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# BALF and BLOOD NK- cells in different stages of pulmonary sarcoidosis

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**Abstract. Background and objective:** Data on natural killer (NK)- and natural killer T (NKT)- like cells in the immunopathogenesis of sarcoidosis remain limited. The aim was to assess NK- and NKT-like cells across different stages in bronchoalveolar lavage (BALF) versus peripheral blood (PB) in comparison to controls. **Methods***:* Forty four patients (32 women and 12 men, mean age 46.6±14.4 years) with biopsy-proven sarcoidosis and 10 healthy individuals (6 women, 4 men mean age 52.6±19.1 years) were submitted to BALF. Total cells and cell differentials were counted, while CD45+, CD3+, CD4+, CD8+, CD19+, CD3-CD16/56 (NK cells) and CD3+CD16/56+ (NKT-like cells) were determined by dual flow cytometry in BALF and PB. **Results***:* A significantly lower percentage of both NK and NKT-like cells was observed in BALF of controls and sarcoid patients (SP) compared to PB. Both BALF NK and NKT-cell counts were significantly higher in SP than in controls (NK: p=0.046, NKT-like: p=0.012) In addition BALF NK cell percentage differed among sarcoidosis stages (p=0.005). In PB NK-cell count was lower in sarcoidosis patients but the difference did not reach statistical significance. Also, in sarcoid patients' BALF NK-cell percentage negatively correlated with lymphocyte percentage (r=-0.962, p<0.001). **Conclusions***:* The increased count of BALF NK and NKT-like cells in sarcoidosis compared to controls along with the increase of NK cells with stage progression are in line with a growing number of investigations suggesting the involvement of NK- and NKT-like cells in the pathogenesis of sarcoidosis.

**Key words:** Bronchoalveolar lavage, Lymphocytes, Natural killer cells, Natural killer T-like cells, Sarcoidosis

## Introduction

More than one hundred years after its initial description and despite advanced genomic, cytometric and immunologic research, sarcoidosis is still considered a disease of unknown origin [1,2]. Despite the advances that have been made over the last 20 years [3,4], a variety of issues regarding its pathogenesis such as genetic predisposition, responsible antigens and the immunologic process leading to manifestations from different systems, remain elusive. Perhaps even more enigmatic are the factors that determine severity and prognosis leading to confusion about the exact indications of treatment, especially in pulmonary involvement. One of the major advances in our understanding of this complex disease is the recognition of a highly polarized T-helper-1 (Th1)/Th 17

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profile in the early stages as opposed to the failure to down-regulate the inflammation which possibly occurs in chronic disease [4]. It seems that the delicate balance of inflammatory cells of both the innate and the adaptive immune system, the presence of defective Treg function and the Th1/Th2 balance are among the factors which determine whether the granulomatous inflammation resolves, persists or evolves to fibrosis [1,4].

Within this growing body of literature on sarcoidosis immunopathogenesis, data on natural killer (NK) and natural killer T (NKT)-like cells remain rather limited [5-7]. NK cells are efficient producers of both pro- and anti- inflammatory cytokines and important components of the innate immune response against various targets [8-10]. NKT-like cells in turn, are part of both the innate and the adoptive immune systems and they are also able to rapidly produce cytokines and modulate the Th1/Th2 balance [11-13]. Apart from their cytotoxic activity NK and NKT-like cells have been implicated in the pathogenesis of different ILDs such as hypersensitivity pneumonitis and pulmonary involvement in the setting of collagen vascular disease [6,14-17]. Regarding sarcoidosis data are still scarce although during the last five years new data on the presence of NK and NKT-like cell populations in the bronchoalveolar lavage fluid (BALF) have emerged[6,7,17].

In our previous work [18] we studied BALF NK- and NKT-like cells in sarcoid patients (SP) and we suggested an increase with stage progression in sarcoidosis implying that these cells may be involved in the disease's immunopathology. In the present prospective study, we investigated both BALF and peripheral blood (PB) NK and NKT-like cells in different stages of sarcoidosis. The aim of the study was to assess the possible implication of BALF NK- and NKT-like cells compared to PB in stage progression in sarcoidosis in a new cohort of patients.

