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Peripheral blood responses to specific antigens and CD28 in sarcoidosis

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Summary

Background: Potential antigens inducing sarcoid inflammation include mycobacterial and auto-antigens. Paradoxically, peripheral anergy to common recall antigens also occurs, possibly due to impaired dendritic cell or regulatory T-cell responses, or impaired T-cell co-stimulation. The purpose of this study was to compare peripheral blood responses of patients with sarcoidosis to candidate antigens, and examine CD28 T-cell co-stimulation.

Methods: Peripheral blood mononuclear cell (PBMC) responses were examined from patients with sarcoidosis ($n = 16$) and healthy control subjects ($n = 22$) following PBMC stimulation with: anti-CD3/CD28 coated beads; *Mycobacterium tuberculosis* ESAT-6 and KatG peptides; vimentin and lysyl tRNA peptides; and common recall antigens, including cytomegalovirus (CMV) cell lysate as well as CMV, Epstein-Barr virus, influenza virus (CEF) peptides.

Results: ESAT-6/KatG peptide stimulation induced greater numbers of IFN-γ producing T-cells, and elevated IL-2, IL-6 and TNF-α production in sarcoidosis compared to purified protein derivative (PPD)-negative healthy control subjects. PBMCs from patients with sarcoidosis showed reduced IFN-γ producing T-cells following stimulation with CMV lysate, CEF peptides and CD3/CD28 beads; and reduced IL-4 and TNF-α production following CD3/CD28 activation.

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Conclusions: Patients with sarcoidosis exhibit greater PBMC responses to *M. tuberculosis* antigens compared to PPD-negative controls, but reduced T-cell responses to common recall antigens. One contributing mechanism may be impairment of T-cell CD28 co-stimulation.

Introduction

Sarcoidosis is a multi-system disorder of unknown aetiology, characterised by formation of non-caseating granulomas.[1] It is associated with abnormal responses to antigens presented by Major Histocompatibility Complex (MHC) Class II molecules to CD4+ T-Helper type-1 (T_H1) lymphocytes. T-cells from sites of sarcoid inflammation reveal a restricted variable-α (Vα) and Vβ region of the T-cell receptor (TCR), indicating oligoclonal expansion in response to a limited number of antigens.[2] Vβ-specific TCR oligoclonality has been shown in intradermal responses to Kveim reagent (a sarcoid splenic extract) in individuals with sarcoidosis.[2]

Optimal T-cell stimulation also depends on co-stimulatory molecule binding to appropriate ligands on antigen presenting cells (APCs).[3] TCR-antigen presentation in the absence of secondary co-stimulatory signals leads to T-cell anergy or apoptosis.[4] CD28 is an important co-stimulatory molecule, interacting with CD80 and CD86 on APCs, stimulating IL-2 production and T-cell proliferation.[3,5] Flow cytometric analysis has indicated reduced CD28 expression on CD4+ cells in peripheral blood and bronchoalveolar lavage (BAL) of patients with sarcoidosis.[6,7] Persistence of CD28− cells may have a role in influencing regulatory T-cell homeostasis in peripheral tissues.[6] This is significant, as sarcoidosis is characterised by exaggerated inflammation at sites of disease but a suppressed delayed-type hypersensitivity (DTH) skin test and peripheral blood immune responses.[8,9]

Antigens associated with sarcoidosis

Bronchoalveolar lavage (BAL) T-cells from HLA-DRB1*0301 patients with sarcoidosis, expressing the Vα2.3 (AV2S3) TCR have been used to identify strong binding peptides to sarcoid HLA-DRB1∗0301 molecules, including those derived from human vimentin and lysyl tRNA synthetase, which have been proposed as auto-antigens.[10] Subsequent interferon-γ (IFN-γ) enzyme-linked immunospot (ELISpot) assays identified T-cell responses to peptides from vimentin in peripheral blood of DRB1∗0301 patients and lysyl tRNA synthetase in BAL T-cells of both DRB1∗0301 positive and negative patients with sarcoidosis.[11]

