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ORIGINAL ARTICLE

NK and NKT-like cells in granulomatous and fibrotic lung diseases

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Abstract

Background The pathogenetic and regulatory roles of natural killer (NK) and natural killer T-like cells in interstitial lung diseases (ILDs), fibrotic and granulomatous of unknown etiology are unclear. *Objectives* Here we investigated NK and NKT-like cells in peripheral blood (PB) and Bronchoalveolar lavage (BAL) from patients with ILDs. *Method* 190 patients (94 male mean age 61 ± 14.3 years) and 8 controls undergoing bronchoscopy for ILD diagnostic work-up were enrolled consecutively; 115 patients sarcoidosis, 24 chronic fibrotic hypersensitivity pneumonitis and 43 patients other ILDs [32 idiopathic pulmonary fibrosis (IPF) and 11 non-specific interstitial pneumonia (NSIP)]. PB and BAL were processed by flow cytometry using monoclonal antibodies to differentiate NK and NKT-like cells. *Results* NK% in BAL was significantly different among ILDs ($p = 0.02$). Lower NK% was observed in BAL from sarcoidosis than other ILDs ($p < 0.05$). Similar findings were observed for NKT-like, whereas no differences were found for PB NK%. Difference of NK% was observed between BAL and PB in all groups ($p < 0.001$). Sarcoidosis patients reported the best area under the curve for NKT-like (AUC = 0.678, $p = 0.0015$) and NK cells (AUC = 0.61, $p = 0.001$). In the IPF-NSIP subgroup, NK% cell was inversely correlated with FVC% ($r = -0.34$, $p = 0.03$) and DLCO% ($r = -0.47$, $p = 0.0044$). *Conclusions* NK and NKT-like were expressed differently in BAL from patients with different ILD and were significantly depleted in sarcoidosis respect to other ILDs. This suggests that these cells may play a protective role in the pathogenesis of sarcoidosis.

Keywords Natural killer cells · Natural killer T-like cells · Bronchoalveolar lavage · Interstitial lung diseases

Abbreviations

LFT	Lung function tests
BAL	Bronchoalveolar lavage
NK	Natural killer cells
NKT-like	Natural killer T-like cells
IPF	Idiopathic pulmonary fibrosis
NSIP	Non-specific interstitial pneumonia
cHP	Chronic fibrotic hypersensitivity pneumonitis
ILD	Interstitial lung diseases
FVC	Forced vital capacity
FEV1	Forced expiratory volume in the first second
DLco	Diffuse lung carbon monoxide

Introduction

Natural killer cells (NK) ($CD3^-$ $CD16/56^+$) are cells of first-line defense by virtue of their cytotoxic immune activity and ability to release a multitude of mediators involved in fighting neoplastic disorders and viral infections [1–6]. The NK inflammatory response is mediated by TNF-α and IFN-γ activities that influence the Th1 cytokine system. Natural killer T-like cells (NKT-like) ($CD3^+$ $CD16/56^+$) are the only cell line to express both T cell (CD3) and natural killer ($CD16/56^+$) receptors on their surface membranes, influencing Th1 and Th2 cytokine systems and stimulating or suppressing immune responses [7–11].

The frequencies of NK cells in lymphocytes in different tissues are not homogeneous, 4 NK cells specialize in different functions in appropriate tissues.

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In the lung, NK cells showed higher percentage of resident lymphocytes than in other tissues [12], this is critical for maintaining pulmonary homeostasis, due to their ability to respond to pathogens. For this reason, NK cells may maintain the balance between health and pathology in the lung [13].

The pathogenetic and regulatory role of NK and NKT-like cells in interstitial lung diseases (ILDs), fibrotic lung disorders of unknown etiology, are unclear. Very little data are available on this topic, unlike for chronic obstructive lung diseases, lung transplant, infections and cancer [14–17]. NK and NKT-like cells have been evaluated in several biological fluids, including bronchoalveolar lavage (BAL), a biological fluid representative of the alveolar airway compartment that provides important information on the complex immunopathogenesis of ILDs [18]. Assessment of NK cell subtypes by flow cytometry may be useful for discriminating ILDs during diagnosis, particularly because of immune regulatory properties of these cells in the modulation of inflammatory responses in BAL [19].

