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Patients with Chronic Sarcoidosis have Reduced $CD27^+IgM^+IgD^+$ Unswitched Memory B cells and an Expanded Population of Terminal Effector $CD8^+CD27^-CD28^-$ T cells

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Abstract

Granulomas that occur in sarcoidosis are histologically identical to those found in a subset of Common Variable Immunodeficiency (CVID) patients where they are associated with a reduction in class switched memory B lymphocytes. The aim of this study was to investigate whether the abnormalities in peripheral blood lymphocyte populations associated with granulomatous variant CVID (gvCVID) are also present in individuals with sarcoidosis. We examined B lymphocyte populations using flow cytometry and found that the reduction of class switched memory (CSM: $CD19^+CD27^+IgM^-IgD^-$) and unswitched memory ($CD19^+CD27^+IgM^+IgD^+$) B cells in our sarcoidosis cohort was similar to that previously reported in gvCVID patients. The reduction of class switched memory B cells in sarcoidosis patients indicated a possible defect in the T cell repertoire as antibody class switching requires T cell help. We subsequently explored the peripheral blood T cell compartment of our sarcoidosis patients. The results identified a population of terminally differentiated effector $CD8^+$ T cells ($CCR7^-CD45RA^-CD127^-CD27^-CD28^-$) that were significantly expanded in the peripheral blood of sarcoidosis patients. Terminally differentiated effector $CD8^+$ T cells have been defined as cytolytic, inflammatory cells with reduced replicative capacity. The discovery of abnormal peripheral blood B and T cells compartments in sarcoidosis may be of value in clinical diagnosis and could be relevant to the pathogenic process.

Keywords: Sarcoidosis; Common variable immunodeficiency; Immunophenotyping; Flow cytometry; Lymphocytes

Introduction

Sarcoidosis is a multisystem, cell-mediated immunological disorder of unknown aetiology characterised by non-caseating granulomas in affected organs [1-3]. The most common manifestation (90%) of sarcoidosis is granulomatous disease of the lungs and intra-thoracic lymph nodes, although granulomatous disease may be present in any organ system [4]. As well as epitheloid cells and activated macrophages, infiltrated B and T lymphocytes are also found within sarcoid lesions. Sarcoidosis shares some features of systemic autoimmunity; despite this it is not generally classified as an autoimmune disease [5].

Lymphocyte immune responses are dysregulated in sarcoidosis; manifesting as hyperactive immune responses at the site of granuloma formation and anergy in the peripheral blood. Studies of bronchoalveolar lavage (BAL) fluid from sarcoid patients has revealed that sarcoidosis is a disease of enhanced cell-mediated immune processes at the site of granulomatous inflammation, demonstrating that increased proportions of activated $CD4^+$ BAL T lymphocytes are present in pulmonary sarcoidosis [6-8] with a restricted TCR repertoire indicating oligoclonality [9]. The phenotypes of most BAL lymphocytes in sarcoidosis are $CD4^+$ $CD45RO^+$ $CD25^+$ activated memory T cells with a $CD4^+/CD8^+$ ratio of >3.5:1.

Sarcoid T lymphocytes display a type-1 helper (Th1) T cell cytokine phenotype, predominately secreting interleukin 2 (IL2) [8] and interferon gamma (IFNγ) [10]. This cytokine profile promotes macrophage activation and secretion of tumour necrosis factor alpha (TNFα) as well as amplification of the cellular immune response surrounding thegranuloma [11]. The Th1-bias in sarcoidosis may be the result of regulatory T cells dysfunction. CD1d-restricted natural killer T cells (NKT cells) [12], Vα24-invariant NKT cells [13] and natural regulatory T cells (nTregs: defined as $CD3^+CD4^+CD25^+FoxP3^+$) [14] have all been shown to be dysregulated in sarcoidosis patients [15].

Common variable immunodeficiency (CVID) is a primary immunodeficiency characterised by hypogammaglobulinaemia and recurrent sino-pulmonary infections [16]. The clinical spectrum of CVID is broad and includes chronic lung disease (bronchiectasis), inflammatory gastrointestinal disease, autoimmune haemolytic anaemia and lymphoma. Approximately 8-20% of patients with CVID develop non-caseating granulomatous infiltrations that are histologically identical to those seen in sarcoidosis [16-19]. The aetiology of granulomatous disease in CVID is unknown, although it has been proposed that defects in T cell function are responsible for the autoimmune components of the disease [18]. This hypothesis has originated from the defective mitogenic responses observed in CVID patients [18,20,21] as well as the fact that diseases with defined B cell defects, but intact T cell compartments (e.g. X-linked agammaglobulinaemia) do not have granulomatous involvement.

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Additional studies on CVID have found phenotypic abnormalities in the T cell compartment of peripheral bloodincluding a significantly reduced proportion of naive T helper cells ($CD4^+CD45RA^+$) and a $CD8^+CD57^+$ T cell lymphocytosis [22].

Recently, three attempts have been made to classify and sub-divide patients with different clinical manifestations of CVID based on their peripheral blood B lymphocytes populations [23-25]. Of interest were the findings of specific peripheral B cell phenotypes associated with granulomatous disease in CVID. In the Paris classification of CVID [23] individuals were grouped based on the percentage of memory B cells ($CD19^+CD27^+$) and immunoglobulin class-switched memory (CSM) B cells ($CD19^+CD27^+IgD^-IgM^-$) in their peripheral blood. Interestingly, granulomatous disease was observed in CVID patients with less than 11% $CD27^+$ memory B cells (as a % of $CD19^+$ B cells). These individuals were subsequently categorised as group MB0.

