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Activated CD8+ T cells and natural killer T cells in bronchoalveolar lavage fluid in hypersensitivity pneumonitis and sarcoidosis

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Abstract. *Background:* Sarcoidosis and hypersensitivity pneumonitis are diffuse parenchymal lung diseases characterized by formation of non-caseating granulomas with a bronchocentric distribution. Analysis of the white blood cell differential profile in bronchoalveolar lavage fluid can be a useful supplement in the diagnostic work-up. *Objective:* Diagnostic markers that can improve the discrimination of sarcoidosis and hypersensitivity pneumonitis are wanted. *Methods:* Bronchoalveolar lavage fluid fractions of $CD4^+$ and $CD8^+$ T cells expressing the activation marker HLA-DR and fractions of natural killer T cells determined by flow cytometry were investigated in sarcoidosis (N=83), hypersensitivity pneumonitis (N=10) and healthy control subjects (N=15). *Results:* In hypersensitivity pneumonitis, natural killer T cell fractions were over 7-fold greater [median (IQR): 5.5% (3.5-8.1) versus 0.7% (0.5-1.2), p<0.0001], and HLA-DR^+ fractions of $CD8^+$ lymphocytes were almost two fold greater [median (IQR): 79% (75-82) versus 43% (34-52), p<0.0001] than in sarcoidosis. In healthy control subjects, natural killer T cell fractions of leucocytes and HLA-DR^+ fractions of $CD8^+$ lymphocytes were lower [median (IQR): 0.3% (0.3-0.6) and 30% (26-34), p=0.02 and p=0.01 compared to sarcoidosis]. The combined use of these two markers seems to discriminate the diseases very well. *Conclusion:* This study suggests a role for the bronchoalveolar lavage fluid lymphocyte subsets HLA-DR^+ $CD8^+$ T cells and natural killer T cells in the diagnostic work up of sarcoidosis and hypersensitivity pneumonitis. *(Sarcoidosis Vasc Diffuse Lung Dis 2014; 31: 316-324)*

Key words: BALF, hypersensitivity pneumonitis, harcoidosis, NKT cells, HLA-DR, lymphocyte subsets

Abbreviations:
BALF: Bronchoalveolar lavage fluid, DPLD: diffuse parenchymal lung diseases, HP: hypersensitivity pneumonitis, HRCT: High Resolution Computed Tomography, HLA: Human leucocyte antigen, NKT cell: Natural killer T cell, TCR: T cell receptor, IQR: Inter quartile range.

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Introduction

Sarcoidosis and hypersensitivity pneumonitis (HP) are diffuse parenchymal lung diseases (DPLD) characterized by formation of non-caseating granulomas with a bronchocentric distribution. In HP, the inflammation is an immune response to an inhaled antigen in predisposed individuals (1), and over 300 antigens, predominantly organic in nature, have been identified. In contrast, the inciting antigen in sarcoidosis has not yet been identified(2), but an inhaled antigen is suspected (3, 4).

Analysis of the white blood cell differential profile in bronchoalveolar lavage fluid (BALF) can be a

useful supplement in the diagnostic work-up of DPLD (5). Although not diagnostic, relative and absolute number of lymphocytes are increased in sarcoidosis and HP (6). The lymphocytic alveolitis in sarcoidosis is dominated by $CD4^+$ T cells with an increased CD4/CD8 ratio. In contrast, the alveolitis in HP is often dominated by $CD8^+$ T cells, resulting in an inverted CD4/CD8-ratio, together with an increase in relative numbers of mast cells, neutrophils and foamy alveolar macrophages(7). However, considerable overlaps are seen between the diseases, as the CD4/CD8 ratio can also be increased in HP, and different causes of HP may cause different BALF cellular patterns (7, 8)

Lymphocyte subsets in the BALF may be identified and quantified by the use of monoclonal antibodies and flow cytometry. In sarcoidosis and HP, there is an increased expression of the activation marker HLA-DR on T-lymphocytes in the BAL fluid. HLA-DR expression is increased in $CD4^+$ T cells in both diseases (9, 10), and is lower in $CD8^+$ T cells in sarcoidosis than in HP (10, 11). Natural Killer T (NKT) cells, a subset of T cells expressing the NK cell markers CD56 and/or CD16, are increased in HP(12). As far as we are aware, the combined use of these subsets has not been reported previously in HP and we report our findings on these two lymphocyte subsets in sarcoidosis, HP and healthy controls.