The study of NK cells in BAL from sarcoidosis patients began decades ago, revealing distinct phenotypes of NK cells involved in this granulomatous ILD [20]. More recently, significant NK depletion and dysfunction, suggesting altered innate immunity, were observed in two severe ILDs, namely idiopathic pulmonary fibrosis and rheumatoid arthritis associated with fibrotic lung involvement [21]. Morais et al. also reported a significant increase in NK cells in BAL from patients with ILD and lymphocytic alveolitis, suggesting that BAL NK immunophenotyping is useful in the differential diagnosis of ILDs, especially hypersensitivity pneumonitis [22].

The aim of this study was to investigate and compare NK and NKT-like cell percentages in BAL and peripheral blood of patients with different interstitial lung diseases, including granulomatous lung diseases such as chronic hypersensitivity pneumonitis and sarcoidosis, and fibrotic lung disorders, such as idiopathic pulmonary fibrosis and non-specific interstitial pneumonia.

Materials and methods

Study design and population

One hundred and ninety patients (94 male, mean age 61 ± 14.3 years) monitored at Siena Regional Centre for Sarcoidosis and other Interstitial Lung Diseases from 2015 to 2018 and undergoing bronchoscopy for ILD diagnostic work-up were enrolled in the study. Diagnosis of sarcoidosis, idiopathic pulmonary fibrosis (IPF), non-specific interstitial pneumonia (NSIP) and chronic fibrotic hypersensitivity pneumonitis (cHP) was performed according to international criteria [23–26]. The population was composed of: 115 patients with sarcoidosis, 24 with cHP, 43 with other ILDs (including 32 patients with IPF and 11 patients with NSIP) and 8 controls. Data of these consecutive ILD patients including lung function parameters are reported in Table 1. All patients underwent lung function tests, chest X-ray, high-resolution computed tomography of the chest (HRCT), blood sampling and bronchoscopy with BAL procedures in the frame of ILD diagnostic work-up. The data were entered in a specific database. Patients were not in therapy at the time of bronchoscopy. Sarcoidosis and cHP patients had subacute or chronic disease monitored at our referral centre; patients with acute variants, including Lofgren syndrome and acute lymphocytic HP, were excluded. Control subjects were matched for age and gender, had no history of asthma or allergy and were not under therapy of any kind. They had normal lung function parameters and chest X-ray picture. They were monitored for 12 months and did not develop any disease. Patients and controls were all Caucasians and gave their written informed consent to participation in the study, which was approved by our Local Ethics Committee C.E. A. V. S. E. (Code Number 180712).

Table 1 Demographic data, smoking status and functional parameters

	Sarcoidosis ($n=115$)	cHP ($n=24$)	Other ILD ($n=43$)	Healthy controls ($n=8$)	P values
Age, years (M ± S.D.)	56 ± 14.9	66.1 ± 10	71 ± 7.3	53.7 ± 12	< 0.0001*
Gender (f/m)	64/51	10/14	17/26	5/3	0.18
Smoking status (current- former/never)	49/66	11/13	27/16	3/5	0.13
Pulmonary function tests (M ± S.D.)					
FEV1(%) predicted value	96.4 ± 22.6	84.72 ± 22.9	82.6 ± 20	118 ± 14.8	0.0015*
FVC(%) predicted value	104.7 ± 22.8	81.5 ± 22.3	80.3 ± 20	131.4 ± 17.8	< 0.0001*
DLCO(%) predicted value	75.4 ± 18.1	64.6 ± 18	41.8 ± 15.1	85.6 ± 2.2	< 0.0001*
KCO(%) predicted value	87.9 ± 15.4	91 ± 22.3	67.9 ± 19.2	81.5 ± 0.8	< 0.0001*

Data are expressed as Mean ± standard deviation (M ± S.D.)