Polymerase-chain reaction techniques have detected mycobacterial nucleic acids in some sarcoid tissues, although results were inconsistent.[12] Mycobacterial catalase-peroxidase (KatG) was identified in sarcoid tissue homogenates, and anti-KatG IgG found in the circulation of sarcoidosis subjects.[13] Investigations indicate greater peripheral blood mononuclear cell (PBMC) and BAL T_H1 responses in sarcoidosis patients compared to healthy controls, but no differences compared with purified protein derivative-positive (PPD+) subjects in response to: *Mycobacterium tuberculosis* KatG,[14]

heat shock proteins,[15,16] Early Secretory-Antigenic Target-6 (ESAT-6),[17−19] mycolyl-transferase antigen85A[20] and super-oxide dismutase A.[15] Again, these findings have been inconsistent.[21,22]

In this current study we collectively investigate the role of the major candidate antigens in a cohort of patients with sarcoidosis. It was hypothesised that PBMC from patients with sarcoidosis will have greater IFN-γ production following stimulation with *M. tuberculosis* and also auto-antigenic peptides derived from vimentin and lysyl tRNA synthetase than healthy controls. The purpose of this study was thus to further investigate the immunopathogenesis of sarcoidosis, by examining T-cell responses and *ex vivo* cytokine production following PBMC stimulation with previously hypothesised candidate antigens and also to investigate the contribution of CD28 using anti-CD3/CD28 beads.

Materials and methods

Study subjects and design

Patients with sarcoidosis attending the Prince of Wales Hospital Sarcoidosis Clinic and the St. Vincent's Immunology of the Eye Clinic were invited to participate. This study was approved by the Human Research Ethics Committees of the Prince of Wales Hospital and St. Vincent's Hospital, Sydney, Australia. Informed written consent was obtained from all participants.

Inclusion required a diagnosis of sarcoidosis prior to participation, using criteria from the World Association of Sarcoidosis and Other Granulomatous Diseases.[1] All patients had sarcoidosis, determined by symptoms (acute respiratory illness, fatigue, sweats, dyspnoea, and dry cough), chest radiography findings and lung function tests. The diagnosis was confirmed with either positive biopsies showing non-caseating granulomas (in the absence of a known cause); uveitis; a BAL mononuclear cell alveolitis with CD4/CD8 ratio >3.5; or Löfgren's syndrome (acute-onset sarcoidosis characterised by erythema nodosum, bilateral hilar adenopathy and ankle arthritis). Patients with sarcoidosis had PPD testing and were negative. Healthy controls included those with documented skin testing to PPD or self-reported history of vaccination with Bacillus Calmette-Guérin (BCG). Healthy subjects whose PPD skin test and BCG vaccination status were known were also recorded and classified as either skin test positive (PPD+) or negative (PPD−).

Peripheral blood mononuclear cell isolation and storage

Approximately 30 ml of blood was obtained from each subject in heparinised tubes. Purified PBMC were obtained by

Ficoll-Hypaque density gradient centrifugation. Cells were washed in phosphate-buffered saline (PBS) and cryopreserved in 50% autologous serum with RPMI-1640 medium (GIBCO® Invitrogen, Australia) and 10% dimethyl sulphoxide. Cells were frozen at −80 °C for 24 h before transfer to vapour-phase nitrogen at −190 °C until time of analysis.

Selection and synthesis of T-cell stimuli for ex vivo analyses

Candidate antigenic peptide sequences from *M. tuberculosis* ESAT-6 and KatG and human vimentin and lysyl tRNA synthetase, were selected for testing (see Table 1). Additional peptides selected included Cytomegalovirus, Epstein-Barr virus and Influenza (CEF) peptides (National Institute of Health, USA); a panel of 32 pooled HLA-Class I restricted (9-mer) epitopes, stimulating CD8+ T-cell responses in the majority of individuals.[23] Cytomegalovirus (CMV) cell lysate was also used, which includes proteins likely to elicit CD4+ T-cell responses.[24] These common recall antigens were used to distinguish sarcoidosis-unrelated responses in patients with sarcoidosis and healthy control subjects. Pooled *M. tuberculosis* ESAT-6 and KatG peptides, and pooled auto-antigenic peptides derived from vimentin and lysyl transfer RNA synthetase were used (Mimotopes, Melbourne, Australia). These peptides were synthesised using solid-phase 9-fluorenylmethoxycarbonyl chemistry to an immunograde purity of >85%. Identity was confirmed by mass spectroscopy and purity by high-phase liquid chromatography. As a pan T-cell stimulus, human T-activator CD3/CD28 Dynabeads® (Invitrogen, California, USA) were also used. These are superparamagnetic polystyrene beads conjugated with anti-CD3 and anti-CD28 antibodies, mimicking *in vivo* TCR T-cell activation by antigen presenting cells.