Materials and methods

Study population

Between September 2007 and September 2012, bronchoscopy and flow cytometric analysis of BALF samples were performed in all patients investigated for suspicion of DPLD in our institution. Of these patients, 146 were given a clinical diagnosis of sarcoidosis or HP, and were eligible for inclusion in the study. Patients on systemic corticosteroid or other immunosuppressive treatment or active cancer at the time of bronchoalveolar lavage were excluded from the study. In addition, patients who did not meet the diagnostic criteria for sarcoidosis and HP were excluded. The final diagnoses of the included patients were sarcoidosis (N=83) and HP (N=10) (figure 1). Sixteen patients presented with Löfgren's syndrome.

Healthy control subjects (N=15) with no known history of asthma or allergy were recruited by advertisement on the hospital web-site. All subjects had normal CO diffusing capacity. The FEV1/FVC ratio was below 0.7 in one subject (0.67), a second subject had a FEV1 of 76% of predicted value and a third subject was on anti-hypertensive therapy.

Demographics of the study population are presented in table 1.

The Regional Ethics Committee assessed that use of patient data did not require ethical approval as it was considered to be a quality control of a routine clinical investigation (Ref.nr.: 2009/909-2). Ethical approval (Ref.nr.: 2010/1939-4) and written informed consent were obtained for bronchoscopy with bronchoalveolar lavage in control subjects.

Diagnostic criteria

The diagnostic criteria of sarcoidosis were a clinical and radiological pattern consistent with sarcoidosis, presence of non-caseating granulomas on biopsy, and exclusion of other known causes of granulomatous diseases(13). Histological demonstration of granuloma was not required for patients with classic features of Löfgren's syndrome, defined as fever, erythema nodosum and/or ankle arthritis together with bilateral hilar lymphadenopathy.

Patients with HP had at least 4 of the major criteria and 2 of the minor criteria as suggested by Schuyler et al. (14). The major criteria are: symptoms

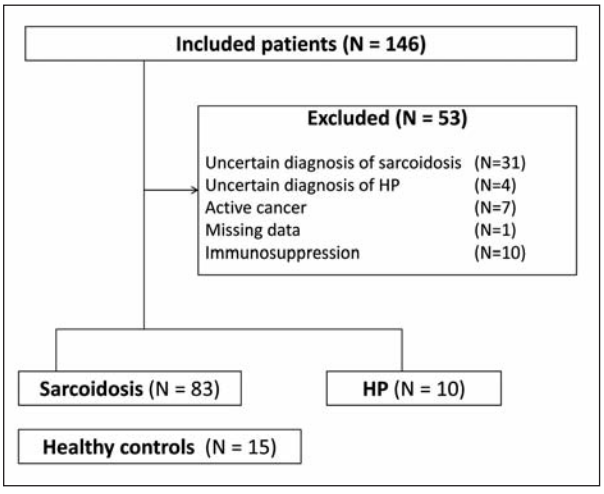

Fig. 1. Study population. HP: Hypersensitivity pneumonitis

Table 1. Demographics and BAL differential cell counts of study population

	Sarcoidosis (N=83)	HP (N=10)	HC (N=15)	P
Age, years*	48 (18-77)	54 (39-72)	35 (21-53)	0.003
Sex (male/female)	61/22	6/4	8/7	0.22
Smoker/non-smoker	6/76	0/10	0/15	0.77
Total cell count (x 10^6/ml)	0.19 (0.14-0.29)	0.39 (0.25-0.53)	0.14 (0.12-0.17)	0.0009
Lymphocytes (%)	31 (21-52)	69 (66-74)	14 (10-25)	<0.0001
Neutrophils (%)	2 (1-6)	5 (3-6)	4 (2-5)	0.35
Eosinophils (%)	0 (0-1)	4 (1-7)	0 (0-0)	0.0009
Alveolar macroph. (%)	57 (39-75)	21 (15-28)	81 (69-85)	<0.0001

Fisher exact test was used for categorical variables and Kruskal-Wallis test for continuous variables. *Data shown as median (range). All other data are shown as median (IQR). HP: Hypersensitivity pneumonitis. HC: Healthy control subjects

compatible with HP, serologic evidence or a history of antigen exposure, chest radiograph or High Resolution Computed Tomography (HRCT) findings compatible with HP, lymphocytosis in the BALF, compatible histologic findings on lung biopsy and reproduction of symptoms after exposure to the antigenic environment. The minor criteria are: bibasilar rales, decreased diffusing capacity and arterial hypoxemia.

BAL procedure and flow cytometry

Bronchoscopy with bronchoalveolar lavage was performed in accordance with recommendations(15), as previously described(16). In brief, lavage was done with 2-3 aliquots of 60 ml phosphate buffered saline (Hospital pharmacy, Haukeland University Hospital, Bergen, Norway) under local anaesthesia with lignocaine and intravenous sedation with midazolam and alfentanyl. The first fraction was used for microbiological or cytological analyses, and the remainder of the lavage fluid was pooled, filtered through nylon gauze and kept at 4° C until processing.