Bronchoalveolar lavage processing and flow cytometric analysis

Bronchoscopy with bronchoalveolar lavage was performed for diagnostic purposes in line with the European Respiratory Society Task Force Group on BAL [27]. Briefly, BAL was filtered through sterile gauze and cell count was determined by cytocentrifuge smear (600 rpm for 5 min) with a Diff quik stain kit (DiaPath, Italy); a total of 500 cells were counted. Cell viability was determined by Trypan blue exclusion in a Burker Chamber. The samples were processed by flow cytometry using a panel of monoclonal antibodies (BD Multitest™ 6-color TBNK, San Jose, CA, USA), including FITC-labeled CD3, PE-labeled CD16 and CD56, PerCP-Cy5.5-labeled CD45, PE-Cy7-labeled CD4, APC-labeled CD19 and APC-Cy7-labeled CD8 according to the manufacturer's instructions. At least 30,000 events were collected for each sample. Data were analyzed using DIVA software (BD-biosciences San Jose, CA, USA). Lymphocytes were distinguished on the basis of forward (FSC) versus side (SSC) scatters, and additional gating was applied using SSC versus CD45 to distinguish lymphocytes from cell debris. Specific panels were subsequently assessed to identify T lymphocytes, B lymphocytes and NK cells. T lymphocyte subpopulations were gated in order to distinguish $CD3^+CD4^+$ (T-helper), $CD3^+CD8^+$ (T-cytotoxic) and $CD3^+$ $CD16/56^+$ (NKT-like) cells. Peripheral lymphocyte subpopulations were likewise analyzed in patients and controls by the above protocol. Gating strategy is shown in Fig. 1.

Lung function tests

The following lung function measurements were taken according to ATS/ERS standard parameters using a Jaeger Body Plethysmograph with corrections for temperature and barometric pressure: forced expiratory volume in the first second (FEV1), forced vital capacity (FVC), carbon monoxide diffusing capacity (DL_{CO}), Tiffeneau index and carbon