## Patients

Forty-four patients with biopsy-proven sarcoidosis were enrolled consecutively in the study during the period 2010-2015. Patients were submitted to chest radiography, high-resolution CT, and fiberoptic bronchoscopy with biopsy and bronchoalveolar lavage (BAL). Twenty one (4 men and 17 women, mean age ± sd 46.5 ± 14.1 years) suffered from sarcoidosis stage I, 13 (4 men and 9 women, mean age ± sd 45.6 ± 12.7 years) from sarcoidosis stage II and 10 (4 men and 6 women, mean age ± sd 40.8 ± 19.1 years) from sarcoidosis stage III. All patients had newly diagnosed, histologically confirmed pulmonary sarcoidosis and were untreated. Broncoscopy and BAL were performed for diagnostic purposes.

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 finally attributed to extra-pulmonary causes. Peripheral blood (PB) was collected from patients and controls on the day that BAL was performed.

## Methods

### *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. 200 mL (4 portions of 50 mL) of sterile phosphate buffered saline solution were aspirated gently after each administration [19]. 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 hemocytometer. 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 analyzed at the time of processing and only technically

appropriate BALF were evaluated. The procedure was also consistent with the BALF guidelines [20]. Approval was obtained from the "G. Papanikolaou" Hospital's Ethical Committee and all patients and controls gave their informed consent.

*Flow cytometry*

BALF lymphocyte subsets were analyzed by dual flow cytometry (FACSscan, Becton-Dickinson Inc, USA). The subsets studied were CD45+, CD3+, CD4+, CD8+, CD19+ CD3-CD16/56+ and CD3+CD16/56+. BALF cells were stained with PerCP –conjugated anti-CD45, 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-like (CD3+CD16/56+) from NK-cells (CD3-CD16/56+). All antibodies were supplied by Becton-Dickinson Inc, USA. More than 5000 cells were analyzed. All cells examined came from the lymphocyte region. The values were expressed as a percentage of lymphocytes and as cells $10^3$/ml.

*Statistical analysis*

All data are expressed as mean ± SD. Biostatistical analysis was performed using SPSS 21.0 software package. The normality of distribution was assessed by Kolmorov-Smirnov test. Non parametric variables were compared by Kruskal Wallis, Wilcoxon and Mann-Whitney tests. For an overall alpha level of 0.05 the Bonferoni's adjustment was used and 0.05 was lowered to 0.0125 (z-value for two-sided testing > 2.5). Correlations among BALF cell types and lymphocyte subsets were assessed using the Spearman's rho correlation coefficient.

## Results

BALF cellularity and cell differential in different stages of sarcoidosis and controls are presented in Table 1. Representative data of flow cytometry strategy appear in Figure 1. A significantly lower percentage of both NK and NKT-like cells was observed in BALF of controls and all sarcoidosis stages compared to PB (controls NK: p=0.005, controls NKT-like: p=0.008, stage I NK: p<0.001, stage I NKT-like: p<0.001, stage II NK: p=0.012, stage II NKT-like: p=0.006, stage III NK: p=0.009, stage III NKT-like: p=0.008). Lymphocyte percentages were statistically significantly higher in SP compared to controls (p<0.001). BALF and PB CD4+ and CD8+ cell counts and percentages are shown in

**Table 1.** Demographics and BALF cells and cell differentials of patients with Sarcoidosis and controls.

| | sarcoidosis | | | | controls |
|---|---|---|---|---|---|
| | **Stage I** | **Stage II** | **Stage III** | **Stages I-III** | |
| Age, years (mean±sd) | 46.5±13.7 | 45.6±12.7 | 40.8±10.6 | 46.4±14.4 | 52.6±19.1 |
| Gender (female/male) | 17/4 | 9/4 | 6/4 | 32/12 | 6/4 |
| Smoker/nonsmoker | 11/10 | 6/7 | 4/6 | 21/23 | 0/10 |
| Total cell count ($10^3$/ml) | 171.3±102.9 | 173.6±112.3 | 166.5±113.4 | 170.9±105.6 | 148.6±105.4 |
| Macrophages (%) | 48.6±18.7* | 48.4±21* | 49.4±22.2* | 48.7±19.7* | 80±6.6 |
| Lymphocytes (%) | 44.5±16.9* | 44±21* | 40.2±21.2* | 43.36±18.8* | 14.7±6.1 |
| Neutrophils (%) | 4.8±4.2 | 6.5±7.4 | 6.6±4.9 | 5.7±5.4 | 3.5±1.8 |
| Eosinophils (%) | 0.7±1.2 | 0.3±0.5 | 2.6±5.6 | 1±2.8 | 0.5±0.7 |

