SARCOIDOSIS VASCULITIS AND DIFFUSE LUNG DISEASES 2014; 31; 142-148

Sarcoidosis and Purified Protein Derivative reactivity

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Abstract. *Background:* The possible association between (tuberculous and nontuberculous) mycobacterial infections and sarcoidosis is still a matter of dispute. Using diagnostic tests for specific T-cell responses, this association can be investigated in an innovative manner. *Objective*: To measure the T-cell responsiveness to the purified protein derivative (PPD) antigen in blood and broncho-alveolar lavage (BAL) fluid in patients with sarcoidosis and patients with other causes of interstitial lung disease. It was hypothesized that if a mycobacterial infection of the lung is of importance for the development of sarcoidosis, T-cell responsiveness towards the PPD antigen would be increased in patients with sarcoidosis when compared to patients with other causes of interstitial lung disease. *Methods*: A single-center study was conducted which included patients with and without sarcoidosis. Venous blood was collected and BAL was performed for, inter alia, Interferon Gamma Release Assay´s (IGRA) with different stimulating antigens, including PPD, ESAT-6, CFP-10 and, as a control, Epstein-Barr virus (EBV). *Results:* A total of 118 patients were included. There is no difference between PPD reactivity in BAL fluid in patients with or without sarcoidosis. In patients without sarcoidosis, ELISpot PPD in blood shows more reactivity compared to patients with sarcoidosis, although this difference is not significant. ELISpot EBV and TB results are not significant different between both groups. *Conclusion:* These results provide no evidence for the involvement of different mycobacteria in the pathogenesis of sarcoidosis. *(Sarcoidosis Vasc Diffuse Lung Dis 2014; 31: 142-148)*

Key words: Sarcoidosis, Mycobacteria, PPD-reactivity, Interferon Gamma Release Assay, Immunodiagnostics

Introduction

Sarcoidosis is a multi-organ granulomatous disorder of unknown etiology, characterized by non-caseating granulomas involving the lung, lymph-nodes or other organs. Several possible causes are discussed in the literature, including genetic, immunological, environmental and infectious influences. The association between (*tuberculous and nontuberculous*) mycobacterial infections and sarcoidosis has been investigated in different studies, leading to varied and inconclusive results (1,2). Most of these studies use acid-fast stains, cultures or identification of bacterial nucleic acids for detection of mycobacteria.

With cellular immune assays, the association between different mycobacteria and sarcoidosis can be investigated in an innovative manner. Interferon-γ release assay´s (IGRA) stimulate T-cells using mycobacterial specific antigens, resulting in interferon-γ production when patients are already sensitized to mycobacterial antigens (3). During active infection, T-cells are clonally increased and recruited to the site of

Received: 13 June 2013
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infection (4,5). Therefore, IGRA's on mononuclear cells obtained from the site of infection might be of additional diagnostic value in patients with active infection. Wilkinson et al. (6) showed a much higher concentration of Early Secreted Antigen Targeted-6 (ESAT-6) specific T-cells in pleural effusions compared to peripheral blood in patients with pleural tuberculosis (TB). Jaffari et al. (7) demonstrated that IGRA's might be of additional diagnostic value for the diagnosis of pulmonary TB when performed on specimens from the site of infection, such as cells obtained from bronchoalveolar lavage (BAL). In our laboratory, we developed an IGRA on BAL mononuclear cells, using purified protein derivative (PPD) as stimulating antigen for rapid detection of Bacillus Calmette-Guérin (BCG) related pulmonary disease resulting from intravesical BCG therapy in patients with bladder malignancy (8).

In this current study, we measured the PPD specific T-cell response in both blood and BAL fluid in patients with interstitial pulmonary disease. We hypothesized that if mycobacterial infection of the lung is of importance for the development of sarcoidosis, T-cell responsiveness towards the PPD antigen, especially in alveolar mononuclear cells, should be at a higher level in patients with sarcoidosis when compared to patients with other causes of interstitial pulmonary disease.