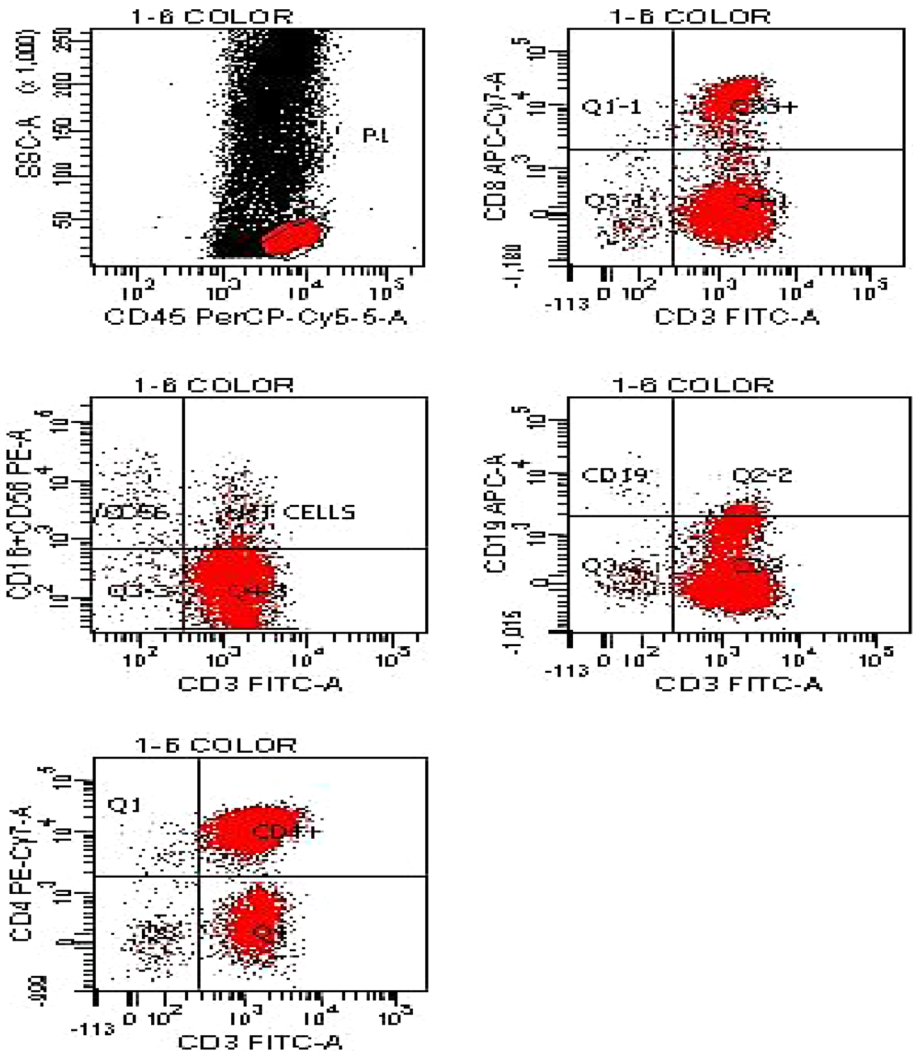

Fig. 1 Gating strategy of FACS measurements of lymphocytes subsets

monoxide transfer coefficient (KCO). All parameters were expressed as percentages of predicted values.

Statistical analysis

Results were expressed as mean and standard deviations (SD) or medians and quartiles (25th and 75th percentiles) for continuous variables. Data were not normally distributed. One-way ANOVA nonparametric test (Kruskal–Wallis test) and Dunn test were performed for multiple comparisons. Chi-squared test was used for categorical variables as appropriate. BAL cell population percentages were compared between groups. NK and NKT-like cell lymphocyte subpopulation percentages were also compared between groups, assessing areas under (AUC) the receiver operating characteristic curves (ROC). The Spearman test was used to look for correlations between variables. A p value less than 0.05 was considered statistically significant. Statistical analysis was performed by SPS Software (SPSS Inc., Chicago, IL, USA) and graphic representation of the data by GraphPad Prism 4.0 software.

Results

Study population

Demographic and functional parameters are reported in Table 1. No significant differences were observed with regard to gender and smoking habits between groups.

As expected, patients with other ILD groups were prevalently ex-smokers, over 65 years of age and male, whereas sarcoidosis patients were significatively younger than cHP and other ILD groups ($p < 0.05$, $p < 0.0001$, respectively), prevalently females who had never smoked. All data are shown in supplementary materials (S-Table 1).

Pulmonary function tests analysis

As expected, significant differences of FEV1(%) and FVC(%) were observed between sarcoidosis and other ILD ($p < 0.01$) and between other ILD and healthy controls ($p < 0.001$); differences were also observed for DLCO (%) between sarcoidosis and cHP ($p < 0.0001$), and other ILD ($p < 0.001$); and other ILD with healthy controls ($p < 0.05$). All data are reported in supplementary materials (S-Table 1).

Different percentages of NK and NKT-like cells in peripheral blood and BAL of ILD patients

Comparative analysis of BAL cell populations and T lymphocyte immunophenotypes are reported in Table 2. These data showed higher lymphocyte percentages in the sarcoidosis group than in the cHP and other ILD groups ($p = 0.0003$). BAL neutrophil and eosinophil percentages were significantly higher in the other ILD groups than in sarcoidosis and cHP patients ($p < 0.0001$). The percentages of BAL CD4 and CD8 cells and the CD4/CD8 ratio were significantly different in sarcoidosis patients than in cHP and other ILD groups ($p < 0.0001$). BAL NK cell percentages were significantly different between other ILD groups ($p = 0.02$). All data are reported in supplementary materials (S-Table 2).