The Freiburg classification scheme of CVID patients [24] examined CSM B cells as well as immature CD21lo B cells but did not examine their association with granulomatous disease. More recently, a combined classification system for CVID known as EUROclass [25] assessed CSM B cells, CD21lo immature B cells (CD21lo CD38lo CD19hi) and transitional B cells (CD38hi IgMhi). In the EUROclass study, multivariate analysis identified both a reduced number of CSM B cells (≤2% as a percentage of B cells) and an expanded population of CD21lo B cells (≥10% as a percentage of B cells) as significantly associated with granulomatous disease. These groups were classified as SmB- and CD21lo and contained the largest number of CVID patients with granulomatous involvement.

The Paris and EUROclass classifications showed that the peripheral B cell phenotype of a CVID patient can be used to predict the presence or development of granulomas that are histologically identical to those seen in sarcoidosis. Whether these B cell abnormalities in CVID are the cause of granuloma development or a consequence of defects in another cell lineages (such as T lymphocytes) is not currently known. However, B cell abnormalities remain a useful and easily measured variable that correlates with the presence of granulomas.

We hypothesised that the B cell memory defects observed in the peripheral blood of individuals with granulomatous variant CVID (gvCVID) [23,25] are also present in sarcoidosis and that this defect is key to the formation of non-caseating granulomas by an, as yet, undetermined mechanism.

Patients and Methods

Patients

A diagnosis of sarcoidosis was made by a consultant chest physician (V.V) and a consultant clinical immunologist (A.B) in accordance with current guidelines [26]. All patients had chronic sarcoidosis with no evidence of immunodeficiency. A total of 27 sarcoidosis patients (mean age 56 years, male:female ratio 0.6:1) were recruited from St Helier Hospital Chest Clinic in addition to 18 healthy volunteers (mean age 45 years, male:female ratio 0.8:1). This research project was granted ethical approval by the London-Surrey Borders Research Ethics Committee. Sarcoidosis was diagnosed on the basis of a positive CXR/CT scan of the chest, a positive Kveim test or lymph node biopsy showing non-caseating granulomas. Informed consent was given by all subjects via approved consent forms. A summary of the patient demographics, including disease duration, diagnosis and steroid treatments is provided in Table 1.The majority of the patients were not on steroid immunosuppression, 3 were on low dose prednisolone at 5, 10 and 10 mg per day respectively and 1 was on 20 mg per day of prednisolone.

Patient Number	Age	Disease duration (Years)	Method of Diagnosis	Patient on Prednisolone (mg/day)
1	44	8	CXR, bronchoscopy	No
2	74	45	Bilateral hilar nodes, Kveim	No
3	71	7	Biopsy, CT	No
4	47	12	Biopsy, CXR	No
5	66	21	CT	No
6	41	7	Biopsy, mediastinoscopy	No
7	58	12	Kveim test, CT	Yes (10)
8	40	1	CXR	Yes (20)
9	69	5	Node biopsy	Yes (5)
10	78	>30	CXR, Kveim	In the first year only
11	50	2	biopsy	No
12	59	29	CXR, Kveim test	No
13	45	18	Kveim test , bilateral hilar nodes	No
14	41	2	Bronchial biopsy, CXR	No
15	53	19	CXR, biopsy, Kveim test	Yes only for 2 yrs at 1989-1991
16	48	7	CXR, biopsy	No
17	49	7	Biopsy, mediastinoscopy, CT, CXR	No
18	68	22	CXR, CT, Kveim	No
19	65	12	Mediastinoscopy and biopsy	No
20	49	22	Erythema nodosum, Kveim	Occular sarcoid steroid drops only
21	77	4	CT	No
22	44	7	Lymph node Biopsy, CT	No
23	52	15	Biopsy, CXR, Kveim test	No
24	51	2	CXR, No biopsy, iritis, Hilar nodes	No
25	42	0.5	Biopsy, mediastinoscopy ,CT	No
26	61	7	CT	Yes (10)
27	42	1	Biopsy	No

Table 1: Summary of the sarcoidosis patient cohort
A diagnosis of sarcoidosis was made in accordance with current guidelines. A total of 27 sarcoidosis patients (mean age 56 years, male:female ratio 0.6:1) were recruited from St Helier Hospital Chest Clinic.

Abbreviations: CXR: Chest X-ray; CT: Computerised Tomography scan.

Flow cytometry studies

Peripheral venous blood was collected from each patient by venesection using EDTA coated vacutainers (Becton Dickinson, NJ, United States). For B lymphocyte memory phenotyping whole blood was processed and stained as previously described [27] using anti-IgM-Cy5 (Jackson Immuno-research, PA, United States), anti-CD27-fluorescein isothiocyanate (FITC; Dako, Glostrup, Denmark), anti-IgD-phycoerythrin (PE; Cambridge Bioscience, Cambridge, United Kingdom), anti-CD19-PC5 (Becton Dickinson), anti-CD21-FITC (Becton Dickinson) and anti-CD38-PE (Becton Dickinson). All samples were analysed on a FACS-Caliber flow cytometer (Becton Dickinson) using a previously described analysis and gating strategy to enumerate B cell memory populations [25,28]. The gating strategies are illustrated in Figure 1.