The total cell count was made by ADVIA 120 Hematology System (Siemens AG, Erlangen, Germany). Differential cell counts of a minimum of 300 cells were performed on cytospin preparations stained with May Grünwald/Giemsa.

Cell pellets were prepared by centrifugation for 5 min at 1500 rpm. Tubes containing 0.5-1.0 x 10^6 cells per tube were incubated for 15 minutes with selected antibodies as detailed in table 2. The cells were then washed twice in 2 ml phosphate buffered saline containing 0.1% bovine serum albumin (Dulbecco A, Sigma-Aldrich, St. Louis, USA).

Table 2. Antibody panel

Tube	Antibody conjugates			
	FITC	PE	PerCP-Cy5.5	APC
1	Isotype control[1]	Isotype control[1]	Isotype control[2]	Isotype control[1]
2	CD8[1]	CD4[1]	HLA-DR[2]	CD3[1]
3	CD3[3]	CD16[3] and CD56[3]	CD45[3]	CD19[3]

[1] Multimix, Dako Denmark AS, Glostrup Denmark, [2] BD Biosciences, San Jose, USA, [3]MultiTEST™, BD Biosciences. FITC: Fluorescein isothiocyanate, PE: phycoerythrobilin, PerCP-Cy5.5: Peridinin chlorophyll protein with cyanine dye (Cy5.5), APC: Allophycocyanin

Flow cytometry of a minimum of 10.000 cells was performed with a FACS Canto I flow cytometer with FACS DIVA software (BD Biosciences, Mountain view, CA, USA). Lymphocytes were identified as $CD45^+$ cells with low side and forward scatter. An isotype control tube was used to set the gates to discriminate HLA-DR^+ and HLA-DR^- lymphocytes. NKT cells were defined as $CD3^+$ lymphocytes with expression of CD16/CD56, and the gates were set by visual comparison to the dominating population of CD16/$CD56^-$ T cells. In October 2010, the tube for identification of NKT cells was changed in our laboratory, and NKT cells in subsequent patients (Sarcoidosis: N=30, HP: N=4, HC: N=15) were defined as $CD3^+$ lymphocytes with expression of CD56 (CD16 was omitted from the antibody panel). Gating strategy is presented in figure 2.

Statistical methods

Group comparison of continuous data was done with Kruskal-Wallis test. Post hoc analyses were performed with pairwise Wilcoxon rank sum test and

