NK cell populations in collagen vascular disease

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Abstract

Objectives

Pulmonary involvement of varying etiology is common in collagen vascular diseases (CVDs). Bronchoalveolar lavage fluid (BALF) cell differentials reveal information on the immune mechanisms involved in the CVDs. The aim of the present study was to evaluate BALF cell populations in CVD-associated ILD and to investigate possible correlation with pulmonary function.

Methods

Fifty-seven patients (26 male and 31 female, mean age ± SD: 54.68±12.18 years) with CVD-associated interstitial lung disease were studied. Patients were divided into 6 groups based on underlying CVD. The study population also included a group of 10 healthy controls. BALF was examined in all individuals. Cell density, total cell number and differential cell count were recorded. BALF lymphocyte subsets were analysed by dual flow cytometry. Pulmonary function was assessed in all patients.

Results

BALF differential cell count did not differ significantly among the different groups. Scleroderma patients showed the highest percentage of CD19 cells ($p<0.001$). The NK and NKT cell percentages were significantly higher in systemic lupus erythematosus and in Sjögren, respectively, compared to other CVDs and controls ($p=0.001$ and $p<0.001$). Also BALF neutrophil percentage correlated negatively with FVC ($r=-0.356$, $p=0.011$) and FEV1 ($r=-0.336$, $p=0.017$) and BALF NKT cell percentage correlated negatively with pO_2 ($r=-0.415$, $p=0.003$).

Conclusion

Important variations observed in BALF cell populations suggest the implication of NK and NKT cells in the pathogenesis of lung involvement in CVDs.

Key words

collagen vascular diseases (CVDs), bronchoalveolar lavage fluid (BALF)

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Introduction

Collagen vascular diseases (CVDs) include a group of heterogeneous disorders characterised by immune-mediated involvement of various organs. Pulmonary complications are common during the course of CVDs either as a result of the immune process which often leads to interstitial lung disease or because of infection or drug toxicity. Lung involvement ranges from trivial to life threatening and is a common cause of morbidity and death in CVDs (1, 2).

The assessment of a CVD-associated interstitial lung disease (ILD) is challenging, since many issues arise. Two or more clinical entities may coexist in the same patient. Another issue is subclinical alveolitis detected by sensitive diagnostic procedures such as high-resolution computed tomography (HRCT) and bronchoalveolar lavage (BAL) (3). Furthermore, the severity of symptoms in a patient with CVD-associated ILD is variable and not always correlated to pulmonary function (3).

BAL is a safe and minimally invasive procedure, used for the diagnosis of ILDs (4). Bronchoalveolar lavage fluid (BALF) cell differentials reveal information on the immune mechanisms involved in the CVDs as well as on the progression of lung involvement from inflammation to fibrosis. BALF may also detect subclinical alveolitis and provide significant information in case of alveolar haemorrhage, alveolar proteinosis, infection or drug-induced ILD (1, 3, 5). Moreover, BALF seems to have a prognostic value, since patients with lymphocytic alveolitis have been found to stabilise or improve with corticosteroids. On the contrary, symptomatic patients with neutrophilic alveolitis, with or without increased eosinophils exhibit rapid disease progression (5).

In several CVDs, such as rheumatoid arthritis and Sjögren's syndrome, a subclinical lymphocytic alveolitis frequently precedes neutrophilic alveolitis present when chest x-ray manifestations confirm lung involvement (5). The CD4/CD8 ratio may be variable. In the setting of lymphocytic alveolitis, usually CD4 are the dominant cell population in subclinical cases, whereas CD8 seem to predominate in established CVD-associated ILD (1). Low peripheral blood NK cell number and activity have been found in CVDs (6, 7). In addition, NKT cells, which are important regulatory cells of auto-immunity, seem to be reduced in number and defective in function in the above disorders (8, 9, 10). The aim of the present study was to evaluate BALF cell populations in CVD-associated ILD. Also a possible correlation with pulmonary function was investigated.