Methods

Patients

A single-center study was conducted in our hospital which included all patients from the pulmonary outpatient department scheduled for BAL as a result of suspected interstitial pulmonary disease. BAL was performed for *Mycobacterium tuberculosis* (MTB) Nucleic Acid Amplification Technique (NAAT), (mycobacterial) culture, immunophenotyping and IGRA. In addition, venous blood was collected for immunophenotyping and IGRA.

Patients were divided into two groups. The first group included patients diagnosed with sarcoidosis, based on international accepted criteria (9,10), with or without histological confirmation. Group 2 involved all other patients with a varied group of non-sarcoidosis diagnoses (*table 3*), which functioned as a control group.

IGRA (ELISpot)

MTB-specific IGRA (ELISpot TB) on peripheral blood mononuclear cells (PBMCs) was performed at the TSPOT®.TB platform, according to the manufacturers instructions (Oxford Immunotec Ltd., Abingdon, UK). Briefly, 2.5 x 10^5 PBMCs were incubated with 50 µL of AIM-V medium (negative control), phytohemagglutinin (PHA, positive control) and two MTB specific antigens (ESAT-6 / panel A, and CFP-10 / panel B, respectively). After 16-20 hours incubation at 37°C, the microtitre plates were washed and a conjugate incubation and detection step were carried out to visualize the IFN-γ production by sensitized T-cells. IGRA for PPD was performed using the TSPOT®.TB platform, except that the TB-specific antigens were replaced by a mixture of PPD (tuberculin RT50 3 µg/mL, Statens Serum Institute, Denmark). In addition, an EBV ELISpot assay was performed using an EBV antigen which was kindly provided by Debby van Baarle (WKZ Hospital, Utrecht, the Netherlands) (11) and was used in a concentration of 0.5 µg/mL. In the absence of an evidence based cut-off value, ELISpot PPD and EBV were defined as reactive in the case of one or more spots. Spot formation was enumerated on an ELISpot reader (Auto Immun Diagnostika GmbH, Strassberg, Germany). ELISpot results were valid when ≥ 20 spots were visible in the positive control well. When spots in the positive control well were lower, the test result was scored as indeterminate.

BAL was performed with 150 ml saline fluid placed into an affected lung segment. Sample debris was discarded by passing the BAL fluid through a stainless steel sieve. ELISpot assays were performed as previously described (7) and explained above.

Individual patient-data meta-analysis

The results of the current study were extended in an individual patient-data meta-analysis (described separately in the results paragraph) combining our data with the data of Hörster et al (12). Those authors also investigated the interferon-γ production by enzyme-linked immunospot in response to PPD, ESAT-6 and CFP-10 by mononuclear cells both from BAL fluid and blood, but defined a different patient population (17 patients with

pulmonary sarcoidosis compared to 33 patients with smear-negative tuberculosis and a varied group of 35 controls).

Another difference is that Hörster et al defined the response of stimulated cultures to be positive when the antigen well contained more than five spots and at least twice the number of spots compared to the negative control. With current limited evidence about PPD specific T-cell response, we preferred to show the absolute amounts of spots. However, only in this individual patient-data meta-analysis, to be in agreement with the study-design of Hörster et al, we applied a cut-off value of > 5 spots for PPD reactivity in our population.

Statistical analysis

A description of clinical characteristics was made using mean and standard deviation in variables with a normal distribution (unpaired T-test), median and range in non-normal distributed variables (Mann-Whitney U test) and percentages for dichotomized variables (Chi-squared test, reporting exact significance). To test for normality, Kolmogorov-Smirnov test (n > 50) and Shapiro-Wilk test (n < 50) were applied.

To compare lymphocytes in BAL fluid and the concomitant number of spots in EBV and PPD reactive samples (blood and BAL fluid), logarithmic transformation was applied, resulting in geometric mean with 95% confidence interval of the difference. To compare ELISpot results between both groups, p-values were calculated using Pearson Chi-Square test (2-sided). In the individual patient-data meta-analysis, logistic regression was applied to compare both studies. This analysis was performed with respect to the percentage of sarcoidosis patients in both groups, the association between PPD outcome and the occurrence of sarcoidosis.