Table 3 reported the differences of NK and NKT-like cells percentages among groups in BAL and PB. Lower NK cell percentages were observed in sarcoidosis patients than in the other ILD groups (IPF and NSIP) ($p < 0.05$) (Fig. 2a). Similar findings were obtained for NKT-like cells which were expressed differently in the different groups ($p = 0.004$) in particular between sarcoidosis and other ILD patients

Table 2 Bronchoalveolar lavage analysis and lymphocytes phenotyping

	Sarcoidosis ($n = 115$)	cHP ($n = 24$)	Other ILD ($N = 43$)	Healthy controls ($n = 8$)	P values
Total cell count ($\times 10^5$)	6.5 ± 3.8	9.5 ± 7.3	7.8 ± 4.3	4.0 ± 1.7	0.003*
Cell/mL ($\times 10^3$)	11.9 ± 17.9	12.8 ± 8.4	11.9 ± 5.6	17.6 ± 24.7	0.09
Macrophages (%)	71 (52–85)	78 (60–87.5)	69 (54.5–80.5)	79 (74.5–87)	0.08
Lymphocytes (%)	24 (11–40)	7.5 (5–17.5)	13 (6–21)	11 (7–19.5)	0.0004*
Neutrophils (%)	2 (0–4)	4 (1.5–10)	7 (3–16)	2 (0–5.5)	< 0.0001*
Eosinophils (%)	0 (0–1)	1 (0–4.5)	4 (1–10.5)	0 (0–6)	< 0.0001*
BAL lymphocyte phenotyping					
CD3 T cell (%)	94.8 (90.7–96.6)	93.9 (86.2–97.1)	92.2 (86–96.9)	92.5 (88.5–94)	0.26
CD4 T cell (%)	71.8 (57.8–80.8)	54.3 (36.6–63.4)	54.2 (42–68.8)	56 (52.9–66.5)	< 0.0001*
CD8 T cell (%)	19 (13.5–32)	40.3 (29.1–49.9)	32.2 (23.3–50)	30 (21.9–40.7)	< 0.0001*
CD4/CD8 Ratio	3.7 (1.8–6)	1.3 (0.8–2)	1.7 (1–3)	1.9 (1.2–2.9)	< 0.0001*
CD19 B cell (%)	0.8 (0.3–1.3)	1.25 (0.2–2)	1.4 (0.5–1.9)	0.7 (0.3–1.2)	0.11

Total cell count and cell/ml are expressed as M ± S.D., all other variables are expressed as median (25th and 75th percentiles)

Table 3 Comparison of BAL NK cells (%), BAL NK T-like cells (%) and peripheral blood lymphocytes CD16/56 NK Cells (%) among groups

	Sarcoidosis ($n=115$)	cHP ($n=24$)	Other ILD ($N=43$)	Healthy controls ($n=8$)	P values
BAL NK cells (%)	1.8 (0.8–3.3)	1.5 (1–4.5)	3 (1.1–6.3)	2.4 (2.2–3.7)	0.02*
BAL NK T-like cells (%)	4.1 (2.6–6.1)	5.5 (3.4–9)	6.3 (6.7–8.4)	5.7 (4.6–12)	0.004*
Peripheral blood lymphocyte CD16/56 NK cells (%)	12.2 (7.5–18.1)	10 (7.8–17.3)	14.2 (10.1–18.1)	7.3 (6–10.5)	0.3

All variables are expressed as median (25th and 75th percentiles)