The same washing and staining protocols were used for T cell phenotyping except with the following fluorescent antibodies: Anti-CD25-PE (Becton Dickinson), anti-CD45RO-PE (Becton Dickinson), anti-CD45RA-FITC (Becton Dickinson), anti-CD127-PE (Becton Dickinson), anti-CD28-PE (Becton Dickinson), anti-CD4-APC

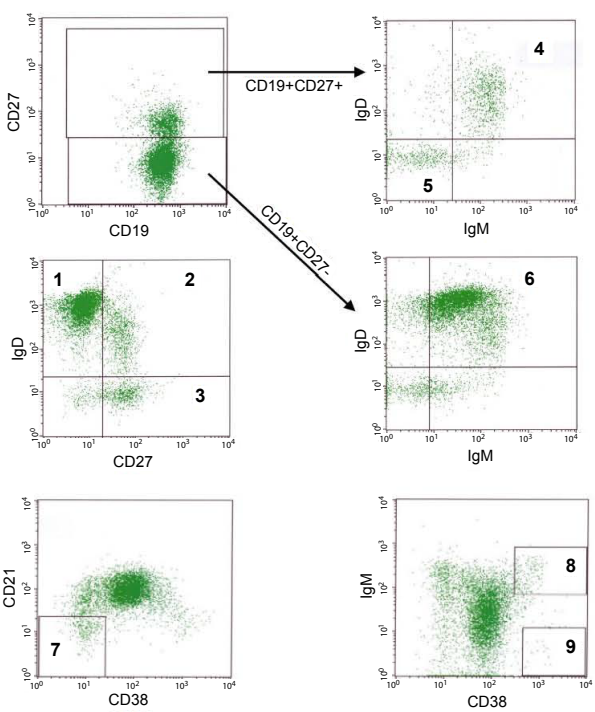

Figure 1: Flow cytometric analysis of B cell sub-populations
After gating on lymphocytes according to forward (FSC) and side (SSC) scatter B cells were selected by CD19 surface staining. B cells were then analysed for CD27 and IgD expression to assess the percentage of IgD$^+$ CD27$^-$ B cells (1), IgD$^+$ CD27$^+$ B cells (2) and IgD$^-$ CD27$^+$ B cells (3). In addition, CD27$^+$ and CD27$^-$ B cells were selected and further assessed for surface IgD and IgM staining to determine the percentages of IgD$^+$IgM$^+$CD27$^+$ unswitched memory B cells (4), IgD$^-$IgM$^-$CD27$^+$ class switched memory B cells (5) and IgD$^+$IgM$^+$CD27$^-$ naive B cells (6). B cells were further analysed for CD21 and CD38 surface expression to identify CD38loCD21lo B cells (7). In addition, B cells were analysed for IgM and CD38 surface expression to identify CD38^{++}IgMhi transitional B cells (8) and CD38^{++}IgM- plasmablasts (9).

(Becton Dickinson), anti-CD3-PCP (Becton Dickinson), anti-CD31-PE (Becton Dickinson), anti-CCR7-PE (R&D Systems, MN, United States) and anti-CD27-FITC (Dako). These antibodies were used to investigate CD4$^+$ and CD8$^+$ T cells, central and effector memory T cells, naive T cells, recent thymic emigrants [29,30] and regulatory CD4$^+$ T cells [31].

The CD8$^+$ T cell population was identified using a CD3$^+$CD4$^-$ gating strategy. The authors acknowledge that this population may also contain a proportion of rare CD3$^+$CD4$^-$CD8$^-$ T cells including cells of the gamma-delta T cell lineage, invariant NK T cells and double negative (CD4$^-$CD8$^-$) alpha-beta T cells.

Statistical analysis and B cell memory classification

All subjects were classified according to the Paris [23] Freiburg [24] and EUROclass [25] CVID B cell memory classification systems as summarised below:

1. Freiburg Classification (calculated as a percentage of lymphocytes)

I: <0.4% antibody class-switched memory (CSM) B cells (CD27$^+$IgD$^-$IgM$^-$)

Ia: >20% CD21lo B cells (as a percentage of CD19$^+$ B cells)

Ib: <20% CD21lo B cells (as a percentage of CD19$^+$ B cells)

II: >0.4% antibody CSM B cells (CD27$^+$IgD$^-$IgM$^-$)

2. Paris Classification (calculated as a percentage of CD19$^+$ B cells)

MB0: <11% CD27$^+$ memory B cells

MBI: >11% CD27$^+$ memory B cells, <8% CD27$^+$IgD$^-$IgM$^-$ CSM B cells

MBII: >11% CD27$^+$ memory B cells, >8% CD27$^+$IgD$^-$IgM$^-$ CSM B cells

3. EUROclass Classification (calculated as a percentage of CD19$^+$ B cells). Individuals with ≤ 1% B cells as a percentage of lymphocytes are not included (**B-**)

SmB+: >2% CD27$^+$IgD$^-$IgM$^-$ CSM B cells

SmB-: ≤ 2% CD27$^+$IgD$^-$IgM$^-$ CSM B cells

(SmB-)(Trhi): ≥ 9% CD38hi IgMhi Transitional B cells

(SmB-)(Trnorm): <9% CD38hi IgMhi Transitional B cells

SmB+ or **SmB-**

(SmB+/-)(CD21lo): ≥ 10% CD21lo B cells

(SmB+/-)(CD21norm): <10% CD21lo B cells

Differences in classification between the two groups were compared using Chi-Square and Fisher-Exact tests. Lymphocyte sub-populations were compared between groups and either *t*-test or Mann-Whitney U tests were performed depending on the results of the Kolmogorov-Smirnov normality test. All statistical analysis was performed in Excel Data Analysis Suite (Microsoft) and SigmaStat 3.11 (Systat Software). Multiple testing corrections (Benjamini-Hochberg) were applied to the results of statistical tests where appropriate.

Results

B cells: Sarcoidosis patients have reduced proportions of class switched memory and unswitched memory B cells compared to healthy control subjects

Statistical analyses were performed comparing the proportions of various B cell populations between the healthy controls and sarcoidosis patients as summarised in Table 2 and Figure 2. The majority of tests were not significantly different between the two groups at a 95% confidence level. Four tests generated *p*-values of <0.05.

• There was no significant difference in the total % of CD19$^+$ B cells between the sarcoidosis patients and the healthy controls (Figure 2.A, p=0.24).

• The total proportion of memory B cells (CD27$^+$CD19$^+$) was lower in the sarcoid patients (Figure 2.B) compared to the controls, however this difference did not reach statistical significance (p=0.14).