Patients

Fifty-seven patients (26 male and 31 female, mean age±SD: 54.68±12.18 years) with CVD-associated ILD admitted to the Pulmonary Department, Aristotle University of Thessaloniki were studied. Patients were examined for investigation of pulmonary involvement of a known CVD or for initial CVD diagnosis. Patients were divided into 6 groups based on underlying CVD. Seventeen patients suffered from rheumatoid arthritis (RA) diagnosed according to the American Rheumatism Association criteria (11). Eight patients suffered from systemic sclerosis (SSc), 8 patients from systemic lupus erythematosus (SLE) and 7 patients from and Sjögren's syndrome (SS) based on international guidelines (12-15). Six patients suffered from Wegener's granulomatosis (WG) based on the American College of Rheumatology criteria (16) and the presence of c-ANCA. Eleven patients who did not meet the criteria for a specific CVD were classified as undifferentiated and were included in a separate group. The majority of patients were untreated except for five patients (2 with RA, 2 with SLE and 1 with SSc) who were treated with immunosuppressive drugs (prednisone and cyclophosphamide). The study population also included a group of 10 healthy controls (4 male and 6 female, mean age±SD 52.6±19.1 years). Controls were individuals with normal radiographic findings and pulmonary function tests who were submitted to bronchoscopy and BAL for the investigation of chronic cough. These individuals were followed up for a period of at least 3 months and

their cough was attributed to extra-pulmonary causes. Approval was obtained from the Hospital's Ethics Committee and all patients and controls gave their informed consent. Individual demographics are presented in Table I. Respiratory symptoms and HRCT findings were recorded for all patients.

Table I. Patients' demographics.

Disease	n	M	F	Age
RA	17	6	11	57.4±8.8
SSc	8	0	8	57.2±7
SLE	8	4	4	53.3±15.3
SS	7	3	4	47.8±13.7
WG	6	5	1	55.5±8.8
UnDif	11	8	3	57.8±18.7
Controls	10	4	6	52.6±19.1

n: number; M: male; F: female; RA: rheumatoid arthritis; SSc: systemic sclerosis; SLE: systemic lupus erythematosus; SS: Sjögren's syndrome; WG: Wegener's granulomatosis; UnDif: undifferentiated CVD.

Table II. Patients' symptoms and HRCT findings.

Disease	Main symptoms	Main HRCT findings
RA	cough, dyspnea	fibrosis, infiltrations
SSc	dyspnea, cough	fibrosis
SLE	dyspnea, cough	effusion, infiltrations
SS	dyspnea	fibrosis
WG	cough, haemoptysis	nodules

RA: rheumatoid arthritis; SSc: systemic sclerosis; SLE: systemic lupus erythematosus; SS: Sjögren's syndrome; WG: Wegener's granulomatosis.

Methods

Pulmonary function tests (PFTs)

Pulmonary function was assessed by an electronic spirometer (Master Screen PFT, Jaeger, Hoechberg, Germany). Forced vital capacity % (FVC), forced expiratory volume in 1 second % (FEV_1), total lung capacity % (TLC), and diffusion capacity % (by the single breath method) were measured. Two valid measurements for each parameter were obtained, and the highest was recorded. Predicted values of the American Thoracic Society criteria were used (17). Arterial pO_2 and pCO_2 were also measured by ABL 510 Radiometer (Copenhagen, Denmark).

Bronchoalveolar lavage

BAL was performed in areas of lung parenchyma exhibiting the most marked infiltrations on HRCT (guided BAL), by an Olympus BF XT40 6.2 mm flexible fiberoptic bronchoscope. Two hundred mL of sterile phosphate buffered saline solution 37°C were used, divided in 4 portions of 50 mL, which were aspirated gently after each administration (18). After evaluation of the total volume of recovered BALF and filtration of the fluid through two layers of sterile gauze, cell density was determined on the unconcentrated lavage fluid by a Malassez haemocytometer. Differential cell count was determined by cytological examination of at least 500 cells after centrifugation in a cytospin (Shandon) and May-Grünwald-Giemsa staining. Cell density (cells/ml) total cell number (cells x 10^6) and differential cell count were recorded for every patient. The rest of BALF was centrifuged at 400g for 10 min and the pellet was processed for lymphocyte subset determination. All BALF were analysed at the time of processing and only technically appropriate BALF were retrospectively reviewed.

Flow cytometry

BALF lymphocyte subsets were analysed by dual flow cytometry (FACSscan, Becton-Dickinson Inc, USA). The subsets studied were CD3+, CD4+, CD8+, CD19+ CD3-CD16/56+ and CD3+CD16/56+. BALF cells were double stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD3 and phycoerythrin (PE)-conjugated anti-CD19 to determine T- and B-cells, FITC-conjugated anti-CD3 and PE-conjugated anti-CD4 to determine helper T-cells, FITC-conjugated anti-CD3 and PE-conjugated anti-CD8 to determine suppressor/cytotoxic T-cells and FITC-conjugated anti-CD3 and PE-conjugated anti-CD16/56 to differentiate NKT (CD3+CD16/56+) from NK-cells (CD3-CD16/56+). All antibodies were supplied by Becton-Dickinson Inc, USA. More than 5000 cells were analysed. All cells examined came from the lymphocyte region. The values were expressed as a percentage of lymphocytes.