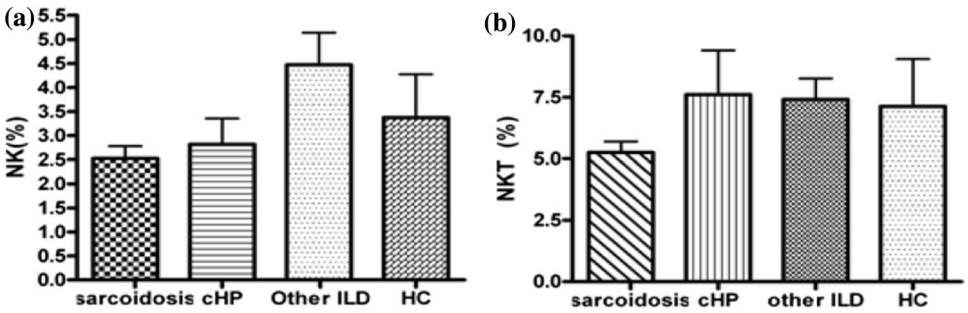

Fig. 2 **a** NK cells in sarcoidosis, cHP, other ILD, HC, **b** NKT-like cells in sarcoidosis, cHP, other ILD, HC

($p<0.05$) (Fig. 2b). Analysis of peripheral blood NK cells showed no significant differences between groups (Table 3). All data are reported in supplementary materials (S-Table 2). Different percentages of NK cells were also observed between BAL and peripheral blood in sarcoidosis (2.9 ± 4.1 and 13.6 ± 8.4, respectively) ($p<0.001$), in HP (2.8 ± 2.6 and 13.2 ± 8.7, respectively) ($p<0.001$) and in other ILDs (4.6 ± 4.7 and 17 ± 10.3, respectively) ($p<0.001$). Healthy controls showed normal BAL cell features including lymphocyte phenotypes. Analysis of NK and NKT-like cells percentages in BAL from controls revealed a pattern intermediate between the pathological groups, although the population was small. Chronic granulomatous lung disorders (sarcoidosis and cHP) showed median NK percentages of < 1.8%, in contrast with controls 2.4% and severe ILD (IPF and NSIP) 3% (Table 2). Median percentages of NKT-like cells were 4.8% in granulomatous lung diseases, 5.7% in controls and 6.2% in other ILDs (IPF and NSIP).

ROC curve analysis

ROC curves were plotted to assess the diagnostic value of NK and NKT-like cell percentages and to determine cutoff values with sufficient sensitivity and specificity between different ILD groups. Sarcoidosis patients showed the best area under the curve for NKT-like (AUC = 0.678, $p=0.0015$) and NK cells (AUC = 0.61, $p=0.001$) than cHP and other ILD patients. Cutoff values for NKT-like < 5.45% showed 70% and 56% sensitivity and specificity, respectively, in discriminating sarcoidosis patients from other groups. NK cutoff values < 1.65% showed sensitivity and specificity of 46% and 66%, respectively, in discriminating sarcoidosis patients from those of other groups.

Correlation with BAL features and functional parameters

We found a significant inverse correlation between NK and CD4 percentages ($r=-0.32$, $p=0.0004$) (Fig. 3a) and NKT-like and CD8 percentages ($r=-0.29$, $p=0.006$) (Fig. 3b) in BAL of sarcoidosis patients. As expected, the CD4/CD8 ratio in sarcoidosis was inversely correlated with NK and NKT-like cell percentages ($r=-0.21$ $p=0.04$ and $r=-0.34$ $p=0.001$, respectively). In our population of patients, NK and NKT-like cell percentages showed an inverse correlation with FVC ($r=-0.24$, $p=0.042$; $r=-0.30$, $p=0.0034$) (Fig. 4a), FEV1 ($r=-0.19$, $p=0.019$; $r=-0.30$, $p=0.0033$), DLCO ($r=-0.24$, $p=0.0043$; $r=-0.31$, $p=0.0024$) (Fig. 4b) and KCO ($r=-0.18$, $p=0.027$; $r=-0.21$, $p=0.05$). In the IPF-NSIP subgroup, NK cell percentages were inversely correlated with those of FVC ($r=-0.34$, $p=0.03$) and DLCO ($r=-0.47$, $p=0.0044$), while NKT-like cell percentages were inversely correlated with those of FEV1 ($r=-0.42$, $p=0.04$). No other significant correlations were found between NK or NKT-like cells and functional parameters.