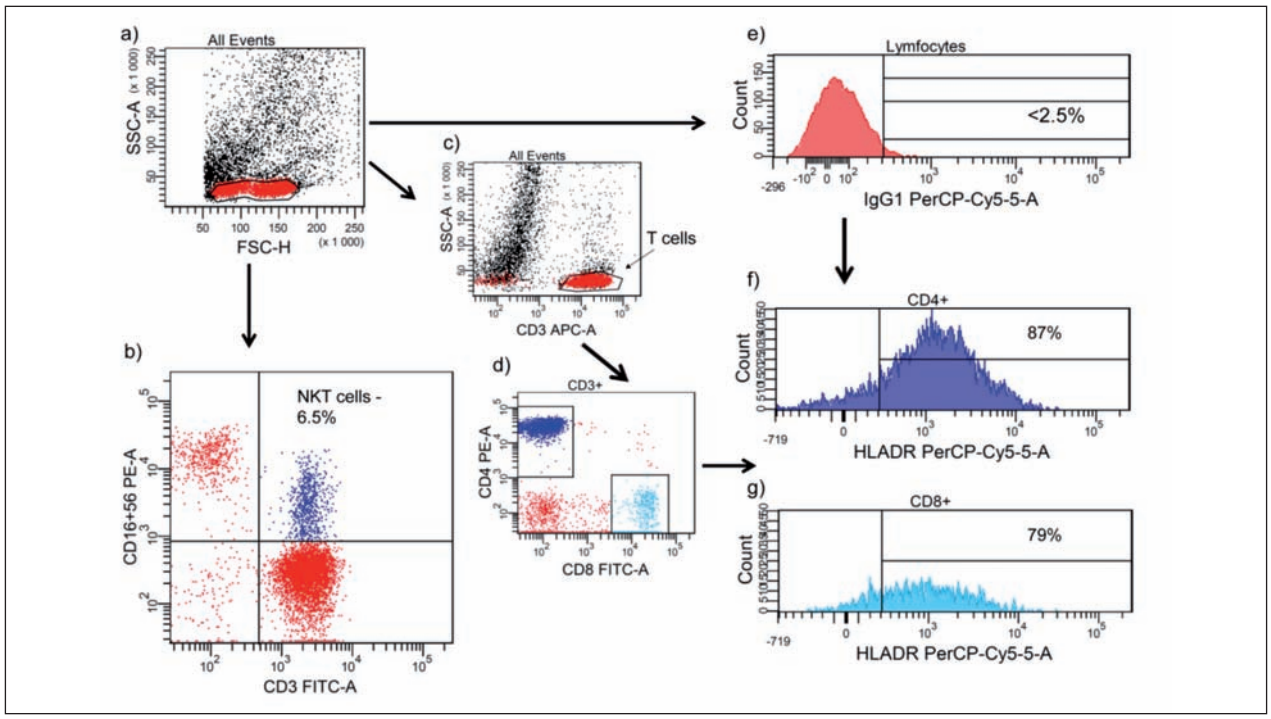

Fig. 2. Gating strategy. The example is a typical HP patient. a) Lymphocytes are gated by their low forward and side scatter signal. b) From the lymphocytes, the T-cells, NKT cells and NK cells (CD3-CD16/56+) are gated by expression of CD3 and CD16/CD56. Numbers represent fractions of leucocytes (CD45+ BALF cells). c) T cells are gated as $CD3^+$ lymphocytes. d) CD4+ and CD8+ T cells. e) In the isotype control tube, the gates for HLA-DR were set so that > 97.5% of the lymphocytes was assigned negative. f) and g) HLA-DR-gates from e) were used to discriminate HLA-DR^+ CD4+ and CD8+ T cells. In October 2010, the tube for identification of NKT cells was changed in our laboratory, and NKT cells in subsequent patients (Sarcoidosis: N=30, HP: N=4, HC: N=15) were defined as $CD3^+$ lymphocytes with expression of CD56 (CD16 was omitted from the antibody panel).

the Bonferroni correction for multiple comparisons. Categorical data were analyzed with Fishers exact test. A p value of < 0.05 was considered to be statistically significant. Statistical analyses were done in R: A Language and Environment for Statistical computing (R Core Team, R Foundation for Statistical Computing, Vienna, Austria).

Results

In HP, the BALF NKT cell fraction was over 7-fold and 18 -fold greater, respectively, than in sarcoidosis and controls [median (IQR): 5.5% (3.5-8.1) versus 0.7% (0.5-1.2), p<0.0001, and versus 0.3% (0.3-0.6), p=0.0001]. The HLA-DR^+ fraction of $CD8^+$ lymphocytes were almost two- fold and 2.5-fold greater, respectively, than in sarcoidosis and controls [median (IQR): 79% (75-82) versus 43% (34-52), p<0.0001, and versus 30% (26-34), p=0.0001]. HLA-DR $CD4^+$ lymphocyte fraction was almost one and a half- fold and two- fold greater, respectively, than in sarcoidosis and controls [median (IQR): 89% (81-93) versus 69% (60-79), p=0.0002, and versus 45% (36-57), p=0.0001] (Figure 3).

Typical patterns of NKT cells and HLA-DR^+ $CD8^+$ cells in HP and sarcoidosis are shown in figure 4. The difference in HLA-DR expression on $CD4^+$ T cells in HP compared to sarcoidosis patients was less striking in comparison to that on $CD8^+$ cells (figure 5b). The combination of high fractions of NKT cells and HLA-DR^+ $CD8^+$ T cells was more suggestive of HP than sarcoidosis (figure 5).

The NKT cell fraction and HLA-DR^+ fraction of $CD8^+$ and $CD4^+$ lymphocytes were higher in sarcoidosis compared to controls (p=0.02, p=0.01 and p < 0.0001).

As expected, the CD4/CD8- ratio was higher in sarcoidosis compared to HP and healthy controls (p=0.006 and p=0.010) (Fig. 3).