Statistical analysis

All data are expressed as mean±SD. Biostatistical analysis was performed using SPSS for Windows release 17.0.1 (Standard version, SPSS Inc). Parametric variables were compared by analysis of variance (one-way ANOVA). Bonferoni adjustment was performed in order to correct the significant differences among multiple comparisons. For multiple comparisons among BALF cell types and lymphocyte subsets, the Pearson's bivariate correlation coefficient was calculated. Bonferoni adjustment for an alpha level of 0.05 for 4 tests was lowered to 0.0125.

Results

Forty out of 57 patients (70.2%) with CVD-associated ILD presented with respiratory symptoms. The main symptoms were dyspnea mainly on exertion (22 patients), dry cough (15 patients), haemoptysis (4 patients) and chest pain (2 patients). The remaining 15 patients (26.3%) had no respiratory complaints and the ILD was diagnosed by imaging techniques. The commonest HRCT findings were alveolar infiltrations, consolidation, ground glass areas, pleural effusion, nodules, reticulation and honeycombing. Patients' symptoms and HRCT findings are presented in Table II.

In PFTs, patients exhibited a restrictive defect with reduced DLCO. Statistically significant differences in pulmonary function parameters were observed among different groups (Table III).

Table III. Pulmonary function tests.

Disease	FEV_1 (%)	FVC(%)	TLC (%)	DLCO (%)	pO_2	pCO_2
RA	82.3 ± 13.1	78.3 ± 12.4	60.6 ± 17.4	88.4 ± 18.1	78.9 ± 8.7	36.7 ± 3.8
SSc	70.4 ± 3	66.5 ± 11.2	64.5 ± 2.5	71.8 ± 9.9	68.2 ± 8.1	40.5 ± 2.5
SLE	51.8 ± 4.8	54.7 ± 6	47.8 ± 3.3	50.2 ± 2.9	68.2 ± 6,3	36,2 ± 4
SS	61.5 ± 1	61 ± 0.8	84 ± 11.2	44 ± 10.7	48.5 ± 1.3	44.5 ± 4.2
WG	86.5 ± 25.5	83.6 ± 21.3	70 ± 15.4	77.4 ± 22	69.7 ± 2.4	35.3 ± 4.8
UnDif	60.8 ± 5.2	64.3 ± 11.6	67.7 ± 14.7	69.6 ± 18.6	69.6 ± 9.7	36.5 ± 4,4
ANOVA	$p<0.001$	$p<0.001$	$p=0.005$	$p<0.001$	$p<0.001$	$p=0.001$

RA: rheumatoid arthritis; SSc: systemic sclerosis; SLE: systemic lupus erythematosus; SS: Sjögren's syndrome; WG: Wegener's granulomatosis; UnDif: undifferentiated CVD.

Table IV. Differential cell count.

Disease	Macrophages	Lymphocytes	Neutrophils	Eosinophils
RA	69.5 ± 21.5	17.1 ± 18.2	11.4 ± 16.9	2.2 ± 3.7
SSc	77.7 ± 6.3	7.5 ± 6.1	11.9 ± 17.2	2.6 ± 2
SLE	71.5 ± 17.2	12.2 ± 9.9	14 ± 12.9	2.1 ± 4.4
SS	82.3 ± 18.5	8.3 ± 3.3	6 ± 9.4	3.4 ± 5.8
WG	84.2 ± 12.4	8.2 ± 7.9	8.3 ± 13.2	0.9 ± 1.6
UnDif	80.5 ± 12.1	6.3 ± 3.7	11.6 ± 12.5	2.2 ± 4.4
Controls	80 ± 6.6	14.7 ± 6.1	3.5 ± 1.8	0.5 ± 0.7
ANOVA	$p=0.310$	$p=0.203$	$p=0.643$	$p=0.778$

RA: rheumatoid arthritis; SSc: systemic sclerosis; SLE: systemic lupus erythematosus; SS: Sjögren's syndrome; WG: Wegener's granulomatosis; UnDif: undifferentiated CVD.

Table V. Lymphocyte subsets.