Discussion

This study investigated NK and NKT-like cell percentages in BAL and peripheral blood of patients with different interstitial lung diseases, including granulomatous lung diseases

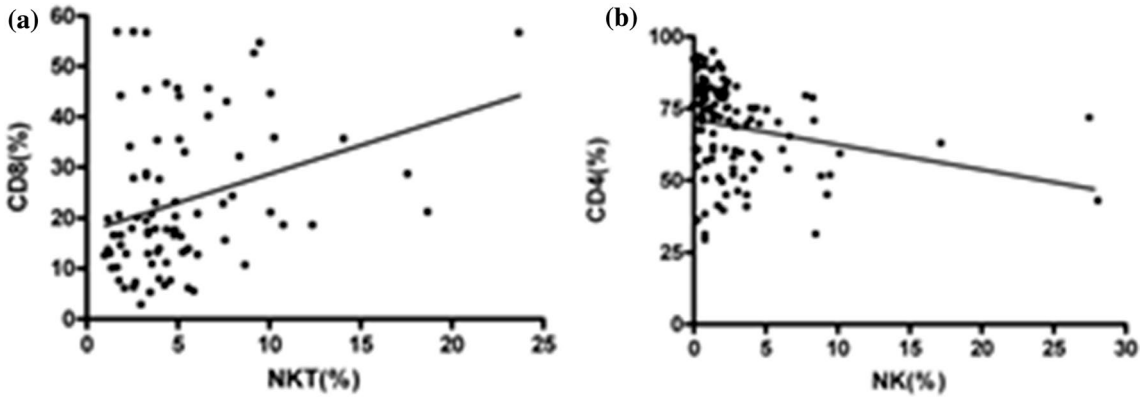

Fig. 3 **a** Correlation NKT–CD8$^+$ in sarcoidosis **b** correlation NK–**CD4**$^+$ in sarcoidosis

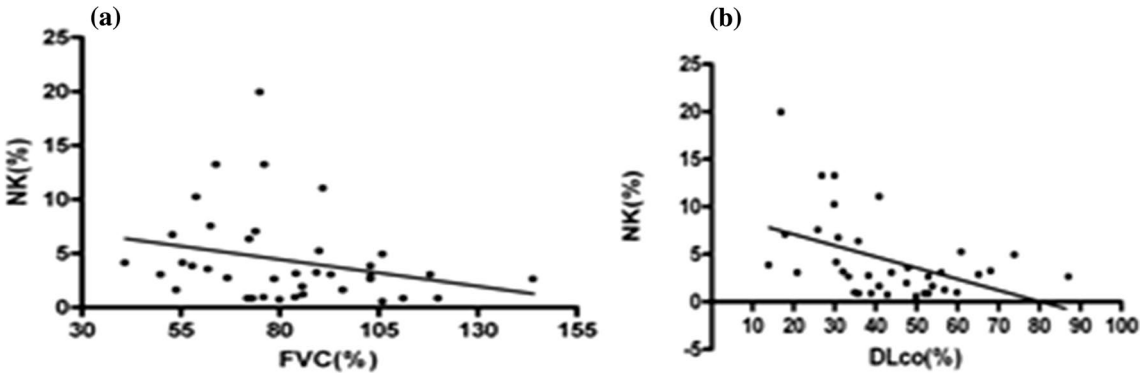

Fig. 4 **a** Correlation NK–FVC in other ILD group **b** correlation NK–**DLco** in other ILD