*statistically significantly different from controls (p<0.05)
I: sarcoidosis stage I, II: sarcoidosis stage II, III: sarcoidosis stage III, I-III: sarcoidosis stages I, II and III.

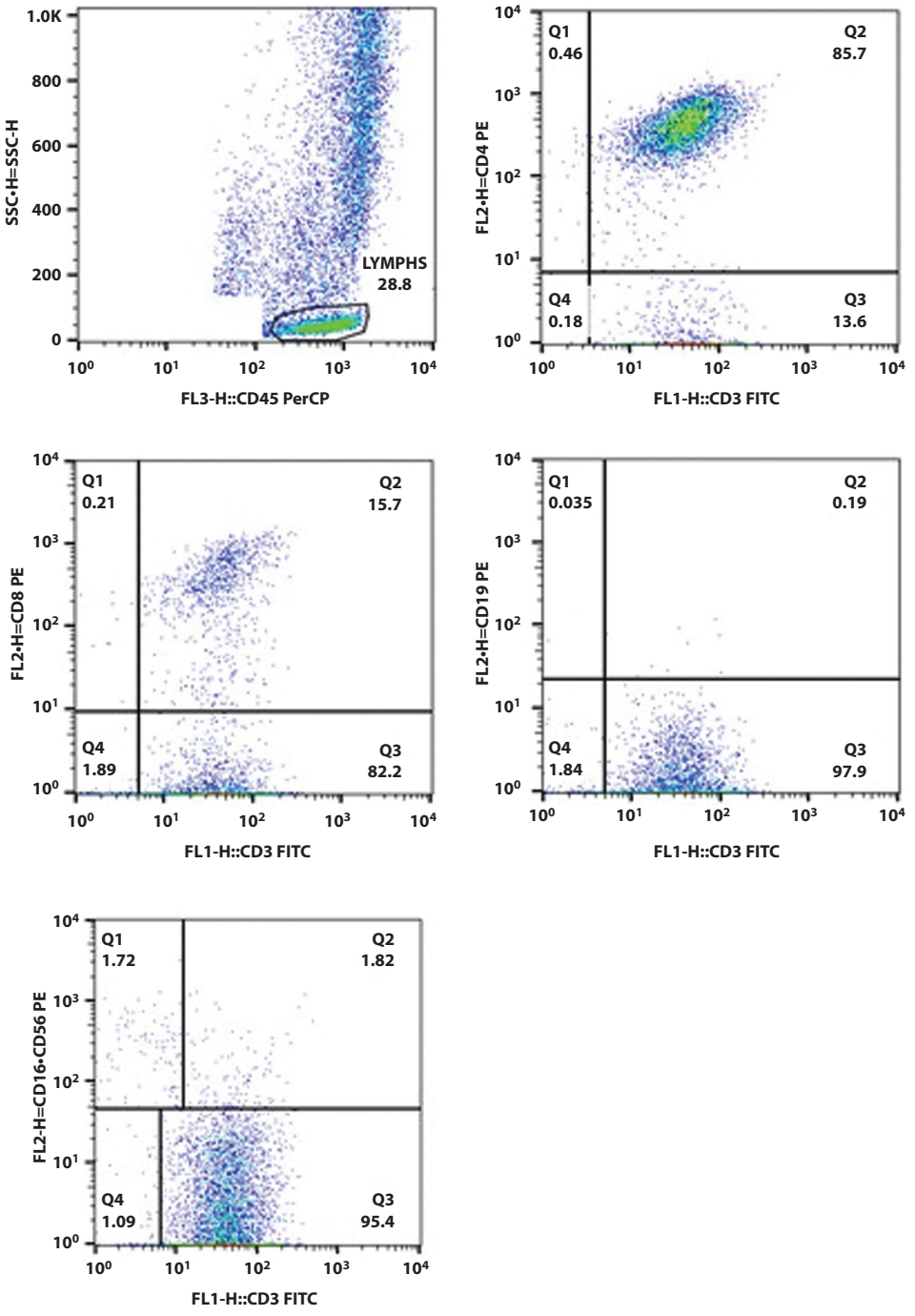


**Figure 1.** Representative data of flow cytometry strategy in a sarcoidosis patient A) in BALF sample B) in peripheral blood sample.