Disease	CD3	CD19	NK	CD4/CD8	NKT
RA	92.9 ± 4.2	2.7 ± 2.1	5.3 ± 2.8	1.3 ± 0.9	4.4 ± 1.6
SSc	89.5 ± 1.9	5.4 ± 3.1*	4.7 ± 1.8	0.8 ± 0.3	4.2 ± 2.8
SLE	88.8 ± 6.1	0.5 ± 0.5	10.4 ± 6†	0.8 ± 0.4	4.4 ± 1
SS	97.5 ± 0.6	1.1 ± 0.3	1.5 ± 0.4	2.8 ± 1.8	21.8 ± 18.1*
WG	92.7 ± 2	2.9 ± 1	4.3 ± 2.2	0.4 ± 0.4*	10.2 ± 4.1
UnDif	91.6 ± 3.3	1.3 ± 0.5	7 ± 2.8	0.8 ± 0.3	3.1 ± 1.6
Controls	92.1 ± 2.6	2.3 ± 1.6	5.6 ± 1.9	1.9 ± 0.5	3.2 ± 3.8
ANOVA	$p=0.011$	$p<0.001$	$p=0.001$	$p<0.001$	$p<0.001$

CD3: T-lymphocytes (% total lymphocytes); CD19: B-lymphocytes (% total lymphocytes); NK: NK cells (% total lymphocytes); NKT: NKT cells (% T-lymphocytes); nd: not done; RA: rheumatoid arthritis; SSc: systemic sclerosis; SLE: systemic lupus erythematosus; SS: Sjögren's syndrome; WG: Wegener's granulomatosis; UnDif: undifferentiated CVD. *$p<0.05$ *vs*. controls, †$p=0.05$ *vs*. controls.

BALF differential cell count data and lymphocyte subsets are presented in Tables IV and V. BALF differential cell count did not differ significantly among different groups. On the contrary, statistically significant differences in BALF lymphocyte subsets were observed. SSc patients showed the highest value of CD19 cells, which was significantly higher than all groups of patients and controls (SSc *vs*. RA: $p=0.005$, *vs*. SS: $p=0.004$, *vs*. SLE: $p<0.001$, *vs*. UnDif: $p<0.001$ and *vs*. controls: $p=0.01$). In addition, all CVDs except for SS showed a low CD4/CD8 ratio. The NK cell percentage was significantly higher in SLE than other CVD groups and controls (SLE *vs*. RA: $p=0.01$, *vs*. SSc: $p=0.02$, *vs*. SS: $p<0.001$, *vs*. WG: $p=0.02$ and *vs*. controls: $p=0.05$). The NKT cell percentage was significantly higher in SS than other CVD groups and controls (SS *vs*. RA: $p<0,001$, *vs*. SSc: $p<0.001$, *vs*. SLE: $p<0.001$, *vs*. WG: $p=0.01$, *vs*. UnDif: $p<0.001$ and *vs*. controls: $p<0.001$). The following correlations were observed. The percentage of BALF neutrophils correlated negatively with FVC (r=-0.356, $p=0.011$) and FEV1 (r=-0.336, $p=0.017$). The percentage of BALF NKT (r=-0.415, $p=0.003$) correlated negatively with pO_2 (Figs. 1, 2).

Discussion

Detection of pulmonary involvement is of great importance for the prognosis of CVDs (19). In the present study, BALF findings of patients with CVD-associated ILD were analysed. Important differences were observed in lymphocyte subsets and more specifically in CD19, NK, NKT cell percentages and CD4/CD8 ratio. The most interesting observation was that SLE exhibited the highest NK cell percentage and SS the highest NKT cell percentage compared to other CVDs and controls.

All patients presented HRCT evidence of ILD and frequently fibrosis. However, one third of the patients did not complain of any respiratory symptoms. As expected (2), patients exhibited a restrictive pattern in pulmonary function and reduced DLCO and pO_2. Moreover, correlations between BALF findings and respiratory function were found.

A neutrophilic alveolitis (>4%) was observed in all CVDs. Mildly increased BALF lymphocytes were observed in RA. No significant lymphocytosis consistent with drug-induced ILD was observed in the very small number of treated patients. Increased BALF neutrophils and eosinophils and increased lymphocytes in RA have been previously reported in several studies (5, 20). An absence of lymphocytosis was observed in SS patients in contrast with previous reports (5). This finding might be explained by the fact that SS was mainly secondary to fibrotic RA. No significant differences in BALF differential cell count among different CVDs were observed in this study. A considerable overlap was detected among CVDs both in the present and in previous studies (5). Increased number of neutrophils and eosinophils is correlated to ongoing fibrosis and subsequently to worse prognosis (19). Nevertheless, alveolitis cannot predict patients more likely to benefit from treatment (21). Negative correlations have already been reported between