(such as chronic hypersensitivity pneumonitis and sarcoidosis) and fibrotic lung disorders (idiopathic pulmonary fibrosis and non-specific interstitial pneumonia). Comparative analysis of BAL cell populations and T lymphocyte immunophenotypes confirmed our previous findings and reports in the literature of a prevalent increase in BAL CD4 + lymphocytes in sarcoidosis and BAL neutrophils and eosinophils in the IPF and NSIP groups [28–32], NK and NKT-like cell percentages were expressed differentially in sarcoidosis and in fibrotic IPF and NSIP. Granulomatous lung diseases (chronic hypersensitivity pneumonitis cHP and sarcoidosis) were associated with few NK and NKT-like cells in BAL, unlike fibrotic progressive lung disorders (IPF and NSIP) where these cells were abundant. Healthy controls showed intermediate values but were necessarily a limited population because bronchoscopy is an invasive procedure.

Depletion of NKT-like cells in blood and BAL of patients with sarcoidosis has already been discussed in the literature [33, 34] and it is supposed that loss of NKT-like immunoregulatory functions may facilitate CD4 + lymphocyte overactivity, enabling granuloma formation (26). In our population of sarcoidosis patients, the good diagnostic sensitivity and specificity of NK and NKT-like cell percentages in discriminating sarcoidosis from other ILDs were documented and BAL, NK and NKT-like cell cutoff values were also determined. Moreover, NK cell percentages in BAL of sarcoidosis population were inversely correlated with CD4 + cell percentages, supporting the hypothesis that granulomatous inflammation is favored by NK deficiency [35], while the depletion of natural killer T cells in this disease results in CD8-mediated proinflammatory activity at alveolar level [31]. According to most studies, NKT-like cell percentages in BAL are unlikely to play a major role in the etiopathogenesis of HP since they are similar to those counted in healthy subjects (17, 31). On the contrary, significantly higher BAL CD3 + CD16/56 + NKT-like cell frequencies have been reported in BAL of HP patients than in patients with other ILDs [33], and recently there was an interesting report suggesting the potential utility of NK evaluation in BAL of lymphocytic HP patients to differentiate this lung disorder from other ILDs [22]. In the latter study, significantly higher NK cell counts were found in BAL from a population of Portuguese HP patients than in controls. Unfortunately, the results cannot be compared

with ours as the authors selected HP patients with lymphocytic alveolitis (BAL lymphocytes > 15%), also including acute forms of HP. Moreover, all the patients came from a region in which avian and mold antigen levels incomparably higher than in our patient population have been documented [22, 36]. Another important difference was that the authors excluded OP patients from their cohort, whereas we included all chronic HP without distinction of lymphocytic alveolitis and radiological HRCT patterns, only excluding acute forms. They underlined the need for standardization of diagnostic guidelines for HP to avoid such heterogeneity that prevents comparison of groups. As already demonstrated, peripheral blood NK and NKT-like cell percentages did not reflect immunoinflammatory reactions occurring in ILD, and there were no significant differences between NK and NKT-like cell percentages [22, 33]. Only BAL represented the alveolar microenvironment and pathogenic alterations occurring in ILD, since most of these diseases are limited to the lungs [37]. NK and NKT-like cell percentages in BAL of ILD patients proved to be potential biomarkers, being negatively correlated with several lung function parameters; in particular NK cells were overexpressed in patients with low FVC and DLCO percentages affected by IPF-NSIP [38–40] and this result calls for further investigation. In conclusion, NK and NKT-like cell populations are expressed differently in BAL from patients with interstitial lung diseases, being significantly depleted in sarcoidosis patients with respect to other ILDs. A protective role of these cells in the pathogenesis of sarcoidosis may be suggested by our study, which also demonstrates a pathogenic role of these natural killer subgroups in fibrotic lung disorders.

Author contributions LB conducted the study. LB and PC helped to define the study objectives and to coordinate the study. LB, PC and Md performed the statistical analysis and interpreted the results, CL, RMR and MP collected the data, MS and CV performed experiment, LB, EB and PS wrote the first draft of the manuscript. All authors critically revised the manuscript and approved its final version.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (Local Ethics Committee C.E. A. V. S. E. (Code Number 180712) and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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