similar specifity with the cut off value >3.5 and higher sensitivity (52%, 41 % respectively). Whereas an elevated CD4/CD8 ratio would obviate biopsy in patients with a typical clinical/radiological picture for sarcoidosis but this occurs in only approximately 50% of the patients with sarcoidosis because of the low sensitivity (6). In the present study, CD4/CD8 ratio was lower than 3.5 in 57% of the patients and lower than 1.0 in 16% of the patients with biopsy proven sarcoidosis. Smilarly Kantrow et al (25) reported that the CD4/CD8 ratio was >4.0 in 42%, 2.5–4.0 in 21%, 1.0–2.5 in 26% and <1 in 12% of sarcoidosis patients. Thus sarcoidosis cannot be excluded if the CD4/CD8 ratio is normal or even decreased below 1.0.

The proportion of CD103+ T cells in the BAL within the CD4+ T-cell population increases significantly in interstitial lung diseases. The integrin CD103 is also expressed by lymphocytes in mucosal areas, such as bronchi, by some alveolar wall lymphocytes, and by bronchoalveolar fluid T cells (21, 26). However, in bronchoalveolar lavage fluid, the relative amount of CD103 expressing T cells is very different between CD4+ and CD8+ T cells. Most of the CD8+ T cells express this integrin independently of the disease of the lung (26). In contrast, the proportion of CD4+ T cells expressing CD103 is significantly higher in diseases associated with pulmonary fibrosis than in non-fibrotic diseases or healthy controls (26). In the present study we found that BAL CD103+ lymphocytes percentage was lower in sarcoidosis than nonsarcoidosis but the difference was not significant. However, we found also that CD4+CD103+ was significanty lower in sarcoidosis group than nonsarcoidosis DPLD group but CD4+ CD103+/CD4+ratio, reflecting the relative number of CD4+ T-lymphocytes that express CD103 within the total CD4+ subpopulation, was not significantly different between two groups. In contrast to our results, Caetano Mota et al. reported that the CD4+CD103+/CD4+ ratio, was associated with a better diagnostic performance (sensitivity: 81%; specificity: 78%) for a cut-off point of 0.45. They also reported that as compared to other ILD, BAL CD4+CD103+/CD4+, was also lower in sarcoidosis patients. In accordance with our results Hydalgaard et al. (15) reported that CD103+CD4+/CD4+ was not statistically significant and did not correlate with radiographic staging. When they combined cut-off levels of 0.22 and 3.8 for CD103+CD4+/CD4+ and CD4+/CD8+, respectively, the sensitivity was 42% and the specificity was 91% for sarcoidosis diagnosis.

In sarcoidosis, NKT cells have been found at reduced levels in blood and BAL fluid. The majority of NKT cells are CD4 positive, and they express an invariant T-cell receptor (TCR) (27). In the present study, we found TCR gamma delta NKT cells are lower in sarcoidosis group but difference was not significant. In contrast to our results Tondell et al. (10) reported that in the subgroup of 44 sarcoidosis and 60 nonsarcoidosis patients with BAL lymphocyte fraction above 15%, the median NKT cell fraction was lower in sarcoidosis than in non-sarcoidosis patients significantly. This difference may be due to the selection of the patients. They selected the patients with a high lymphocyte percentage.

Our study had some limitations. First the study was retrospective and patient population was relatively small. We pooled the data of all nonsarcoidosis patients in only one group and analysed as sarcodiosis and nonsarcoidosis. If we compare sarcoidosis and specific subgroups of DPLD seperately results might be different. In our clinical practice we used only second aliqouts of BAL fluid retrieved but ERS/ATS/WASOG guideline recommends to use all not only the second aliquot for flow cytometric analyse. This may have affected our results.

Conclusion

The BAL CD4/CD8 ratio seems useful and specific but not sensitive for sarcoidosis. But, BAL flow cytometry does not fully discriminate sarcoidosis from other DPLD. Sarcoidosis diagnosis should be based on appropriate clinicoradialogical and/or histopathological findings in addition to BAL cytometry. Additional investigation is required to further elucidate the role of CD3+16+56+, CD103+, CD4+CD103+ lymphocytes in sarcoidosis

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