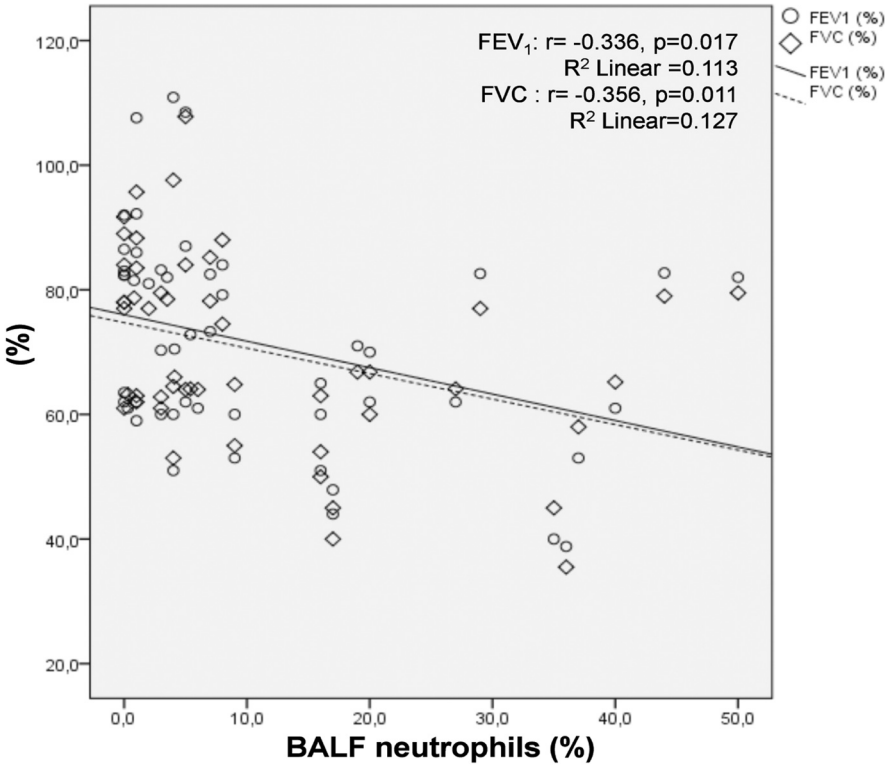

Fig. 1. Correlation between pulmonary function (FVC and FEV1) and BALF neutrophil percentage.

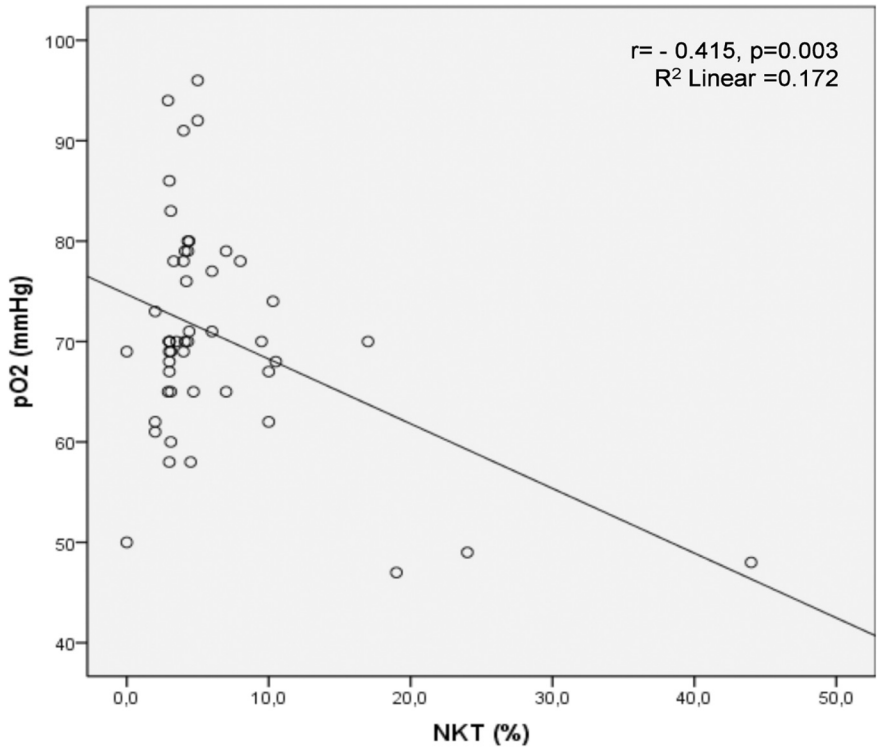

Fig. 2. Correlation between arterial pO_2 and BALF NKT cell percentage.

pulmonary function parameters such as FVC and DLCO and BALF neutrophils, lymphocytes (including CD8) and eosinophils (5, 19, 22, 23, 24). In the present study, BALF neutrophils correlated negatively with pulmonary function, a fact also observed in previous reports (22, 23, 25).