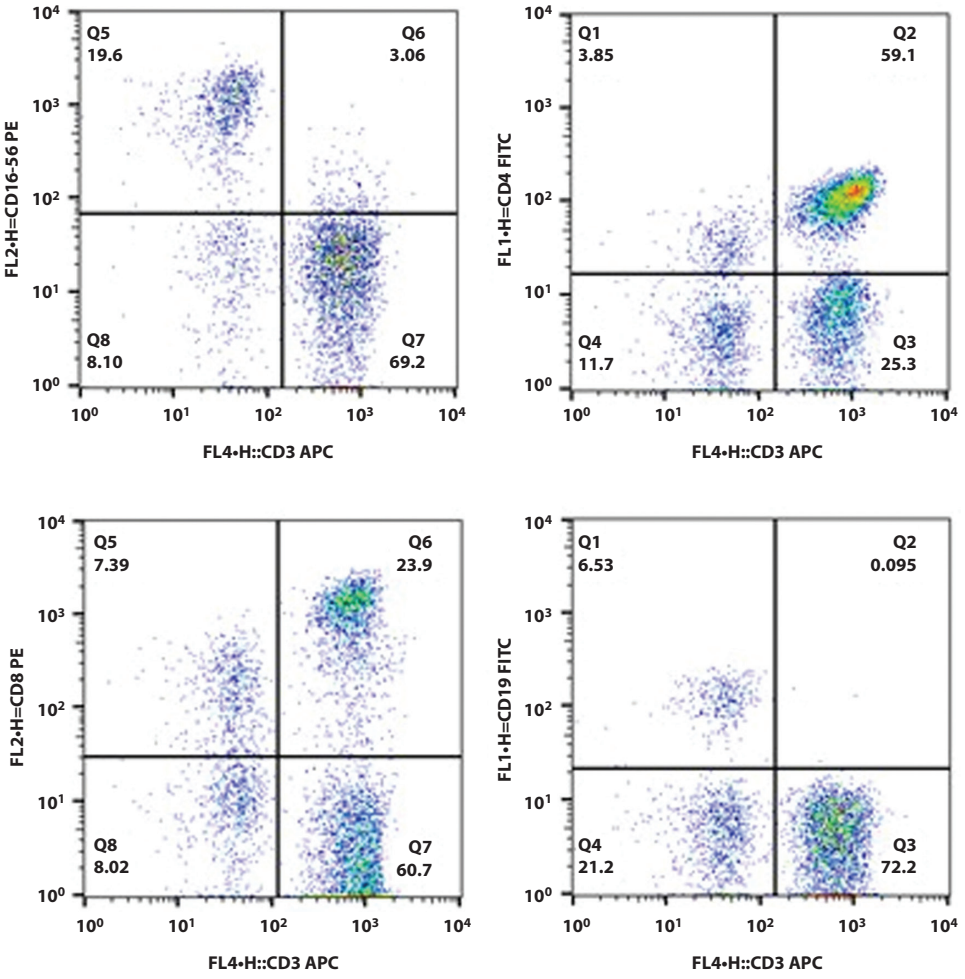


**Figure 1.** (*continued*)

**Table 2.** BALF and peripheral blood T-lymphocyte subsets.

| | BALF | | | | |
|---|---|---|---|---|---|
| | **$CD_4$ %** | **$CD_4$ ($10^3$/ml)** | **$CD_8$ %** | **$CD_8$ ($10^3$/ml)** | **$CD_4/CD_8$** |
| I | 75.5±18.1 | 60.1±55.5 | 24.1±17.5 | 14±9.2 | 5.3±4.7 |
| II | 76.2±17.3 | 60.5±53.1 | 20.3±10.1 | 12.2±9.5 | 6.4±7.9 |
| III | 67.8±16.2 | 42.6±34.6 | 29.7±14.4 | 20.2±28.8 | 1.8±0.6 |
| I-III | 74±17.4* | 56.2±50.3* | 24.2±15.1 | 14.3±15.7* | 5.3±5.5* |
| controls | 62±5.9 | 12.7±11 | 33.2±5.4 | 7.4±7.5 | 1.9±0.5 |
| | **Peripheral blood** | | | | |
| | **CD4 %** | **CD4 ($10^3$/ml)** | **CD8 %** | **CD8 ($10^3$/ml)** | **CD4/CD8** |
| I | 60.6±13.9 | 773.1±555.2 | 39.4±13.6 | 478.5±207.8 | 2±1.4 |
| II | 52.3±15.9 | 502.3±252.4 | 41.6±15.3 | 373.1±147 | 1.7±1.5 |
| III | 62.3±15.3 | 513.7±293.9 | 35.7±13.4 | 304.1±162.5* | 2.1±1.3 |
| I-III | 58.6±15.1 | 627±436.1 | 39.1±14 | 403.3±190.9 | 1.9±1.4 |
| controls | 61±16.7 | 990.1±514.8 | 39.6±14.4 | 634.7±396.2 | 1.9±1.2 |

$CD_4$ and $CD_8$ cells are expressed as percentages of T-lymphocytes and as absolute counts.