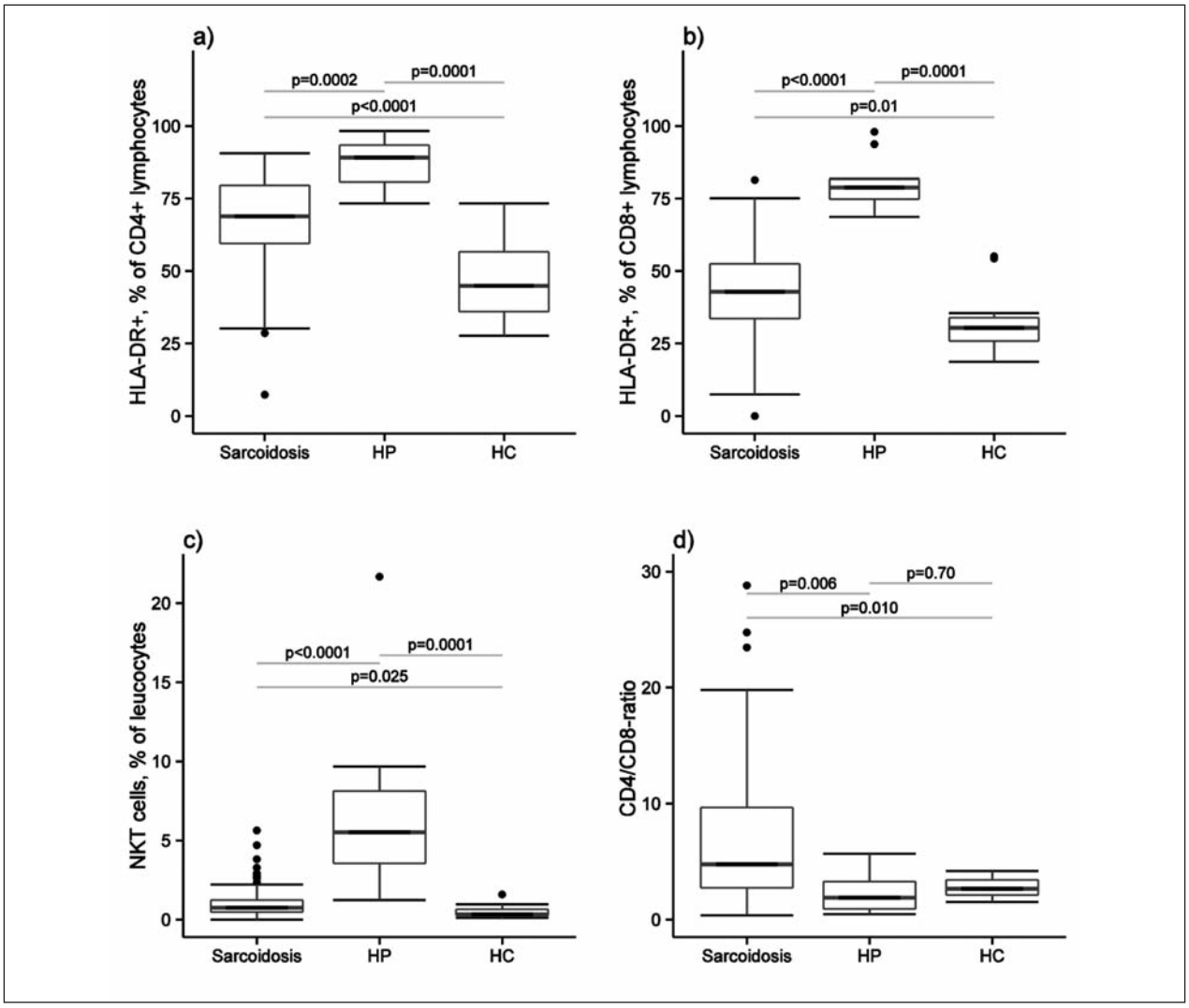

Fig. 3. Higher fractions of NKT cells and HLA-DR$^+$ CD4$^+$ and HLA-DR$^+$ CD8$^+$ T cells in hypersensitivity pneumonitis than in sarcoidosis and healthy controls. a) HLA-DR expression on CD4$^+$ was higher in sarcoidosis than healthy control subjects, while HP patients had highest expression of HLA-DR on CD4$^+$ T cells of all groups. b) HLA-DR$^+$ fractions of CD8$^+$ T cells, and c) fraction of NKT cells were highly significant raised in HP versus sarcoidosis and HC, and significantly higher in sarcoidosis compared to healthy control subjects. d) HP patients have lower CD4/CD8-ratio than patients with sarcoidosis. Kruskal-Wallis with post hoc multiple comparison and p-value adjustment with Bonferroni correction. HP: Hypersensitivity pneumonitis; HC: Healthy control subjects.

Differential cell counts in BALF are presented in table 1. BALF lymphocyte fractions were more than two-fold greater in patients with HP compared to sarcoidosis (p=0.0001). Fractions of eosinophils were higher in HP patients (p=0.008), and fractions of alveolar macrophages were almost threefold greater in patients with sarcoidosis compared to HP (p<0.0001). There was a strong trend towards higher total cell count in HP compared to sarcoidosis, although this was not significant after multiple comparisons correction (p=0.052).