BALF T-lymphocyte percentage (CD3) was high whereas B-lymphocyte percentage (CD19) was low in all CVDs and controls, as reported in the literature (18). SSc patients presented with a CD19 percentage significantly higher compared to the groups studied. This finding could be attributed to the role of B-cells in the evolution of fibrosis, although data on B-cells in SSc are scarce. B-cells are currently under investigation as therapeutic targets in SSc (26, 27). CD8 lymphocyte predominance in CVDs has been observed in previous studies (5, 23, 28). BALF CD8 cells were related to fibrotic lung pattern in various CVDs (29). In the present study, a reduced CD4/CD8 ratio was observed in all CVDs with the exception of SS.

The most important finding of the present study is the significant difference of NK cell populations among different CVDs. Patients with SLE exhibited higher NK cells compared to other CVDs and controls. BALF NK cell population is low and its activity varies in healthy individuals (30). There are very few reports in the literature concerning NK cell populations in CVDs. NK cell number and activity have been found reduced in CVDs in peripheral blood (6, 7, 31). NK cells have been shown to participate in the initiation of autoimmunity (32). Gudbjornsson *et al*. have studied BALF NK cells in SSc and primary SS and found no differences between the two diseases (20). A negative correlation between BALF NK cell number and DLCO has been reported in SLE (24). In the present study, it is worth mentioning that the significant increase of NK cells in SLE was observed despite the limited number of SLE patients. This finding is compatible with the role of NK cells in regulation of autoimmunity in SLE.

Another interesting finding of the present study is the difference of BALF NKT cell population among CVDs. Patients with SS exhibited the highest NKT cell percentage compared to other CVDs and controls. A negative correlation between BALF NKT cells and pO_2 was also observed. NKT cells participate in various kinds of immu-

noregulation due to a potent ability to produce various cytokines (8, 9, 10, 33, 34). Although this capacity is beneficial in combating environmental pathogens, NKT cells could play a deleterious role in autoimmunity by producing disease-promoting cytokines (35). Alterations in both NKT number and activity in peripheral blood have been observed in SSc, SLE, RA and WG (9, 10, 36, 37, 38, 39). Importantly, according to recent studies, NKT cells could be used as therapeutic targets in different CVDs (37, 40). The correlation between BALF NKT cells and pO_2 found in the present study could be attributed to an increased activity in the lung implicating their involvement in the pathogenesis of lung damage in CVDs.

Very few data exist on BALF NK and NKT cells in various ILDs. NK and NKT cell populations have been implicated in the pathogenesis of various pulmonary disorders, such as IPF (41, 42), hypersensitivity pneumonitis (43), eosinophilic pneumonia (44) and sarcoidoisis (41, 45). More specifically, in sarcoidosis NK and NKT cells were increased in more advanced stages implicating an excess of cytotoxic activity associated with stage progression and fibrosis (45). Moreover, it is important to mention that also in this study all SS patients presented with advanced stage disease and fibrosis indicating that increased NKT cells might be implicated in the fibrotic procedure.

Very few studies have focused on BALF NK and NKT cells in patients with CVDs. According to the present study, the above cells seem to participate in the immune response in the lung. More studies in BALF are needed to verify cell function in CVD-associated ILD and to clarify the role of NK cell populations in disease prognosis. An even more interesting perspective would be a possible therapeutic intervention through these cell types, before the establishment of pulmonary fibrosis. Until the pathogenetic mechanisms of CVDs are fully clarified BAL will remain a useful tool for the investigation of the underlying lung inflammation and fibrosis.