IFN-γ ELISpot assay

Stimulation of antigen-specific T-cells was assessed using the human IFN-γ ELISpot assay. IFN-γ assays were performed as per the manufacturer's instructions (R&D Systems, Sydney, Australia) with thawed PBMCs added at a concentration of 2×10^5 cells per well of the IFN-γ antibody-coated filter plate. Cells were incubated in duplicate per stimulus. CEF peptides were added at a concentration of 2 μg/ml, CMV lysate at a 1:125 dilution in RPMI-1640 medium and mycobacterial and auto-antigen peptides at 10 μg/ml as described previously.[11,18] CD3/CD28 beads were also added in separate wells. Optimisation experiments on healthy subjects revealed that a ratio of 1 bead:80 cells induced a suitable number of spots per well to allow reliable counting and statistical analysis. Phytohaemagglutinin A (Sigma—Aldrich, Sydney, Australia) was used as a positive assay control at 5 μg/ml, and non-stimulated wells containing media only as a negative control. As a measure of inter-assay variability, cells from one subject found to respond strongly to CEF peptides were tested in each plate (the inter-assay coefficient of variance was <6%).

Cells were incubated with each stimulus at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h, and subsequently developed according to manufacturer's instructions (R&D Systems, Sydney, Australia). Plate membranes displaying spots were scanned and counted using the AID ELISpot plate reader system and software version 5.0 (AID Diagnostika, Germany). Results are presented as the mean spot-forming cell count (SFC) for duplicate wells after subtraction of the mean negative control (background) number of spots. Negative wells had <50 SFC per 2×10^5 input cells. A positive response was defined as a concentration of at least 50 SFC per 10^6 PBMCs after subtraction of background, being at least three times higher than the background level.

Multiplex cytokine analysis of PBMC culture supernatants

Following 24 h ELISpot incubation, supernatants were extracted and stored at −80 °C until time of analysis. Supernatants were collected from cultures stimulated with CEF peptides, CD3/CD28 beads, ESAT-6/KatG peptides and non-stimulated wells. A Bio-Plex assay (Bio-Rad Laboratories, Hercules, USA) was used to quantify supernatant levels of IL-2, IL-4, IL-6, IL-10 and TNF-α according to the manufacturer's instructions. Cytokine measurement was performed using the Luminex 100 xMAP™ system (Luminex, Austin, USA) and data analysis with Bio-Plex Manager software version 5.0 (Bio-Rad Laboratories).

Statistical analyses

Data was analysed using GraphPad Prism version 5.03 (GraphPad, La Jolla, USA). Non-parametric comparisons of differences between healthy control subjects and patients were performed using the Mann—Whitney *U*-test for independent data, and the Kruskal—Wallis test followed by Dunn's post-test for comparison between groups. Categorical comparisons were analysed using Fisher's exact test. All *p*-values are two-tailed and *p*-values <0.05 considered significant.

Table 1 Candidate antigenic peptides in sarcoidosis. Peptides previously determined to be antigenic in some patients with sarcoidosis. Accession numbers obtained from NCBI BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Antigenic peptide	Amino acid sequence	Amino acid position (codons)	GenBank accession number	Gene ID
M. tuberculosis ESAT-6	NNALQNLARTISEAG	66—80	NP338543	922546
M. tuberculosis KatG	WTNTPTKWDNSFLEI	321—335	NP216424	885638
Homo sapiens vimentin	DSLPLVDTHSKRTLL	263—277	AAA61282	7431
Homo sapiens lysyl transfer RNA synthetase	GPEGQAYDVDFTPPFR	363—378	EAW95623	3735

Results

Thirty-eight subjects were recruited, including sixteen with sarcoidosis (see Table 2). Subjects with sarcoidosis exhibited relative peripheral blood lymphopenia, a feature of this disease,[5] with a mean white cell count (±standard deviation) of $5.7 \pm 2.1 \times 10^6$/ml compared to $6.4 \pm 1.5 \times 10^6$/ml in healthy subjects ($p = 0.27$), of which the lymphocyte percentage was $24\% \pm 0.08\%$ in sarcoidosis patients and $35\% \pm 0.05\%$ in healthy subjects ($p < 0.0001$). Additionally, patients had elevated median serum ACE of 54.5U/L (normal range < 42U/L). Patients with sarcoidosis had lower percentage predicted FEV_1 values of $86.1\% \pm 17.8\%$ compared to $98.8\% \pm 9.7\%$ in controls ($p < 0.05$); and lower percentage predicted FEV_1/FVC ratios of $93.3\% \pm 15.6\%$ compared to $102.8\% \pm 4.4\%$ in healthy controls ($p < 0.05$).