• The percentage of CSM B cells (CD27$^+$IgM$^-$IgD$^-$) expressed as a proportion of lymphocytes and the proportion of memory B cells that had not class switched (CD27$^+$IgD$^+$, Figure 2.C) were found to be significantly higher in controls subjects than sarcoidosis patients (p=0.034 and p=0.05 respectively). CSM B cells were not significantly different between the two groups when expressed as a percentage of B cells (Figure 2.D, p=0.28).

• The percentage of unswitched memory CD27$^+$IgM$^+$IgD$^+$ B cells

Cell population compared	Cell surface markers	Health controls (n=18) Median % (Interquartile range)	Sarcoids (n=27) Median % (Interquartile range)	Control versus sarcoidosis group p-value
B cells (% lymphocytes)	$CD19^+$	9.79 (4.97)	8.00 (7.10)	0.24
Memory B cells (% B cells)	$CD19+CD27^+$	28.70 (23.87)	13.50 (16.92)	0.14
Switched memory B cells (% lymphocytes)	$CD19+CD27^+IgD^-IgM^-$	0.82 (0.80)	0.47 (0.60)	0.03
Switched memory B cells (% B cells)	$CD19^+CD27^+IgD^-IgM^-$	8.75 (8.80)	5.67 (10.66)	0.28
Unswitched memory B cells (% lymphocytes)	$CD19^+CD27^+IgD^+IgM^+$	1.30 (0.99)	0.58 (0.57)	0.01
Unswitched memory B cells (% B cells)	$CD19^+CD27^+IgD^+IgM^+$	12.41 (15.46)	6.28 (8.43)	0.02
naive-B cells (% lymphocytes)	$CD19^+CD27^-IgD^+IgM^+$	5.26 (1.88)	4.26 (3.96)	0.62
naive-B cells (% B cells)	$CD19^+CD27^-IgD^+IgM^+$	59.19 (37.22)	61.96 (25.34)	0.48
CD38low CD21low cells (% B cells)	$CD19^+$CD38loCD21lo	2.82 (6.62)	2.32 (3.15)	0.18
Transitional B cells (% B cells)	$CD19^+CD38^{++}$IgMhi	3.76 (2.74)	2.77 (4.36)	0.37
Plasmablasts (% B cells)	$CD19^+CD38^{++}IgM^-$	1.05 (1.35)	0.89 (1.24)	0.42
IgD+ memory B cells (% B cells)	$CD19^+CD27^+IgD^+$	18.87 (16.69)	8.38 (7.99)	0.05

Table 2: Statistical analysis on the proportions of peripheral blood B cell populations compared between healthy controls and sarcoidosis patients
All statistical tests were performed in both Excel and Sigma-Stat 3.1. All values are expressed as a percentage of either total lymphocytes determined by FSC/SSC gating or B cells determined by CD19 gating. The Mann-Whitney U test was used for statistical analysis; the median, interquartile range and p-values for each of the groups are shown.
It should be noted that the application of a multiple testing correction (Benjamini-Hochberg) to the analysis resulted in a loss of significance at 0.05 in the IgD+ memory B cells (%B cells).

(sometimes referred to as Marginal Zone-like B cells) were significantly reduced in the sarcoidosis patients compared to the controls when expressed as both a percentage of lymphocytes (p=0.009) and as a percentage of B cells (Figure 2.E, p=0.022).

- There was no significant difference between the sarcoid or control groups when either naive B cells ($CD27^-IgM^+IgD^+$, Figure 2.F), CD21low B cells (CD21loCD38lo, Figure 2.G), Transitional B cells ($CD38^{++}$IgMhi, Figure 2.H) or Plasmablasts ($CD38^{++}IgM^-$, Figure 2.I) were analysed.

Re-analysis of the data using only age-matched sarcoidosis (n=12) and healthy control (n=12) subjects produced similar results with a significant reduction in the class switched memory (p=0.02) and unswitched memory (p=0.02) B cells in the sarcoidosis patients compared to the controls. This confirmed the findings were not the result of a significant difference in patient age between the sarcoid subjects and the controls. The re-analysis also excluded all patients receiving Prednisolone therapy (n=4) confirming that the results were not a consequence of immunosuppressive therapy.

There were some patients with reduced immunoglobulin levels. A separate analysis of data showed that the significant difference observed between sarcoid patients and controls was essentially unaltered by the exclusion of patients with reduced serum concentrations of one or more immunoglobulin isotype (IgG, IgA and IgM), except in the case of non-switched memory B cells ($CD27^+IgD^+$) where significance which was at p=0.05 was lost. This was attributed possibly to the lower numbers of patients (n=14) in the latter analysis and hence the full data set is presented. Importantly none of the patients with reduced immunoglobulin levels showed a pre-disposition to recurrent infections involving the upper and lower respiratory tracts.

Application of the Freiburg CVID classification system (see "Statistical analysis and B cell memory classification" in the Methods section) to our sarcoidosis data revealed a significant difference in group II and Ib between the sarcoidosis patients and controls (Fisher Exact test, p=0.014). Specifically, 17/18 of the controls were classified as II with 1/18 classified as Ib whilst the sarcoidosis patients were classified as II in 16/27, with 11/27 as Ib. This indicated that all but one of the healthy controls had >0.4% CSM B cells whilst 41% of the sarcoidosis patients had <0.4% CSM B cells (as a percentage of lymphocytes) in their peripheral blood. No statistically significant differences between sarcoidosis patient and control B cells were found when the Paris and EUROclass CVID B cell classification systems were used.