Statistical analyses were performed with the aid of computer software (SPSS 17.0, SPSS Inc. Chicago, Illinois, USA).

Results

A total of 118 patients were included. Baseline characteristics are described in table 1, for patients diagnosed with sarcoidosis (n = 32) and patients without sarcoidosis (n = 86). Staging of sarcoidosis patients is described in table 2. The classifying diagnoses in patients without sarcoidosis are described in table 3. Of all patients with sarcoidosis, 19 patients (59%) were diagnosed based on clinical, biochemical (ACE / Lysozyme / Soluble IL-2 receptor), cytological (BAL fluid) and / or radiological evidence without histological confirmation. In the remaining 13 patients (41%), sarcoidosis was histologically confirmed (9,10). Sarcoidosis patients were significantly younger compared to patients without sarcoidosis. In addition, sarcoidosis patients had significantly more lymphocytes in the BAL fluid, resulting in a (geometric) mean difference of 2.0 x10^4 lymphocytes [CI 1.22;3.35], (p = 0.007) between both groups. Other baseline characteristics were comparable.

ELISpot PPD on blood was reactive in 13 (41%) sarcoidosis patients, compared to 50 (58%) patients without sarcoidosis, (p = 0.262). ELISpot TB on blood was positive in none of the sarcoidosis patients, in comparison with 13 (17%) patients without sarcoidosis, (p = 0.065). ELISpot EBV on blood was reactive in 17 (53%) of sarcoidosis patients, compared to 62 (72%)

Table 1. Baseline Table

	Sarcoidosis	No Sarcoidosis	
Number of subjects	32	86	
Male (%) *	18 (56%)	54 (63%)	p = 0.531
Mean age [Standard deviation] †	40.9 [14.9]	60.3 [14.6]	p < 0.0005
Dutch nationality (%) *	28 (88%)	71 (83%)	p = 0.516
Smoking (%) *	9 (28%)	25 (29%)	p = 0.821
Lymphocytes in BAL, Geometric Mean (x 10^4), [Min;Max] †	4.3 [3.0-49.4]	2.1 [0.0-99.4]	p = 0.007

** For this analysis the Chi-squared test was used*

† For this analysis the Independent samples T-test was used

Table 2. Staging of patients with sarcoidosis

Thoracic Stage	Stage I	6 (19%)
	Stage II	22 (69%)
	Stage III	2 (6%)
	Stage IV	2 (6%)
Extrapulmonary localization *		16 (50%)
Skin (Including erythema nodosum)		8
Bone		3
Spleen		2
Liver		2
Joint		2
Eye (Uveitis)		2
Neurological involvement		1

** Some patients had ≥ 1 extrapulmonary localization*

Table 3. Classifying diagnosis in patients without sarcoidosis

Diffuse Lung Disease and/or fibrosis without classifying diagnosis	29
Infection	16
Extrinsic Allergic Alveolitis	10
Smoking Related ILD	7
Medication Induced Interstitial Pneumonia	4
Eosinophilic pneumonia	4
Organizing pneumonia	3
Idiopathic Pulmonary Fibrosis	3
Silicosis / Asbestosis	3
Immune Mediated Interstitial Pneumonia	2
Bronchiectasis e.c.i.	2
Non-specific Interstitial Pneumonia	1
Malignancy	1
Allergic Bronchopulmonary Aspergillosis	1

patients without sarcoidosis, (p = 0.092). ELISpot PPD on BAL mononuclear cells was reactive in 5 (16%) sarcoidosis patients compared to 13 (15%) patients without sarcoidosis, (p = 0.356). All ELISpot TB samples on BAL cells were negative in both groups. ELISpot EBV on BAL cells was reactive in 5 (16%) patients compared to 16 (19%) patients without sarcoidosis, (p = 0.409). Above results are summarized in figure 1. Although not significant, there seems to be a trend to elevated PPD, TB and EBV reactivity in blood in patients without sarcoidosis. Similar analyses were performed using different cutoff points for positivity of ELISpot PPD and EBV, again resulting in no significant differences between both groups (data not shown).