All subjects responded to CD3/CD28 bead stimulation at varying levels, but not all responded to peptide stimulation (see Table 1 in Supplementary material). A greater median IFN-γ SFC was found in healthy subjects compared to patients with sarcoidosis following stimulation with CEF peptides ($p = 0.037$, Fig. 1A), with 14/22 (63.6%) healthy subjects with positive ELISpot responses compared with 6/16 (37.5%) patients with sarcoidosis. Similar findings were observed with CMV lysate stimulation ($p = 0.01$, Fig. 1B), with 13/22 (59%) positive responses in healthy controls compared with 5/16 (31.3%) responses in patients with sarcoidosis.

PBMCs from patients with sarcoidosis had greater numbers of IFN-γ producing cells reactive to pooled *M. tuberculosis* ESAT-6 and KatG peptides compared to PPD− controls ($p = 0.019$, Fig. 2A). There was also a significant difference in the frequencies of positive responders, with

Table 2 Subject characteristics data are presented as mean ± one standard deviation. Significant differences between patients with sarcoidosis and healthy control subjects (**$p < 0.0001$, *$p < 0.05$), p-values based on two-tailed independent t-tests, with significance value at 0.05. ACE, Angiotensin converting enzyme; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; WCC, white cell count; N/A, not assessed.

	Sarcoidosis ($n = 16$)	Healthy controls ($n = 22$)	p-Value
Female/male	5/11	9/13	ns
Age (years); median (min, max)	49 (32, 83)	39 (22, 82)	ns
Disease duration prior to this study (years)	7.5 ± 6.7		
Serum ACE (U/L) median (min, max) (Normal < 42U/L)	54.5 (7.2, 107)	N/A	
Blood WCC ($\times 10^6$/ml)	5.7 ± 2.1	6.4 ± 1.5	ns
Lymphocytes (%)**	$24\% \pm 0.08\%$	$35\% \pm 0.05\%$	<0.0001
Monocytes (%)	$6.0\% \pm 0.02\%$	$6.0\% \pm 0.02\%$	ns
Neutrophils & eosinophils (%)**	$70\% \pm 0.09\%$	$59\% \pm 0.06\%$	<0.0001
Pulmonary function			
Mean FEV_1 (% predicted)*	86.1 ± 17.8	98.8 ± 9.7	< 0.05
Mean FVC (% predicted)	93.7 ± 15.4	98.4 ± 9.5	ns
FEV_1/FVC ratio (% predicted)*	93.3 ± 15.6	102.8 ± 4.4	< 0.05
Chest X-ray stage (0/I/II/III/IV)[a]	1/6/6/1/2		
Smoking status (current/past/never)	0/9/7	1/5/16	
Affected organs			
Lung	15		
Eye (uveitis)	10		
Lymph nodes	13		
Skin	5		
Neurosarcoid	3		
Others[b]	5		
Löfgren's syndrome	2		
Immunosuppressive treatment (yes/no)	4/12	None	
Mantoux skin test & BCG status (PPD+/PPD−)[c]	0/16	5/17	

[a] Pulmonary disease was scored using the 1961 Scadding sarcoidosis chest X-ray staging system, Stage 0 disease (no intra-thoracic involvement), Stage I (bilateral hilar lymphadenopathy), Stage II (bilateral hilar lymphadenopathy with parenchymal infiltrates), Stage III (parenchymal infiltrates) and Stage IV (pulmonary fibrosis).

[b] Others is defined as liver, kidney, spleen, lacrimal and salivary glands.