Discussion

This study investigated the HLA-DR$^+$ fractions of CD8$^+$ and CD4$^+$ T cells and fractions of NKT cells in BALF in patients with sarcoidosis, HP and

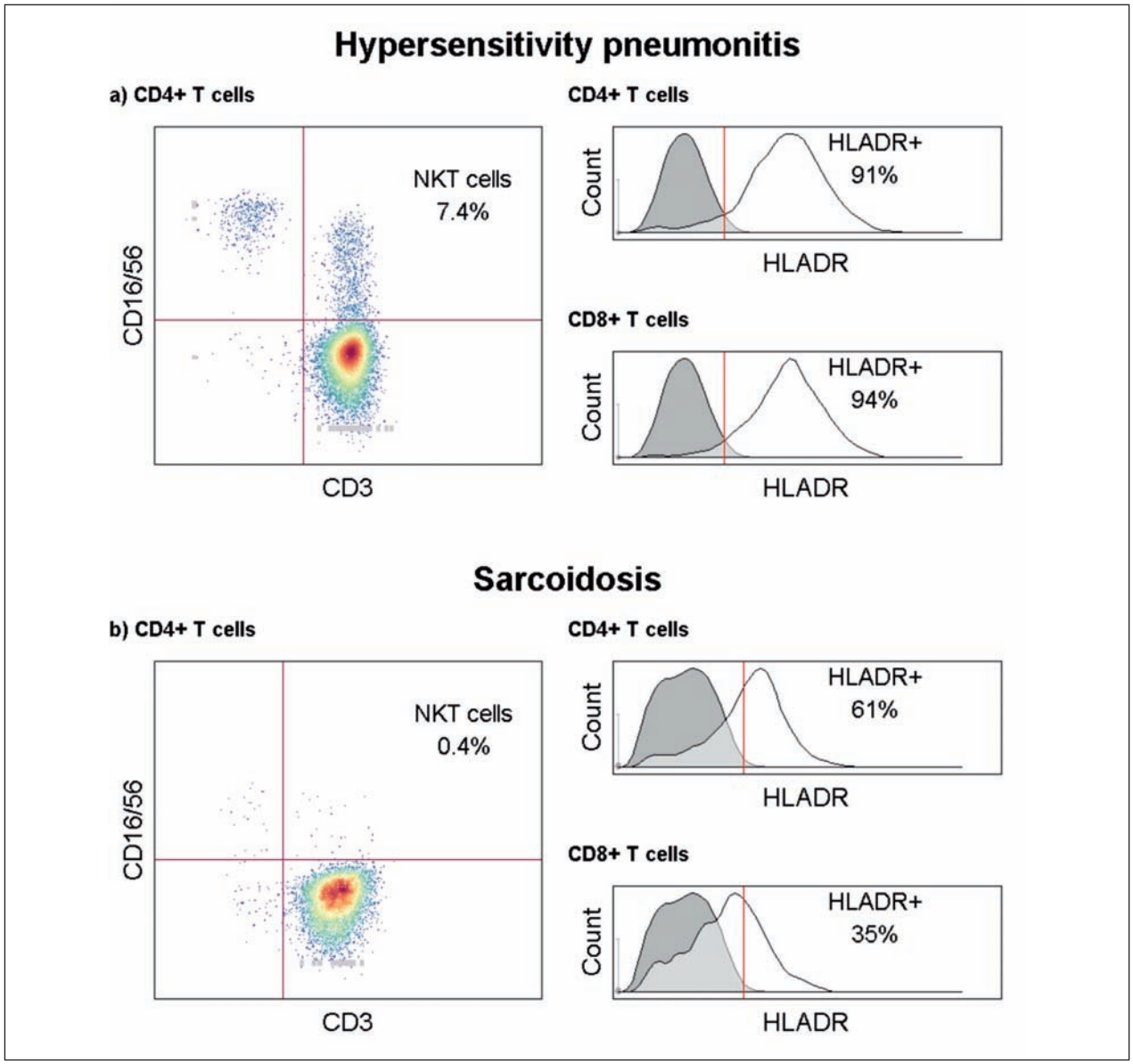

Fig. 4. Flow cytometry scatter plots and histograms in Hypersensitivity pneumonitis and Sarcoidosis. Scatterplot of NKT cells and histograms displaying HLA-DR$^+$ CD4$^+$ and CD8$^+$ lymphocytes for a) a typical hypersensitivity pneumonitis patient, and b) a typical sarcoidosis patient. NKT cells are displayed in fraction of leucocytes. HLA-DR$^+$ CD4$^+$ and CD8$^+$ T cells are fractions of CD4$^+$ and CD8$^+$ T cells, respectively.

healthy control subjects. In addition to a decrease in the CD4/CD8 ratio and an increase in lymphocytes and eosinophils, HLA-DR CD8$^+$ and NKT lymphocyte subsets were augmented in HP compared to sarcoidosis patients. To our knowledge, these subsets have not previously been investigated simultaneously. Together, fractions of HLA-DR$^+$ CD8$^+$ T cells and NKT cells in BALF seem to discriminate very well between HP and sarcoidosis, suggesting that these markers may be a valuable tool in the diagnosis of HP and sarcoidosis. Fractions of HLA-DR$^+$ CD4$^+$ T cells, on the other hand, may be less useful in discriminating HP from sarcoidosis.