* statistically significantly different from controls. I: sarcoidosis stage I, II: sarcoidosis stage II, III: sarcoidosis stage III, I-III: sarcoidosis stages I, II and III.

**Table 3.** BALF and peripheral blood NK- and NKT-cells.

| | BALF | | | | Peripheral blood | | | |
|---|---|---|---|---|---|---|---|---|
| | NK (%) | NK ($10^3$/ml) | NKT-like (%) | NKT-like ($10^3$/ml) | NK (%) | NK ($10^3$/ml) | NKT-like (%) | NKT-like ($10^3$/ml) |
| I | 3.1±1.8 | 2.3±1.9 | 3.6±3 | 2.5±2.3 | 20.7±11.4 | 330.8±151 | 13.5±9.8 | 159.4±107.5 |
| II | 5.7±8.2 | 2.9±3.9 | 3.2±3.4 | 1.9±2.5 | 19.1±11.7 | 328±331.3 | 16±10.2 | 138.3±92.7 |
| III | 7.2±4.5 | 4.6±4.1 | 4.7±3.4 | 2.3±2.1 | 25±9.9 | 266.4±133.2 | 16.3±9.7 | 144.6±97.4 |
| I-III | 4.8±5.2 | 3±3.2* | 3.7±3.2 | 2.3±2.8* | 21.3±11.1 | 313.8±213.9 | 15±9.7 | 149.4±98.7 |
| controls | 5.6±1.9 | 1.4±1.8 | 3.2±3.8 | 1.1±2.2 | 20.1±9.7 | 479.9±231.6 | 12.2±7.5 | 216.3±184.2 |

NK cells are expressed as percentage of lymphocytes and as absolute count. NKT-like cells are expressed as percentage of T-lymphocytes and as absolute count.
*statistically significantly different from controls. I: sarcoidosis stage I, II: sarcoidosis stage II, III: sarcoidosis stage III, I-III: sarcoidosis stages I, II and III.