T cells: Both CD4+ and CD4- T lymphocytes that did not express CD27 and CD28 were expanded in sarcoidosis whilst lymph-node homing naïve and memory T cells were reduced

Statistical analysis of proportions of $CD3^+$ T cells, $CD4^+$ T cells and

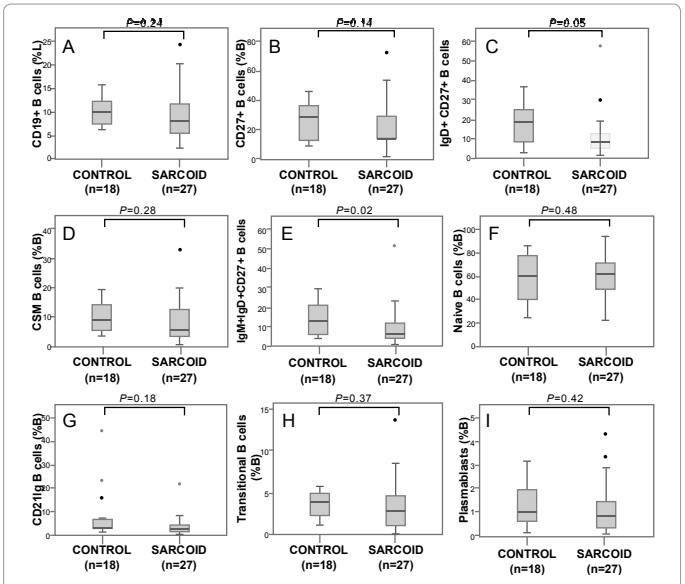

Figure 2: Sarcoidosis patient exhibited reduced peripheral blood B cell memory cell populations
B lymphocytes were analysed in whole blood using forward and side scatter with surface CD19 staining (A). Total B cell memory was analysed using $CD19^+CD27^+$ staining (B). Class switched memory (CSM) B cells were identified using $CD19^+CD27^+IgD^-IgM^-$ surface staining (D). Unswitched memory cells were identified using $CD19^+CD27^+IgD^+$ (IgM^+) (C & E).
Naive B cells were identified as $CD19^+CD27^-IgM^+IgD^+$ cells (F).
$CD19^+$ B cells were further analysed for CD21 and CD38 surface expression to identify CD38loCD21lo B cells (G). B cells were also analysed for IgM and CD38 surface expression to identify $CD38^{++}$IgMhi transitional B cells (H) and $CD38^{++}$IgM- plasmablasts (I).
B cell populations were expressed as either a percentage of lymphocytes (%L) or as a percentage of $CD19^+$ B cells (%B).

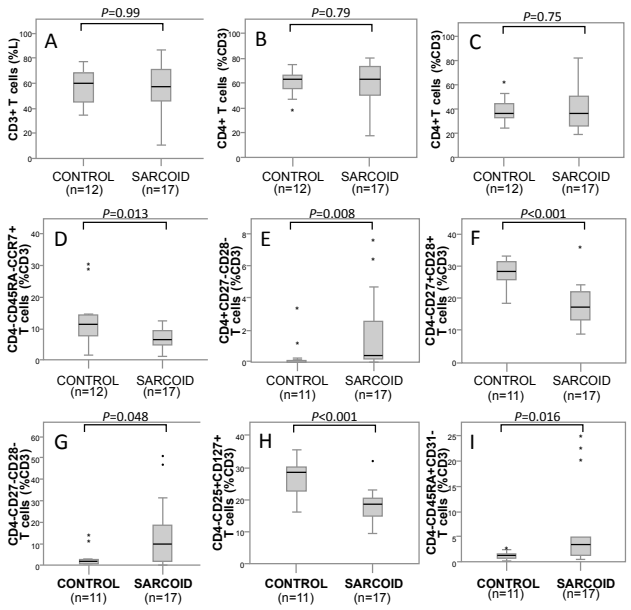

Figure 3: Sarcoidosis patients exhibited reduced naïve T cells and an expanded population of CD27-CD28- T cells when compared to a healthy population

T lymphocytes were analysed in whole blood using forward and side scatter with surface CD3 staining (A). Total helper and cytotoxic T cells memory were analysed by the presence or absence of CD4 surface staining (B & C). Naïve T cells were identified by the surface expression of CD45RA and CCR7 (D). The co-stimulatory molecule CD27 and CD28 were used to identify activated and terminally differentiated T cells (E, F & G). Box plots H and I show $CD4^-CD25^-CD127^+$ and $CD4^-CD45RA^+CD31^-$ T cells respectively.

$CD4^-$ T cells revealed no significant differences between the sarcoidosis patients and healthy controls (Figures 3A-3C). This contradicts recent reports that described lymphopaenia in the peripheral blood of sarcoidosis patients [32-34]. A total of seven populations of T cells were found to be significantly different between the sarcoidosis patients and controls (Table 3). Representative flow cytometric dot-plots of the results found to be significantly different between the two groups are illustrated in Figure 4.

The majority of T cell subsets that were significantly different between the sarcoidosis patients and healthy controls belonged to the $CD3^+CD4^-$ population. Of the $CD3^+CD4^-$ T cell populations identified, the memory population ($CD45RO^+$) was found to be reduced in the sarcoidosis group (Table 3, Figure 4.1). Lymph-node homing $CD8^+$ naive T cells (based on the expression of CD45RA and CCR7) were present in the sarcoidosis group at approximately half the percentage found in the controls (Table 3, Figures 3D and 4.2). $CD27^+CD28^+$ T cells were under-represented in the sarcoidosis group compared to the controls, whilst a population of $CD27^-CD28^-$ T cells was expanded in the sarcoidosis patients and almost absent in the controls (Figures 3F, 3G, 4.4 and 4.5).

A highly significant reduction in the $CD8^+$ T cell population expressing CD127, but not CD25 ($CD25^-CD127^+$) was also found in the sarcoidosis group (Figures 3H and 4.6).