The combined use of NKT cells and HLA-DR expression on CD8$^+$ T cells in the BALF as a diagnostic tool in HP has not been addressed in prior

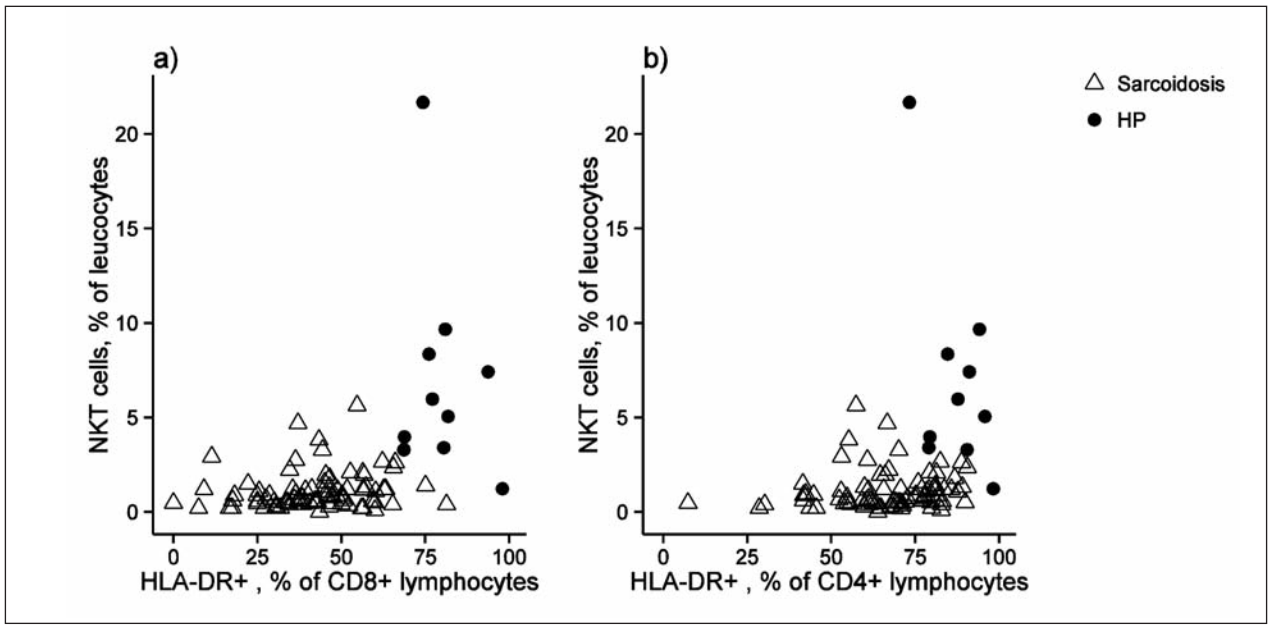

Fig. 5. Fractions of HLA-DR$^+$ CD8$^+$ T cells and NKT cells discriminate patients with hypersensitivity pneumonitis from patients with sarcoidosis. Scatterplot of a) HLADR$^+$CD8$^+$ lymphocytes, and b) HLADR$^+$CD4$^+$ lymphocytes versus NKT cell fraction grouped for patients with hypersensitivity pneumonitis (HP) or sarcoidosis.

studies. We previously reported that the use of these markers improve diagnostic accuracy in sarcoidosis(16). A greater fraction of NKT cells in HP than in sarcoidosis has been previously reported in a study by Korosec et al. (12) who like us defined NKT cells as CD3$^+$CD16/56$^+$ lymphocytes. In that study, NKT cells are reported as a proportion of lymphocytes, while we found it more suitable to report NKT cells as a fraction of leucocytes. In patients with low lymphocyte fractions, noise in the flow cytometry gates has a greater influence on the NKT cell fractions, if reported as fractions of the lymphocytes. When recalculated as fractions of lymphocytes, we found a median of 2.0% NKT cells in patients with sarcoidosis and 7.6% in patients with HP (p=0.0004), while they reported a median fraction of NKT cells of 3% and 11% in sarcoidosis and HP.

Higher fractions of HLA-DR$^+$ CD8$^+$ T cells in HP compared to sarcoidosis, has been reported in one study(11). The difference in HLADR$^+$ fractions of CD8$^+$ T cells in HP compared to sarcoidosis reported by Heron et al. was less pronounced (median 96% versus 80%), although the principle findings were similar. Interestingly, they reported a higher fraction of HLA-DR$^+$ CD8$^+$ T cells in patients with sarcoidosis and parenchymal involvement (radiologic stage II and higher) versus patients without parenchymal involvement (median: 93% versus 77%), while this was not found in our study (median: 47% versus 42%, p=0.20). Dissimilarities in the patient selection, anti-HLA-DR antibodies and flow cytometry set-up may account for the variations.