Figure 2 demonstrates the *absolute number of spots* (in BAL fluid and blood) for ELISpot PPD and

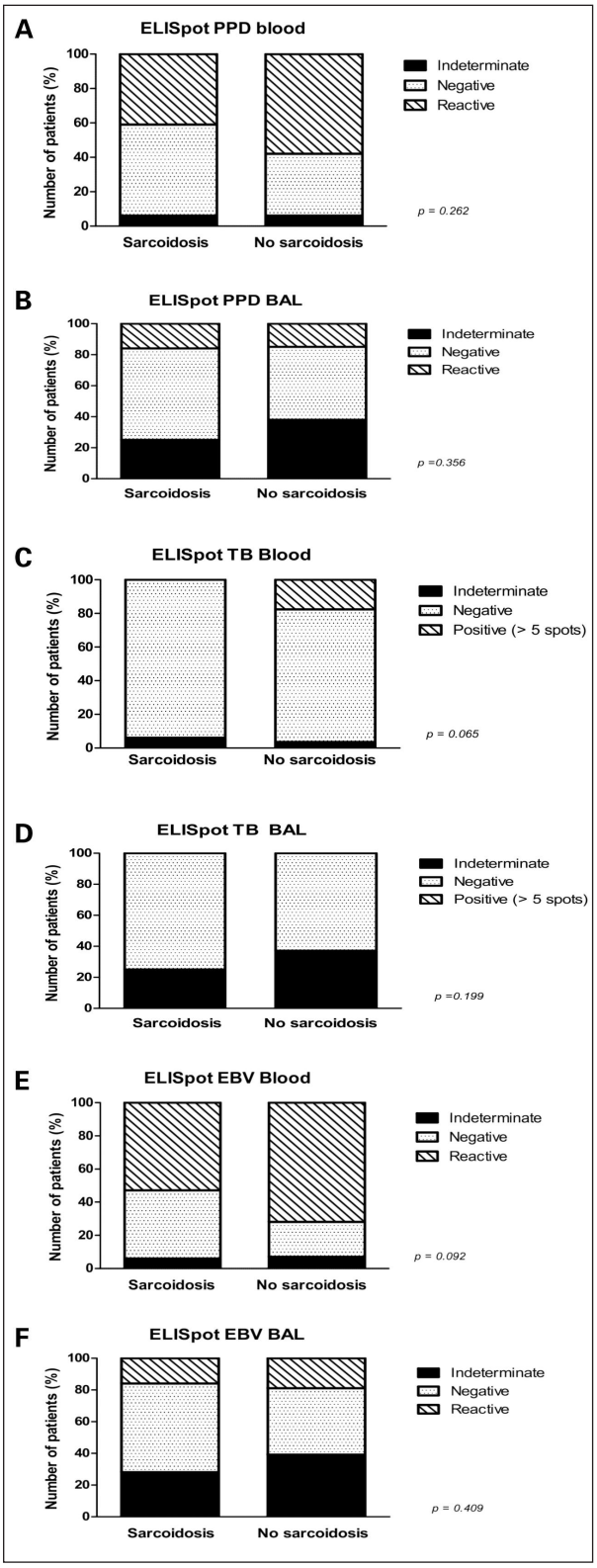

Fig. 1. ELISpot results

EBV *reactive* samples. In PPD reactive patients without sarcoidosis, there are significantly more PPD specific spots in blood (mean difference 2.12 [0.23;0.99], p = 0.046) and more PPD specific spots in BAL fluid (mean difference 1.79 [0.14-2.24], p = 0.387), compared to PPD reactive patients with sarcoidosis. Concerning ELISpot EBV reactive samples, there are no differences in absolute number of EBV specific spots between patients with and without sarcoidosis in blood (mean difference 1.07 [0.53;2.14], p = 0.848) and BAL fluid (mean difference 1.28 [0.32;5.17], p = 0.712).