For the $CD4^+$ cell populations, a similar pattern regarding the expression of CD27 and CD28 was observed: the sarcoidosis patients exhibited a distinct population of $CD27^-CD28^-$ cells in the $CD4^+$ population that was almost absent in the healthy controls (Figures 4.3 and 3E). Finally, the population of naive $CD8^+$ T cells that had not recently exited the thymus ($CD31^-$) was increased in the peripheral blood of the sarcoid patients by 2-3 folds (Figure 4.7 and 3I).

Discussion

A comparison of the B cell sub-populations of sarcoidosis patients and healthy controls revealed a number of significant differences between the two groups. Statistically significant differences were restricted to two main cell populations: Class-Switched Memory (CSM) ($CD19^+CD27^+IgD^-IgM^-$) and unswitched memory ($CD19^+CD27^+IgD^+IgM^+$) B cells.

The reduced levels of peripheral blood CSM B cells observed in the sarcoidosis patients were consistent with reported observations that a lower level of these B cells is associated with granulomatous disease in CVID [23,25]. The reduced level of CSM B cells was only significant when they were expressed as a percentage of lymphocytes, consistent with the results obtained using the Freiburg classification [24].

These results clearly indicate that there is a lower proportion of CSM B cells in the peripheral blood lymphocyte populations of sarcoidosis patients when compared to healthy controls and classification of the two groups showed a significant bias in the sarcoidosis group for Freiburg group Ib (<0.4% CSM B cells). Classification by Paris and EUROclass also revealed a trend for a higher proportion of sarcoid patients to be classified into groups associated with low CSM B cells (**MB0, MB1, SmB-**) when compared to the controls. However, these

Cell surface markers/phenotype	Expressed as Healthy Controls (n=12)	Healthy Controls (n=12) Mean	Sarcoids (n=17) Mean	Control versus sarcoid p-value
CD3+CD4-CD45RO+CD45RA- (memory T cytotoxic)	lymphocytes	9.09	5.97	0.038
	T cells	16.07	10.47	0.028
	CD4- cells	43.13	30.07	0.085
CD3+CD4-CD45RA+CCR7+ (naïve T cytotoxic)	lymphocytes	8.39	4.26	0.034
	T cells	12.79	6.59	0.013
	CD4- cells	30.99	18.40	0.017
CD3+CD4+CD28-CD27- (late differentiated T helper)	lymphocytes	0.02	0.16	0.016
	T cells	0.03	0.36	0.008
	CD4+ cells	0.05	1.45	0.013
CD3+CD4-CD28+CD27+ (early differentiated T cytotoxic)	lymphocytes	17.82	10.59	<0.001
	T cells	28.02	18.24	<0.001
	CD4- cells	78.46	55.90	0.008
CD3+CD4-CD28-CD27- (late differentiated T cytotoxic)	lymphocytes	1.21	6.14	0.054
	T cells	2.08	10.12	0.048*
	CD4- cells	6.27	28.86	0.006
CD3+CD4-CD25-CD127+ (CD127+ T cytotoxic)	lymphocytes	17.43	11.33	<0.001
	T cells	26.74	18.46	<0.001
	CD4- cells	72.84	52.29	0.002
CD3+CD4-CD45RA+CD31- (CD31- naïve T cytotoxic)	lymphocytes	0.83	2.05	0.048*
	T cells	1.29	3.42	0.016
	CD4- cells	2.92	8.16	0.003

Table 3: Statistical analysis of the peripheral blood T cell populations in healthy controls and sarcoidosis patients

All statistical tests were performed in both Excel and SigmaStat 3.1. All values expressed as a percentage of either total lymphocytes determined by FSC/SSC gating, T cells as determined by CD3 staining and as % of either the CD3+CD4+ or CD3+CD4- populations. Lymphocyte sub-populations were compared between groups and either t-test or Mann-Whitney U tests were performed depending on the results of the Kolmogorov-Smirnov normality test.

*These values did not retain significance at a critical value cut-off of 0.05 when a Benjamini-Hochberg multiple testing correction was applied. All other significant values highlighted in bold retained significance at this level after multiple testing corrections were applied.

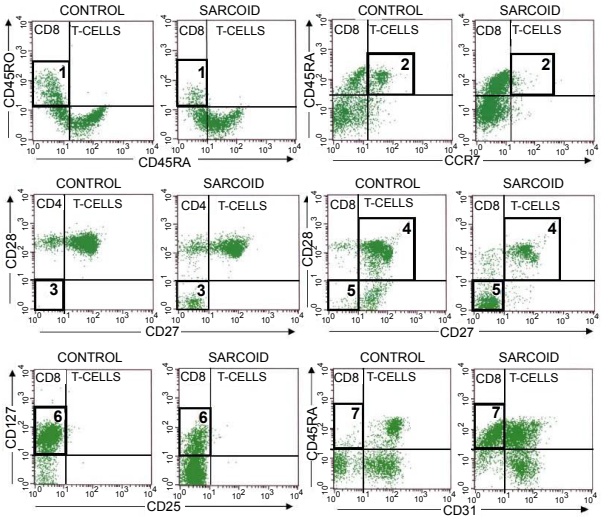

Figure 4: Representative examples of dot-plots for sarcoidosis patients and controls illustrating the T cell populations that were found to be significantly different between the two groups

Examples of the T cell populations are labelled 1-7, referring to the results listed in table 3. The associated population is highlighted with a bold black rectangle. 1: CD3$^+$ CD4$^-$ CD45RO$^+$ CD45RA$^-$. 2: CD3$^+$ CD4$^-$ CD45RA$^+$ CCR7$^+$. 3: CD3$^+$ CD4$^+$ CD28$^-$CD27$^-$. 4: CD3$^+$ CD4$^-$ CD28$^+$ CD27$^+$. 5: CD3$^+$ CD4$^-$ CD28$^-$ CD27$^-$. 6: CD3$^+$ CD4$^-$ CD25$^-$CD127$^+$. 7: CD3$^+$ CD4$^-$ CD45RA$^+$ CD31$^-$

differences were not statistically significant. The higher proportion of Freiburg Ib subjects in the sarcoid group reflects their reduced numbers of CSM B cells compared to the controls (predominantly Freiburg group II). It is not possible to determine the significance of this result with respect to granulomatous disease in sarcoidosis as the original Freiburg classification study in CVID did not examine granulomatous disease [24].