References

1. LAMBLIN C, BERGOIN C, SAELENS T, WALLAERT B: Interstitial lung disease in collagen vascular disease. *Eur Respir J* 2001; 18 (Suppl. 32): 69S-80S.
2. WOODHEAD F, WELLS AU, DESAI SR: Pulmonary Complications of Connective Tissue Diseases. *Clin Chest Med* 2008; 29: 149-64.
3. WELLS AU: Lung disease in association with connective tissue diseases. *Eur Respir Mon* 2000; 14: 137-64.
4. KLECH H, HUTTER C, COSTABEL U: Clinical guidelines and indications for bronchoalveolar lavage (BAL). Report of the European Society of pneumonology Task Group on BAL. *Eur Respir J* 1992; 2: 47-127.
5. WALLAERT B, HOORELBEKE A, SYBILLE Y, ROSSI GA: The central role of bronchoalveolar lavage in collagen-vascular disease. Clinical guidelines and indications for bronchoalveolar lavage (BAL). *Eur Respir Rev* 1992; 2: 64-8.
6. GONZALEZ-AMARO R, ALCOCER-VARELA J, ALARCON-SEGOVIA D: Natural killer cell activity in the systemic connective tissue diseases. *J Rheumatol* 1988; 15: 1223-8.
7. GREEN MR, KENNELL AS, LARCHE MJ *et al*.: Natural killer cell activity in families of patients with systemic lupus erythematosus: demonstration of a killing defect in patients. *Clin Exp Immunol* 2005; 141: 165-73.
8. SIGAL LH: Basic science for the clinician 35: CD1, invariant NKT (iNKT) cells, and gammadelta T-cells. *J Clin Rheumatol* 2005; 11: 336-9.
9. RICCIERI V, PARISI G, SPADARO A *et al*.: Reduced circulating natural killer T cells and gamma/delta T cells in patients with systemic sclerosis. *J Rheumatol* 2005; 32: 283-6.
10. TAKAGI D, IWABUCHI K, IWABUCH C *et al*.: Immunoregulatory defects of V alpha 24V+beta11+NKT cells in the development of Wegener's granulomatosis and relapsing polychondritis. *Clin Exp Immunol* 2004; 136: 591-600.
11. ARNETT FC, EDWORTHY SM, BLOCH DA *et al*.: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-24.
12. MASI AT, RODNAN GP, MEDSGER TAJ *et al*.: Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum* 1980; 23: 581-90.
13. TAN EM, COHEN AS, FRIES JF *et al*.: The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982; 25: 1271-7.
14. HOCHBERG MC: Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1997; 40: 1725.
15. VITALI C, BOMBARDIERI S, MOUTSOPOULOS HM *et al*.: Preliminary criteria for the classification of Sjögren's syndrome: results of a prospective action supported by the European Community. *Arthritis Rheum* 1993; 36: 340-7.
16. LEAVITT RY, FAUCI AS, BLOCH DA *et al*.: The American College of Rheumatology 1990 criteria for the classification of Wegener's granulomatosis. *Arthritis Rheum* 1990; 33: 1101-7.
17. MILLER MR, HANKINSON J, BRUSASCO V *et al*.: Standardisation of spirometry. *Eur Respir J* 2005; 26: 319-38.
18. KLECH H, POHL W: Technical recommendations and guidelines for bronchoalveolar lavage (BAL). *Eur Respir J* 1992; 2: 561-85.
19. BOUROS D, WELLS AU, NICHOLSON AG *et al*.: Histopathologic subsets of fibrosing alveolitis in patients with systemic sclerosis and their relationship to outcome. *Am J Respir Crit Care Med* 2002; 165: 1581.
20. GUDBJORNSSON B, HALLGREN R, NETTELBLADT O *et al*.: Phenotypic and functional activation of alveolar macrophages, T lymphocytes and NK cells in patients with systemic sclerosis and primary Sjögren syndrome. *Ann Rheum Dis* 1994; 53: 574-9.
21. ANTONIOU KM, WELLS AU: Scleroderma lung disease: evolving understanding in light of newer studies. *Curr Opin Rheumatol* 2008; 20: 686-91.
22. SALAFFI F, MANGANELLI P, CAROTTI *et al*.: A longitudinal study of pulmonary involvement in primary Sjögren' syndrome: relationship between alveolitis and subsequent lung changes on high- resolution computed tomography. *Br J Rheumatology* 1998; 37: 263-9.
23. ATAMAS SP, YUROVSKY VV, WISE R *et al*.: Production of type 2 cytokines by $CD8^+$ lung cells is associated with greater decline in pulmonary function in patients with systemic sclerosis. *Arthritis Rheum* 2000; 43: 236-8.
24. GROEN H, ASLANDER M, BOOTSMA H *et al*.: Bronchoalveolar lavage cell analysis and lung function impairment in patients with systemic lupus erythematosus. *Clin Exp Immunol* 1993; 94: 127-33.
25. DOMAGALA-KULAWICK J, HOSER G, DOBOSZYNSKA A, KAWIAK J, DROSZCZ W: Interstitial lung disease in systemic sclerosis: comparison of BALF lymphocyte phenotype and DLCO impairment. *Respir Med* 1998; 92: 1295-301.
26. HASEGAWA M: B lymphocytes: shedding new light on the pathogenesis of systemic sclerosis. *J Dermatol* 2010; 37: 3-10.
27. LEASK A: B cell block: Is rituximab a new possible treatment for systemic sclerosis? *Cell Commun Signal* 2010; 4: 201-2.
28. NAGASAWA Y, TAKADA T, SHIMIZU T *et al*.: Inflammatory cells in lung disease associated with rheumatoid arthritis. *Intern Med* 2009; 48: 1209-17.
29. SALAFFI F, MANGANELLI P, CAROTTI M, BALDELLI S: The differing patterns of subclinical pulmonary involvement in connective tissue diseases as shown by application of factor analysis. *Clin Rheumatol* 2000; 19: 35-41.
30. ROBINSON BW, PINKSTON P, CRYSTAL RG: Natural killer cells are present in the normal lung but are functionally impotent. *J Clin Invest* 1984; 74: 942-50.
31. IZUMI Y, IDA H, HUANG M *et al*.: Characterization of peripheral natural killer cells in primary Sjogren syndrome: impaired NK cell activity and low NK cell number. *J Lab Clin Med* 2006; 147: 242-9.
32. HORIKAWA M, HASEGAWA M, KOMURA K *et al*.: Abnormal natural killer cell function in systemic sclerosis: altered cytokine production and defective killinfg activity. *J Invest*