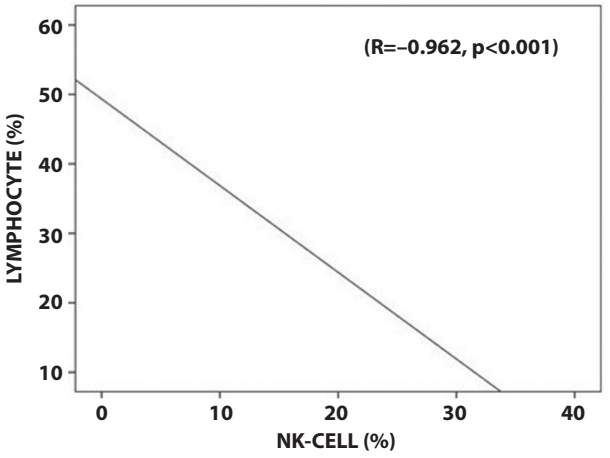


**Figure 2:** Correlation between NK-cell percentage with lymphocyte percentage in patients with sarcoidosis in BALF.

Table 2. In SP, BALF CD4+ percentage and count were significantly higher than in controls (p<0.001, p<0.004 respectively), whereas CD8+ percentage was significantly lower (p<0.007, p<0.027 respectively) resulting in a higher BALF CD4+/CD8+ ratio (p<0.007).

BALF and PB NK and NKT–like cell counts and percentages are shown in Table 3. Both NK and NKT-cell counts were significantly higher in SP than in controls (NK: p=0.046, NKT-like: p=0.012) In addition the NK cell percentage differed among sarcoidosis stages, gradually increasing as stage progressed (p=0.005). In PB NK-cell count was lower in sarcoidosis patients (p= 0.02) and more specifically in stage III compared to controls (p=0.028) but the difference did not reach statistical significance.

In SP' BALF, NK-cell percentage negatively correlated with lymphocyte percentage (r=-0.962, p<0.001) (Figure 2) and NKT-like cell percentage negatively correlated with T-lymphocyte percentage (r=-0.311, p=0.04), which was not the case in controls. In addition, in sarcoidosis, a significant positive correlation between BALF NK-cell count and BALF T-lymphocyte count (r=0.482, p<0.001) was observed. BALF NK-cell count negatively correlated with PB CD8+ count only in sarcoidosis (r=-0.413, p=0.008).

In PB a statistically significant negative correlation between NKT-like cell percentage or count and CD4+/CD8+ ratio was found only in sarcoidosis (r=-0.602, p<0.001 for percentage, r=-0.462, p=0.003).

## Discussion

In the present study we investigated the evolution of NK-cell populations in parallel with stage progression in BALF and PB from corticosteroid naïve patients with pulmonary sarcoidosis. The main findings were: 1) NK-cell count was higher is SP than in controls and NK cell percentage increased with stage progression in BALF. This observation along with the lower PB NK-cell count in SP indicates a compartmentalization of NK cells in sarcoidosis. 2) BALF NKT-like cells were higher in SP compared to controls 3) a

negative correlation between NK cells and lymphocytes was observed in BALF of sarcoidosis patients but not in controls.

The observation that BALF NK-cells increase as stage progresses is consistent with our previous results [18] and also with the results of Tutor-Ureta et al [21]. In this latter study BALF NK cells were associated with poor outcome and higher probability for corticosteroid treatment [21]. In addition, a higher NK-cell count was observed in SP compared with controls in contrast to Liu et al [22]. In a recent study on lymphocytic interstitial lung diseases, Sokhatska et all did not observe significant differences in BALF NK count across different radiologic stages of sarcoidosis. Furthermore, NK cell count did not differ according to pulmonary function tests or disease resolution versus evolution to a chronic condition. Additionally Bergantini et all have recently found that NK cell percentage in a large cohort of subacute and chronic sarcoidosis were lower than in idiopathic pulmonary fibrosis and similar to controls [7]. Interpreting this discrepancy is intriguing particularly since the NK cell percentages in these two studies [6,7] were much lower than in ours (1.3% and 1.8% vs 4.8%). Moreover, in both cohorts [6,7] a lower ratio of stage I disease was included which would be expected to further increase NK cell count. However, interestingly the BALF neutrophil percentages in both studies were lower than in ours (1.6% and 2% respectively vs 5.7% in the total of patients in our study). Elevated neutrophil counts have been observed in severe sarcoidosis cases and are considered as indicators of disease worsening [1,23,24]. Based on this association of neutrophils with progressing disease it could be assumed that our patients were overall at a more advanced, fibrotic state of disease and this difference could be associated with the higher NK cell count.