Similar observations of disturbed peripheral blood B cell homeostasis in sarcoidosis have recently been reported elsewhere in the literature [32]. Lee et al. [32] reported the same phenomena of reduced total memory B cells (CD27$^+$) with specific reductions in both CSM and unswitched memory B cells. However, this study also reported reduced total B cells in patients with sarcoidosis; a phenomenon that was not observed in our cohort. The reason for this disparity is uncertain but may be related to the different make-up of the two sarcoid cohorts (i.e. differences in gender, age, ethnicity, disease severity and treatment protocols).

Previous studies have attributed reduced numbers of B cell memory to either defects in the ability of B cells to activate and differentiate following stimulation [32] or as a result of B cell sequestration to sites of pulmonary inflammation [35,36]. The significance of a reduced peripheral CSM B cell population, as observed previously in granulomatous CVID and here in sarcoidosis, is difficult to determine. Germinal Centre (GC) development and T cell interactions are required for the development of CSM B cells [37] which are classically considered T-dependent follicular B lymphocytes. It has been previously reported that CSM B cells are reduced in monogenetic disorders where T cell genes involved in the antibody class switch process contain mutations. These disorders include X-linked Hyper IgM syndrome (XL-HIGM) where a mutation is present in the CD40 Ligand (CD40L) gene [38] , X-linked Lymphoproliferative disease (XLP) with a mutated SAP gene [39,40] and Inducible Co-stimulator (ICOS) deficiency [41].

XL-HIGM, XLP and ICOS deficiency exhibit abrogation of the GC reaction and reduced numbers of peripheral blood CSM B cells (<2% of B cells) [42-44]. In CVID the reduced numbers of CSM B cells is associated with reduced levels of serum IgG and IgA [23-25]. Surprisingly, no correlation was found between CSM B cell levels and serum immunoglobulin levels in our sarcoidosis cohort (data not shown).

A question that remains unanswered is whether granulomatous disease in gvCVID and sarcoidosis precedes a loss of CSM B cells or vice versa. It is unlikely that a reduction in B cell memory is the cause of granuloma development, as other diseases that exhibit reduced CSM B cells such as XL-HIGM syndrome, X-linked lymphoproliferative disease (XLP) and Wiskott-Aldrich syndrome (WAS) do not include granulomata in their clinical spectrum [43,45,46]. A more plausible explanation is that a defect in the T lymphocyte compartment of both gvCVID and sarcoidosis is responsible for granuloma development as well as GC abrogation and the subsequent loss of CSM B cells.

The other B cell memory population that was significantly different between the sarcoid and control groups was the IgM$^+$IgD$^+$CD27$^+$ B cells. This sub-population was significantly reduced in the sarcoid patients compared to the controls when expressed as both percentage of lymphocytes and B cells. In addition, the proportion of these cells positively correlated with the serum levels of IgM in the sarcoid patients (data not shown). It has been reported that IgM$^+$IgD$^+$CD27$^+$ B cells represent B cells that have undergone somatic hyper-mutation but not immunoglobulin gene class-switching and produce high affinity, IgM antibodies to T-independent (TI) antigens including encapsulated bacteria [47-50]. The development of IgM$^+$IgD$^+$CD27$^+$ B cells is controversial [51]; although they are reported as T cell-, CD40L- and GC-independent, they are markedly reduced in CD40L- and ICOS-deficiencies [43,44] suggesting a requirement for T cell help.

A reduction in IgM$^+$IgD$^+$CD27$^+$ B cells was previously reported in CVID and found to be correlated with the reduction in CSM B cells [23,25]. In the current study, the levels of CSM B cells and IgM$^+$IgD$^+$CD27$^+$ B cells in sarcoidosis patients were also positively correlated (p=0.008). However, the relationship and interactions that occur between these two B cell subsets has yet to be elucidated.

The significance of the reduction in IgM$^+$IgD$^+$CD27$^+$ B cells in sarcoidosis patients found here is difficult to elucidate due to the controversy concerning their role in humans. If IgM$^+$IgD$^+$CD27$^+$ B cells are T-dependent IgM$^+$ memory cells of GC origin then this may be a consequence of disruption to the T lymphocyte compartment and granuloma development as was hypothesised for the reduction in CSM B cells. If IgM$^+$IgD$^+$CD27$^+$ B cells are in fact T cell and GC-independent then the significance of their reduction is difficult to speculate upon because neither B cell nor antibody mediated disease mechanisms have been described in sarcoidosis. In CVID, the reduction of IgM$^+$IgD$^+$CD27$^+$ B cells is clearly associated with clinical symptoms that can be attributed to a subsequent reduction in either serum immunoglobulins or specific antibody responses [52]. Although this observation has been associated with granulomatous disease in CVID [53], the biological mechanism behind this has yet to be elucidated.

An analysis of the peripheral blood T cell compartment of our sarcoidosis cohort revealed a number of significant differences when compared to healthy controls. These differences were apparent in a number of T cell populations defined by cell surface markers whose phenotype has previously been associated with T cell memory, activation, effector function and terminal differentiation.