Dermatol 2005; 125: 731-7.
33. MIYAKE S, YAMAMUTA T: NKT cells and autoimmune diseases: understanding the complexity. *Curr Top Microbiol Immunol* 2007; 314: 251-67.
34. WU L, VAN KAER L: Natural killer T cells and autoimmune disease. *Curr Mol Med* 2009; 9: 4-14.
35. YAMAMURA T, SAKUISHI K, ILLES Z, MIYAKE S: Understanding the behavior of invariant NKT cells in autoimmune disease. *J Neuroimmunol* 2007; 191: 8-15.
36. LINSEN L, THEWISSEN M, BAETEN K: Peripheral blood but not synovial fluid natural killer T cells are biased towards a Th-1 phenotype in rheumatoid arthritis. *Arthritis Res Ther* 2005; 7: 493-502.
37. VAN KAER L: Natural killer T cells as targets for immunotherapy of autoimmune diseases. *Immunol Cell Biol* 2004; 82: 315-22.
38. GABRIEL L, MORLEY BJ, ROGERS NJ: The role of iNKT cells in the immunopathology of systemic lupus erythematosus. *Ann N Y Acd Sci* 2009; 1173: 435-41.
39. NOVAK J, LEHUEN A: Mechanism of regulation of autoimmunity by iNKT cells. *Cytokine* 2011; 53: 263-70.
40. MIYAKE S, YAMAMURA T: Therapeutic potential of glycolipid ligands for natural killer (NK) T cells in the suppression of autoimmune diseases. *Curr Drug Targets Immune Endocr Metabol Disord* 2005; 5: 315-22.
41. GRUBER R, PFORTE A, BEER B, RIETHMULLER G: Determination of gamma/delta and other T-lymphocyte subsets in bronchoalveolar lavage fluid and peripheral blood from patients with sarcoidosis and idiopathic fibrosis of the lung. *APMIS* 1996; 104: 199-205.
42. ESPOSITO I, PERNA F, PONTICIELLO A, PERELLA M, GILLI M, SANDURRI A: Natural killer cells in BAL and peripheral blood of patients with idiopathic pulmonary fibrosis (IPF). *Int J Immunopathol Pharmacol* 2005; 18: 541-5.
43. KOROSEC P, OSOLNIK K, KERN I, SILAR M, MOHORCIC K, KOSNIK M: Expansion of pulmonary $CD8^+CD56^+$ natural killer cells in hypersensitivity pneumonitis. *Chest* 2007; 132: 1291-7.
44. PAPAKOSTA D, MANIKA K, KYRIAZIS G *et al*.: Bronchoalveolar lavage fluid eosinophils are correlated to natural killer cells in eosinophilic pneumonias. *Respiration* 2009; 78: 177-84.
45. PAPAKOSTA D, KYRIAZIS G, GIOULEKAS D *et al*.: Variations in alveolar cell populations, lymphocyte subsets and NK-cells in different stages of active pulmonary sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 2005; 22: 21-6.