[c] Healthy controls were categorised as those who had a positive PPD skin test history or prior BCG vaccination (PPD+/BCG+, hereafter called PPD+) and those who were PPD skin test negative or whose PPD status was unknown, or had no history of BCG vaccination (PPD−/BCG−, hereafter referred to as PPD−).
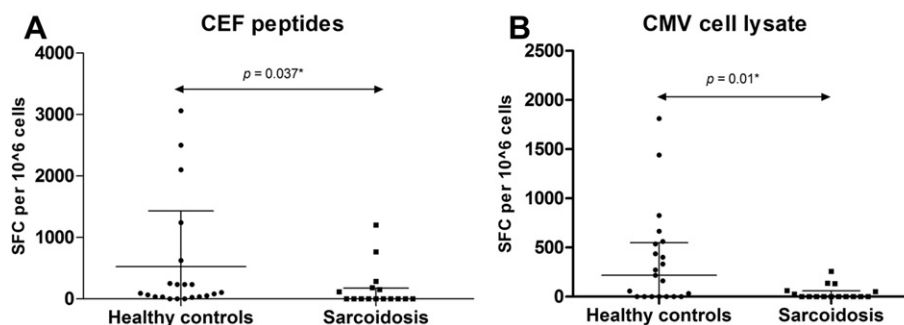
Figure 1 ELISPOT PBMC responses to CEF peptides and CMV-infected cell lysate. (A, B) Number of IFN-γ spot-forming cells per 10^6 input PBMC following stimulation with CEF peptides (A) and CMV lysate (B). Bars represent medians and interquartile ranges.

10/16 patients responding compared to 4/17 PPD− controls ($p = 0.036$). However, there was no difference between patients with sarcoidosis and PPD+ control subjects ($p = 0.32$, Fig. 2A), with 5/5 PPD+ controls responding to pooled ESAT-6/KatG peptides. As expected there was also a significant difference in IFN-γ SFC to these mycobacterial peptides, between PPD− and PPD+ control subjects ($p = 0.01$, Fig. 2A) and also in the frequency of subjects responding ($p < 0.005$, Fisher's exact test). No difference was identified between IFN-γ production following stimulation by pooled peptides from vimentin and lysyl tRNA synthetase ($p = 0.91$), with 1/16 patients and 4/22 control subjects responding (Fig. 2B).

There were significantly fewer IFN-γ SFC per 10^6 PBMC formed by T-cells from patients with sarcoidosis compared to both PPD− and PPD+ healthy control subjects after CD3/CD28 bead activation ($p = 0.0007$, Fig. 3A). Immunosuppressant treatment did not appear to significantly alter CD3/CD28 responses in the patients ($p = 0.43$), although a trend towards lower median SFC was observed in those taking immunosuppressants.

PBMC culture supernatants were available from 10 healthy control subjects (of whom 5 were PPD−), and 11 patients with sarcoidosis. These included all PPD+ subjects, and based on sample availability, PPD-negative control subjects and patients with sarcoidosis. Comparative analyses did not identify any significant differences between these subject groups for unstimulated PBMC

culture supernatants (Table 3A), or following stimulation with CEF peptides (Table 3B). However, in cell cultures of sarcoid PBMCs activated with CD3/CD28 beads, there were lower concentrations of IL-4 and TNF-α ($p = 0.045$ and $p = 0.049$ respectively) compared with control subjects, and a trend for IL-2 and IL-6 ($p = 0.052$ and $p = 0.056$ respectively) (Table 3C). Following ESAT-6/KatG peptide stimulation, there were significant differences in production of IL-2 ($p = 0.02$), IL-6 ($p = 0.04$) and TNF-α ($p = 0.02$) between PPD− control subjects and patients with sarcoidosis (see Table 4), and a trend for IL-10 ($p = 0.07$) between these groups. There were no differences between patients with sarcoidosis and PPD+ control subjects.

Discussion

This study indicates that mycobacterial ESAT-6 and KatG peptides induced greater numbers of IFN-γ producing T-cells from sarcoidosis subjects compared with PPD-negative, but not PPD+ controls. Numbers of IFN-γ producing T-cells and *ex vivo* cytokine production were however, reduced in patients with sarcoidosis following CD3/CD28 activation and stimulation with common recall antigens.