The first observation was a reduction of approximately 50% in the

naive $CD8^+$ population in sarcoidosis patients compared to healthy controls. These cells are defined by the expression of the CD45RA "naive" isoform [54] in conjunction with the lymph node homing chemokine receptor CCR7 [55]. This observation has been previously reported in sarcoidosis patients for the naive $CD4^+CD62L^+CD45RA^+$ T helper cell lineage [33], however the naive $CD8^+$ T cell compartment has not been previously examined. Interestingly, this observation has been made previously in the sub-group of CVID patients with granulomatous disease in the $CD4^+$ lineage [56]. However, in gvCVID this reduction in naive T cells was coupled with an expansion of CD19hiCD21lo B lymphocytes, which was not observed in our sarcoid cohort. In another study, a predominance of $CCR7^-$ $CD8^+$ T cells was observed in CVID patients with deficient proliferative capacity and granulomatous disease [57]. The reduction of $CCR7^+$ cells may go some way to explain the phenomena of peripheral anergy observed in sarcoidosis; T cells lacking the CCR7 surface marker have been defined as antigen experienced effector cells with a reduced proliferative capacity that home to tissues instead of the lymph node [58,59]. It is possible that a dominance of effector T lymphocytes, in conjunction with a reduced number of naïve T cells, contributes to the failure to respond to vaccine antigen previously described in sarcoidosis. T lymphocytes lacking CCR7 have been reported with a reduced capacity to provide help to B cells due to reduced expression of CD40L [60]. This may go some way to explaining the reduced levels of CSM and $IgM^+IgD^+CD27^+$ B cells in sarcoidosis patients as the development of both of these B cell memory populations can be T-dependent [51].

The second principal observation of the T cell analysis was a reduction in $CD4^+CD28^+CD27^+$ T cell population coupled with a significant increase in the $CD4^+CD28^-CD27^-$ and $CD4^-CD28^-CD27^-$ T cells compared with the healthy controls.

The co-stimulatory molecules CD27 and CD28 are respectively involved in the generation of antigen-primed cells and T cell activation [61,62] and have recently been shown to correlate with surface phenotypes of effector T cells (CD45RA-CCR7) and with measures of function [63]. Romero et al. [63] identified four distinct subtypes of $CD8^+$ T cell effector memory populations based on the expression of CD27 and CD28 and defined a population referred to as Effector Memory 3 (EM3) which lacked CD27 and CD28 expression. The functional characteristics of the late differentiated EM3 T cell population included evidence of having reduced proliferative capacity with potent cytolytic function and high IFNγ secretion.

The loss of CD27 and CD28 on $CD8^+$ T cells has previously been reported as a marker of senescence and is observed in persistent viral infections that are characterised by chronic antigenaemia including HIV [64] and CMV [65] disease. In HIV, $CD3^+CD8^+CD28^-$ lymphocytes had repeatedly undergone antigen driven proliferation, cell cycle arrest and possessed short telomeres with low telomerase activity [66]. Other reports have described this subset of effector-memory $CD8^+$ T cells as highly cytotoxic, non-anergic cells with raised IFNγ secretion [67-69] and reduced proliferative capacity [70]. The chronic antigenaemia in HIV is thought to drive the $CD8^+$ T population towards the late stage terminal effector memory cell; a population with a reduced responsiveness to new antigens. In HIV disease late stage effector T cells replace less differentiated cells including naive ($CD45RA^+$) [71,72], central memory ($CCR7^+$) and $CD127^+$ T cells [73].

The same pattern of expansion in terminally differentiated $CD8^+CD28^-CD27^-$ T cell populations with a concurrent reduction in less differentiated T cells ($CD45RA^+$, $CCR7^+$, $CD127^+$) is observed in the peripheral blood of individuals with sarcoidosis. The significance of an expanded, terminally differentiated effector memory T cell population in the context of sarcoidosis is not clear. In diseases defined by high levels and duration of antigenic stimulation (of which sarcoid is thought to be one) terminally differentiated effector $CD8^+$ T cells are described as functionally exhausted [74] and express high levels of the transcription factors T-bet and Blimp1 [32,75-77]. In some chronic viral infections, functionally exhausted effector T cells express markers of senescence including CD57 [75] as well as negative costimulatory molecules including PD-1 [76] and CTLA-4 [77] that are thought to contribute to immune dysregulation in these diseases. The inflation of late differentiated $CD8^+CD27^-CD28^-$ effector memory T cells in chronic conditions has been reviewed and the importance of distinguishing these cells from exhausted, nonfunctioning T cells that express CD57 and high levels of PD-1 emphasised [78].

Recently, other researchers have published similar findings with the identification of both expanded $CD4^+CD27^-$ [33] and CD8+CD28null T cells [79] in the peripheral blood of patients with sarcoidosis. Lee et al. termed the expanded $CD4^+CD27^-$ T cells "differentiated effectors" based on their co-expression of CD95, CD152 and HLA-DR. $CD4^+$ T cells in the peripheral blood of sarcoidosis patients from their cohort were found to have reduced T cell receptor (TCR) responsiveness as well as exhibiting reduced expression of molecules involved in T cell activation (including NFκB, NFAT and CD3ζ-chain). They concluded that this unresponsive state was suggestive of anergy similar to that seen in other conditions of chronic antigen stimulation such as in autoimmune conditions and chronic viral infections [33].

Further work should determine whether the atypical T and B cell sub-populations identified in sarcoidosis can be used as biomarkers of disease prognosis or to allow disease stratification in a manner similar to the classification systems used in CVID. In addition, future research on sarcoidosis should seek to further define the population of $CD8^+$ T cells that resemble late differentiated effector memory cells. Further phenotypic examination should include measuring the expression of PD-1 and CD57 to determine whether these expanded T cells demonstrate an "exhausted" phenotype. Functional work should seek to examine their cytokine secretion profiles as well as their cytotoxic and proliferative capacity. Finally their T cell receptor usage should be examined to determine if this expanded population exhibits a restricted repertoire directed against a limited number of immunodominant antigenic epitopes. Identification of their antigenic targets could further expand our knowledge of the pathogenesis of sarcoidosis and potentially put us one step closer to identifying the causative agent(s) of this elusive disease.

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Competing Interests

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