Even more intriguing is the observation that NK cells were lower in PB from sarcoidosis patients compared to controls and the lowest value was detected in stage III. The present study is one of the few to assess lymphocyte counts in both blood and BALF and based on the above one may speculate that stage progression is associated with the sequestration of NK cells in the lung. This observation may indicate that apart from CD4 cells NK cells are also compartmentalized in the lung is sarcoidosis, but at a later stage. Our findings are not in accordance with the results by Bergantini et al [7] who observed an NK cell increase in PB of a cohort of 115 sarcoidosis patients with subacute and chronic disease, not clarified by stage. This discrepancy is difficult to determine but could involve differences in the patient population. The negative correlation between BALF NK cells and lymphocytes observed in the present study only in sarcoidosis further strengthens our hypothesis that NK cells may in fact to a certain degree contribute to the evolution of sarcoidosis in the lung at the more advanced stages.

NK cell function in sarcoidosis has been addressed in several older studies with conflicting results. According to Agostini et al [25] and Kopinski et al [26] NK cells exhibit a diminished function in sarcoidosis and do not seem to participate in INF-γ production. On the contrary Katchar et al [27] demonstrated that the majority of BALF NK cells expressed CD56 bright indicating the ability for cytokine production. Furthermore, an increased proportion of BALF NK-cells produced both IFN-γ and TNFα compared to PB in sarcoidosis [27]. In parallel with our results it could therefore be assumed that NK cells might be implicated in the Th1/Th2 balance across different stages in sarcoidosis.

The involvement of NKT-like cells in the pathogenesis of sarcoidosis is even more intriguing since many conflicting results have been reported regarding their presence in granulomatous lesions [28-30]. Their number in BALF has been a matter of dispute since they have been reported normal [27] or reduced compared to hypersensitivity pneumonitis [15] bronchial asthma [31] and controls [5,7]. However, in our study BALF NKT-like cells were increased in sarcoidosis and the largest count was observed in stage I. This difference in particular with the study by Korosec 2010 [5] may be explained by the fact that we studied all NKT-like and not invariant NKT-cells. Moreover, the NKT-like cell percentage in our study was higher than the one in the study by Tondell et al [17] (2.3 vs 0.7%); however, the percentage in controls was respectively higher (1.1 vs 0.3%). In addition, a negative correlation between NKT-like cell and T-lymphocyte percentages was observed in accordance to previous reports [5].

The immunoregulatory properties of NKT cells are considered to play an important role in sarcoidosis since a reduction either in number or in function may contribute to the amplified and prolonged T-cell activity that is an essential component of the disease [11,28]. The negative correlation between NKT-like cell and T-lymphocyte percentages observed in our study in accordance to previous reports [5].

In PB, NKT-like cells have been found at reduced levels with the exception of Lofgren syndrome [28,30]. Moreover, a reduction in IFN-γ produced by PB CD1d-restricted NKT-like cells in chronic sarcoidosis in contrast to remitting disease has been observed [30]. However, Katchar et al [27] who measured all PB NKT-like cells observed an increase in comparison to controls while Sokhatska et all found no difference between PB NKT-like cells between sarcoidosis and other ILDs [6]. In our study PB NKT- like cells were reduced in sarcoidosis but this difference was not statistically significant.

The results of the present study are in line with a growing number of investigations that suggest the involvement of NK- and NKT-like cells in the pathogenesis and evolution of sarcoidosis. A possible limitation, in respect to other studies, is that no stage IV patients were included in our study population. In addition, T- lymphocytes which are the key players, it seems that other cells such as the NK and NKT cells may also play a significant role. NK-cells accumulate in the lung with stage progression and appear to contribute to the evolution of sarcoidosis in the lung at the more advanced stages. Further studies especially assessing NK cell function and cytokine production as well as their number in BALF and PB across different stages are needed to clarify the role of these intriguing cell populations in the immunopathogenesis of sarcoidosis.

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