Previous findings indicate reduced CD28 signalling pathway function in T-cells from patients with sarcoidosis.[6,7] One study identified increased CD4+CD28− T-cells in BAL and peripheral blood of patients with chronic
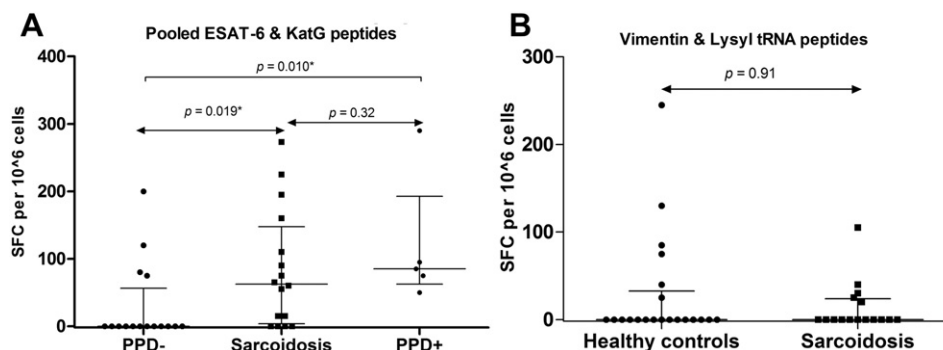

Figure 2 Mycobacterial and auto-antigen responses. (A, B) Measurement of IFN-γ producing PBMCs with ELISPOT, following stimulation with (A) pooled *M. tuberculosis* ESAT-6 and KatG peptides in PBMCs from patients with sarcoidosis and healthy PPD+ or PPD− control subjects. (B) Responses following stimulation with pooled peptides derived from human vimentin and lysyl transfer RNA synthetase in healthy controls and patients.
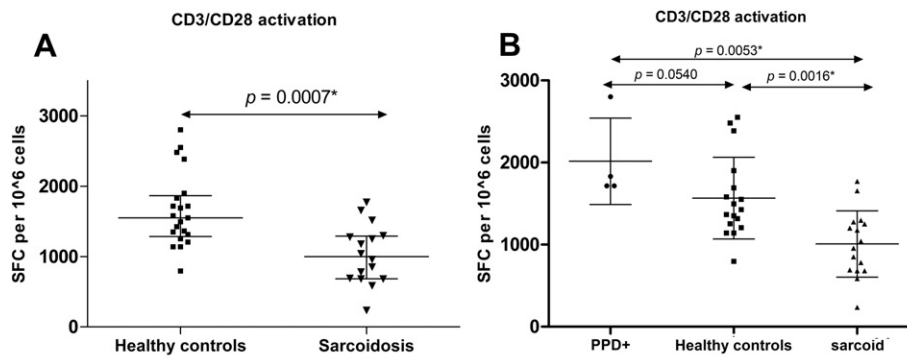
Figure 3 CD28-dependent T-cell activation responses. (A) Number of IFN-γ producing T-cells, following CD3/CD28 bead activation. (B) Number of IFN-γ producing T-cells following CD3/CD28 activation, after subdividing the controls into PPD− healthy controls and PPD+.

sarcoidosis, with reduced baseline IFN-γ and TNF-α production when compared with CD28+ T-cells.[6] The authors did not show that these CD4+CD28− T-cells also had lower capacity for antigenic responses. These findings were however, in contrast to those of Agostini et al.,[25] who identified greater frequencies of BAL T-cells expressing CD28, but not CD152, in patients with untreated sarcoid alveolitis, compared to healthy controls. Agostini et al. demonstrated that enhanced CD28 expression was associated with increased cell surface expression of activation antigens, including CD69, CD103 and HLA-DR. Other studies[7,26] identified reduced CD28 expression by BAL and peripheral blood T-cells from patients with chronic sarcoidosis, along with elevated cell surface activation markers CD26, CD69 and HLA-class II in

BAL. Although these results appear conflicting, the relative expression of CD28 may have clinical significance in sarcoidosis. CD28 elevation in conjunction with reduced CD152, elevated CD80, CD86 and cell surface activation markers in BAL T-cells suggest T-cell activation, leading to compartmentalised lung inflammation. In contrast, CD28 reduction in both peripheral blood and BAL T-cells may indicate chronic systemic T-cell activation leading to T-cell senescence and anergy. Hence the relative expression of CD28 may reflect the activity and chronicity of disease, and may be useful as a marker of acute or ongoing antigen-driven inflammation,[26] although further investigations are warranted.