Our study has limitations. Firstly, the number of patients with suspected HP is small in our clinic, and only 10 patients did fulfill the diagnostic criteria for HP. The frequent inability to make a certain diagnosis of a specific DPLD, may lead to a tendency that only the typical patients with HP are correctly diagnosed, while less typical patients more often are classified as unspecified DPLD. Secondly, our data are insufficient to estimate the diagnostic accuracy of NKT cell and HLA-DR$^+$CD8$^+$ lymphocyte fractions as a test to discriminate HP from sarcoidosis. This would have required a greater number of patients with HP. However, a visual display of these two markers in combination, as shown in figure 5a, suggests that they may discriminate patients with HP from sarcoidosis. Finally, due to a change in the antibody panel in October 2010, NKT cells were de-

fined as T cells expressing CD56 rather than CD16/CD56 in a subset of the patients. This may have resulted in lower fractions of NKT cells in these patients and healthy control subjects. However, we found no significant difference between NKT cell fractions in sarcoidosis or HP patients investigated with the former versus the newer antibody panel ($p=0.60$ and $p=0.48$), whereas the NKT cell fractions were significantly different in HP compared to sarcoidosis also in the subgroup of patients analyzed with the former antibody panel and in sarcoidosis compared to healthy controls analyzed with the newer antibody panel (data not shown). These results show that the change of antibody panel has not influenced our principle findings. In addition, Korosec et al. reported that NKT cells in patients with HP are mainly $CD56^+$ (12), and thus would stain as positive also with the newer antibody panel.

NKT cells are a heterogeneous population of cells with elements of both the innate and the adaptive immune system, capable of rapid responses to antigens with cytotoxic NK cell activity and production of Th1 or Th2 cytokines. They are classified according to their TCR, which can be invariant (CD1d-restricted invariant NKT cells) or have normal variability in antigen specificity. $CD8^+CD56^+$ T cells with an αβ TCR have been implicated in antitumor immunity(17). The contribution of the various NKT cell subsets and activated T cells to the pathogenesis of either sarcoidosis or HP is not yet elucidated. In HP, most of the NKT cells in BALF have αβ TCR(12), and are $CD8^+CD56^+$, capable of both antigen specific and NK-like cytolytic activities(18). On the other hand, deficiencies and impaired INF-γ production of CD1d restricted invariant NKT cells in blood and BALF have been reported in sarcoidosis(19, 20). In this study, the NKT cells are defined as $CD16/CD56^+$ $CD3^+$ lymphocytes. These cells are strikingly increased in patients with HP compared to sarcoidosis and HC, indicating a role of these cells in the immunopathology of HP.

HLA-DR expression on T cells is a well known activation marker, and may play a role in regulating or limiting a specific T cell response to an antigen(21). The fraction of $HLA\text{-}DR^+$ $CD8^+$ lymphocytes increases in some autoimmune diseases, as well as with ageing, possibly as a part of normal immunoregulation(22), although its exact role is unclear. Activated $CD4^+$ T cells of Th1 type produce INF-γ, and is essential for the formation of granulomas in sarcoidosis(23). The finding of increased expression of HLA-DR on $CD4^+$ lymphocytes in sarcoidosis may reflect the prominent role of activated Th1 cells. In contrast, the $HLA\text{-}DR^+$ $CD8^+$ T cells may be involved in the immunology of HP. It may seem surprising that cytolytic T cells exhibit strong activation in HP. The most important biological function of cytolytic T cells is to kill infected cells and cancer cells. Infection is not a central element in HP even though many kinds of fungi and bacteria have been identified as important sensitizing environmental agents. Contrary to this, helper T cells are central both in activating allergen-clearing macrophages and in the production of allergen-specific antibodies, which are among the important characteristics of the disease.

Identifying exposure to an antigen known to cause HP is often difficult, and surgical lung biopsy may be necessary to confirm the diagnosis. However, surgical lung biopsy may not be achievable in all patients, due to procedure related risks, patient preferences or the severity of the disease. Therefore, our demonstration of a BALF lymphocyte subset pattern that may discriminate HP from sarcoidosis may be relevant and helpful in this clinical situation. In addition, the considerable overlap in CD4/CD8 ratio seen in patients with HP and sarcoidosis in the present study emphasize the need for more accurate diagnostic markers.

In conclusion, our study suggests a role for the combined use of NKT cell and $HLA\text{-}DR^+CD8^+$ T cell fractions in BALF as diagnostic markers in HP and sarcoidosis. However, further studies with a greater number of patients with HP are necessary to evaluate the diagnostic accuracy of these lymphocyte subsets. In addition, functional studies on NKT cells and $HLA\text{-}DR^+CD8^+$ T cells in BALF may provide important clues to our understanding of the immunopathogenesis of both diseases.

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