No BAL samples in sarcoidosis patients were positive for mycobacteria in both NAAT and culture. In one patient without sarcoidosis, *Mycobacterium tuberculosis* was cultured from BAL fluid. In this patient, *Mycobacterium tuberculosis* specific NAAT, ELISpot TB and ELISpot PPD on BAL fluid were all negative. In blood, ELISpot TB was positive (33 spots in panel CFP-10, 32 spots in panel ESAT-6) and ELISpot PPD was reactive (26 spots). This patient was treated successfully with anti-tuberculosis drugs. This case emphasizes that *Mycobacterium tuberculosis* specific NAAT and IGRA results can be false negative. In another patient without sarcoidosis, *Mycobacterium intracellulare* was cultured from BAL fluid. In this patient, NAAT for mycobacteria and ELISpot TB in BAL fluid were negative, ELISpot PPD in BAL fluid was slightly reactive with two spots and ELISpot results in blood were indeterminate.

Results Individual patient-data meta-analysis

This additional analysis of current data together with the data of Hörster et al (12) enclosed 49 sarcoidosis patients and 121 patients without sarcoidosis. The ratio of sarcoidosis patients is not different between both studies (p = 0.460). In the logistic regression analysis, there is no interaction between PPD results and the study (p = 0.829 for PPD positive results and p = 0.944 for PPD indeterminate results). ELISpot PPD on blood was reactive (> 5 spots) in 9 (18%) sarcoidosis patients, compared to 45 (37%) patients without sarcoidosis, (p = 0.010). ELISpot PPD on BAL mononuclear cells was reactive (> 5 spots) in 3 (6%) sarcoidosis patients compared to 17 (14%) patients without sarcoidosis, (p = 0.060).