A profile of CD28 down-regulation and anergic T-cells producing less pro-inflammatory cytokines (IL-6 and TNF-α)

Table 3 Measurement of cytokine concentrations PBMC culture supernatants. Cytokine concentrations (in pg/ml) in PBMC culture supernatants: (A) Unstimulated PBMC cultures indicating background responses, (B) stimulation by CEF peptides, (C) cell activation by CD3/CD28 beads. Data are all presented as medians, ranges in parentheses, p-values from Mann–Whitney U-test.

Tested cytokines	Healthy controls (n = 9)	Sarcoidosis patients (n = 11)	p-Value
A. Background responses			
IL-2	2.4 (0.06–3.57)	1.1 (0.06–12.67)	0.22
IL-4	0.2 (0.03–1.36)	0.2 (0.03–1.21)	0.87
IL-6	8748.1 (7.95–11,123.02)	9485.7 (8.6–20,510)	0.36
IL-10	48.9 (0.25–139.33)	50.3 (0.04–173.91)	0.94
TNF-α	4976.2 (5.82–8510.54)	5934.1 (4.5–25,590.79)	0.19
B. CEF peptides			
IL-2	4.9 (0.88–137.29)	1.5 (0.52–36.12)	0.15
IL-4	0.2 (0.03–0.74)	0.2 (0.03–0.57)	0.42
IL-6	9083.1 (9.15–18,798.33)	1406.5 (0.05–22,417.84)	0.23
IL-10	67.2 (0.02–139.57)	68.3 (0.02–174.11)	0.68
TNF-α	4898.5 (7.81–20,091.56)	6972.4 (5.38–28,907.01)	0.34
C. CD3/CD28 activation			
IL-2	1181.7 (598.37–1852.7)	709.4 (142.44–2099.90)	0.052
IL-4	1.4 (0.74–4.54)	0.8 (0.03–1.78)	0.045*
IL-6	13,748.6 (13.84–24,789.87)	1885.2 (6.13–17,815.97)	0.056
IL-10	90.2 (9.06–213.25)	55.1 (0.44–159.54)	0.11
TNF-α	8603.3 (491.99–36,964.04)	2444.3 (56.3–6709.12)	0.049*

* refers to p < 0.05.

Table 4 PBMC culture supernatants cytokine concentrations following ESAT-6/KatG stimulation. Cytokine concentrations (pg/ml) in PBMC culture supernatants following ESAT-6/KatG peptide stimulation. p-Values in the left column are from a Mann—Whitney U-test for comparison between sarcoidosis patients and PPD− controls. One additional PPD+ subject was included for testing mycobacterial peptide responses. p-Values in right column compare responses between PPD+ & sarcoid patients, from Mann—Whitney U-test.

Tested cytokines	Healthy PPD− controls (n = 5)	p-Value	Sarcoidosis patients (n = 11)	p-Value	Healthy PPD+ controls (n = 5)
IL-2	0.08 (0.03—0.61)	0.025*	1.0 (0.06—11.78)	0.92	1.4 (0.03—2.16)
IL-4	0.2 (0.03—0.29)	0.89	0.2 (0.03—0.39)	0.34	0.2 (0.03—0.57)
IL-6	3839.9 (485.5—17,003)	0.039*	15,055.7 (1297.27—21,710.41)	0.99	12,034.3 (10,389.1—19,719.5)
IL-10	0.1 (0.125—261.9)	0.068	117.9 (8.63—258.97)	0.47	202.7 (70.16—212.67)
TNF-α	3180.1 (614.14—6201.6)	0.023*	12,755.0 (1172.6—50,491.1)	0.72	6303.4 (6300.0—8510.7)

* refers to $p < 0.05$.

has been shown in other chronic inflammatory disorders.[6] It has been hypothesised that chronic T-cell stimulation with insoluble mycobacterial antigens complexed as haptens in sarcoid granulomas allowing for slow antigen removal leads to clonal T-cell exhaustion or anergy.[14,27] Similarly, *Mycobacterium leprae* antigens have been shown to induce T-cell anergy *in vitro* by altering TCR/CD28 signalling pathways.[28] Defective TCR/CD28 signalling pathways and T-cell hyporesponsiveness have been identified to occur and is the mechanism by which anergy occurs in patients with leprosy.[29] Stimulation with *M. leprae* total cell wall antigen has been shown to decrease PBMC expression of CD28 and CD80 in patients with leprosy compared with healthy controls.[30] In sarcoidosis, evidence also indicates impairment of signal transduction pathways via the TCR/CD3 complex. Mechanisms include reduced expression of nuclear transcription factor NF-κB/p65 and lymphocyte-specific protein tyrosine kinase (p56^{LCK}) in sarcoid CD4+ T-lymphocytes, with reduced INF-γ and IL-2 production following polyclonal activation.[27] Together with findings from this study and the putative aetiological role of mycobacterial antigens in sarcoidosis,[14,15] this could indicate that certain mycobacterial antigens alter TCR/CD28 signalling pathways inducing T-cell anergy in sarcoidosis.

CD28− T-cells exhibit reduced antigen receptor diversity, defective antigen-induced proliferation and shorter replicative function.[31] This may explain the "all-or-none" type of response observed in sarcoidosis patients to CEF and CMV antigens, however, these factors are also influenced by host immunogenetic factors and presence of specific memory cells. Although not performed due to a limited number of cells available for experimentation, it would have been useful to identify the relative cell surface expression of CD28 in all subjects and correlate this with responses to recall antigens. The functional role of CD28 expression in controlling such responses to common recall antigens in sarcoidosis is hence not well known and requires further investigation.

CD28 plays a critical role in the maintenance of peripheral immunological tolerance, by contributing to development and survival of CD4+CD25^{bright} FOXP3+ T$_{Regs}$[32]. These cells are peripherally expanded in sarcoidosis and have anti-proliferative activity but are insufficient to control exaggerated local granulomatous inflammation.[8,33] This study also indicates for the first time that PBMC from patients with sarcoidosis exhibit reduced CD4+ immune responses after CEF peptide stimulation and reduced CD8+ responses after CMV lysate stimulation, consistent with findings in relation to other antigens.[8,9] Other mechanisms of anergy demonstrated in sarcoidosis include diminished myeloid dendritic cell function, correlating with impaired DTH skin responses.[8] Immunoregulatory invariant natural killer T-cells are also reduced in BAL and blood of patients with sarcoidosis and may have a role in peripheral T$_H$1 down-regulation and, exaggerated local sarcoid immune responses.[9,34] The present study could be extended by examining the relationship between these cell types, along with CD28 signalling and anergy.

In this study PBMC from sarcoidosis patients had greater numbers of IFN-γ producing PBMC and increased production of IL-2, IL-6 and TNF-α following stimulation with *M. tuberculosis* ESAT-6 and KatG peptides compared to PPD− controls. The observed cytokine production following ESAT-6/KatG stimulation[14−19] displays polarised T$_H$1 responses and increased IL-6 and TNF-α production; key factors in granuloma formation.[35] Our findings are consistent with reports of elevated IL-2, IL-6, IFN-γ and TNF-α in sarcoid PBMC culture supernatants following mycobacterial heat shock protein stimulation.[16] The limitations in this study include possibility of selection bias in the cytokine production analysis by using supernatants from a subset of subjects, selected based on sample availability.

Another limitation in this study was that HLA-typing was not performed to verify appropriate alleles for recognition of the candidate antigens studied. It has been found that HLA-DRB1∗0301 BAL T-cells respond to KatG[14] and immune recognition of ESAT-6 and KatG peptides are associated with HLA-DRB1∗1101.[17] The lack of differences in IFN-γ SFC between patients and healthy controls in response to auto-antigenic peptides from vimentin and lysyl tRNA synthetase may have reflected subject HLA genotypes.

Conclusions

These findings indicate that following CD3/CD28 T-cell activation in sarcoidosis, there is less production of IFN-γ,

IL-4 and TNF-α compared to control subjects. Additionally, there were depressed immune responses to recall antigens, indicating peripheral anergy. Significantly greater IFN-γ and TNF-α responses to mycobacterial ESAT-6/KatG peptides were identified in patients with sarcoidosis compared to PPD$-$ controls. The findings also confirmed previous reports that despite negative mycobacterial culture and histology findings in sarcoidosis, certain mycobacterial antigens induce specific immune responses at similar frequencies as PPD$+$ subjects.

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Conflict of interest

The authors do not have any financial conflict of interest related to this manuscript.

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