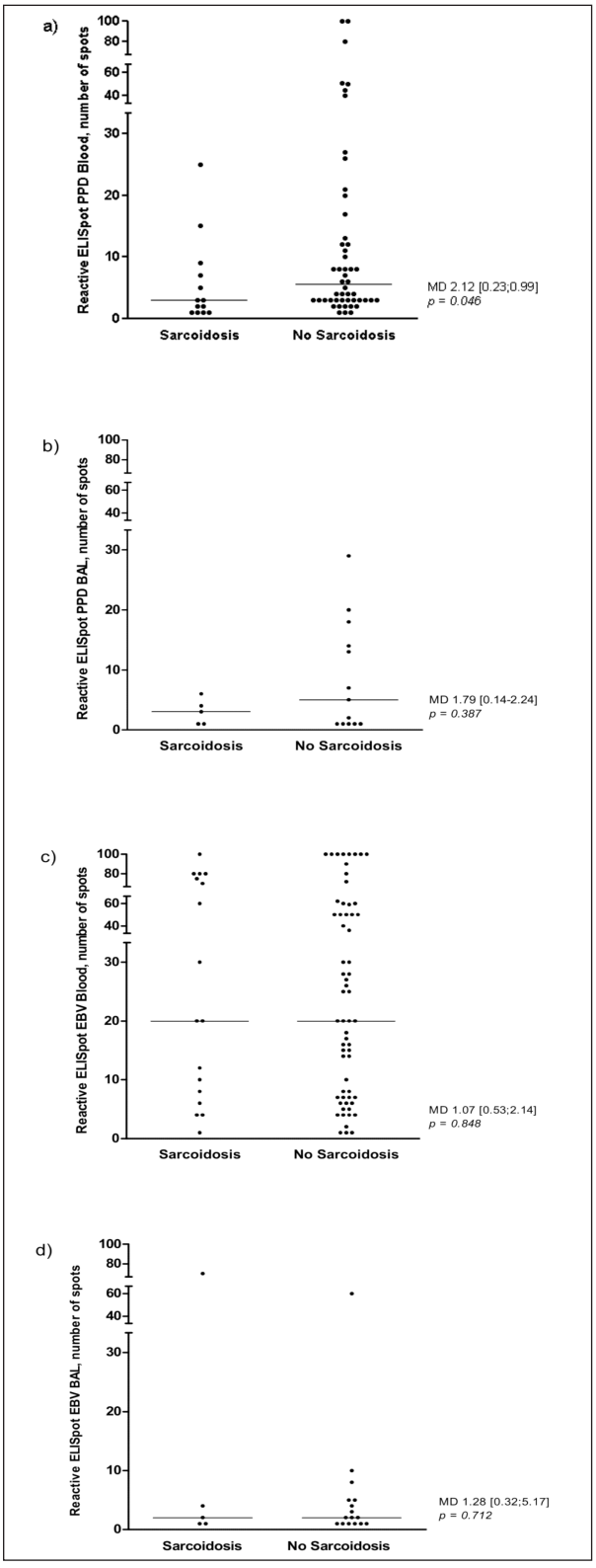

Fig. 2. Reactive ELISpot PPD and EBV (Blood and BAL fluid)
*MD=Mean Difference

Discussion

Here we report on the PPD and *Mycobacteria tuberculosis* specific T-cell response in both blood and BAL fluid in patients with sarcoidosis, compared to patients with other interstitial pulmonary disorders. In contradiction to our hypothesis, T-cell reactivity in BAL fluid is not different between both groups. PPD reactivity in blood in patients without sarcoidosis exceeds the reactivity in patients with sarcoidosis, although this difference is not significant.

We suggest that the significant age-difference between both groups may have influenced the results. During life, there is a cumulative risk of mycobacterial infections, possibly resulting in more PPD reactivity in the older study population of patients without sarcoidosis. It has to be realized that the incidence of mycobacterial infections in the Netherlands is low. For example, the incidence of tuberculosis in Utrecht area is 4-20/100.000 persons/year (13), and the incidence of nontuberculous mycobacterial infections is estimated at 5/100.000 persons/year (14). Therefore, we have to be cautious to extrapolate the results to parts of the world with a higher incidence of mycobacterial infections. Actually, the incidence of sarcoidosis is quite high in North-European climate, while the incidence of mycobacterial infections in these regions is rather low.

Perhaps, T-cell anergy in sarcoidosis patients might have influenced the results (15). T-cell anergy in sarcoidosis patients has been suggested in different papers. To make this effect more unlikely, we included an EBV ELISpot which should be positive in both populations at the same level. In the adult population in the Netherlands, EBV antibodies are common, which we verified with a random sample from all patients, resulting in EBV seropositivity in 100%, (data not shown). In our results, there seems to be a trend to more EBV reactivity in blood in patients without sarcoidosis (figure 1e), although this difference is not significant. EBV reactivity in BAL fluid is not different between both groups (figure 1f). In EBV *reactive* samples (in contradiction to PPD *reactive* samples), the absolute number of spots is comparable in patients with and without sarcoidosis (figure 2c, 2d). These results imply T-cell anergy to be unlikely, but it cannot be ruled out completely.

Interestingly, our ELISpot EBV results do not support the previous reported suggestion about the possible involvement of EBV in pathogenesis of sarcoidosis (16).

Another remaining hypothesis on this topic is that patients with sarcoidosis may have a disturbed immune response against mycobacteria, resulting in negative ELISpot PPD, ESAT-6 and CFP-10 results despite mycobacterial infection, enabling development of granuloma characterizing sarcoidosis, especially in those patients with disturbed immune response.

To the best of our knowledge, this study is the second one measuring PPD specific T-cell responses, utilizing IGRA on both BAL fluid and blood. The results are largely in accordance with the already mentioned report published by Hörster et al (12), concluding that the frequency of mycobacteria-specific local and systemic immune response is not elevated in patients with sarcoidosis when compared to controls.

Both studies include limited number of patients; therefore we performed the individual patient-data meta-analysis, described in the methods and results section. However, these results again do not support our hypothesis and actually shows that mycobacterial immune response is negatively associated with sarcoidosis.

Both studies cannot rule out a causal relation between mycobacteria and sarcoidosis in a small subset of patients. Future research on sarcoidosis should be differentiated to distinguish phenotypes of sarcoidosis, possible resulting in a subgroup of patients with sarcoidosis, each with different pathogenesis.

In conclusion, our data provide no evidence that mycobacteria are involved in pathogenesis of sarcoidosis.

Acknowledgements

We kindly thank Mrs. Ingeborg van der Tweel, PhD, for her advice on statistical analysis and our colleague Mrs. Anya N. Milne, PhD MD, for carefully reading the manuscript.

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