J R Coll Physicians Edinb 2020; 50: 226–32 | doi: 10.4997/JRCPE.2020.304
ORIGINAL RESEARCH PAPER
ANDREW DOUGLAS AWARD 2020 WINNER

Distinct T-cell immunophenotypic signature in a subset of sarcoidosis patients with arthritis

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Abstract

Background: The objective of the study was to assess T-cell subsets in sarcoidosis patients with or without articular involvement.

Methods: Treatment-naïve patients were divided into Group A (articular) and Group B (non-articular) based on joint involvement. Flow cytometric analysis of T-cell subsets and pro-inflammatory cytokines were carried out in the peripheral blood.

Results: Patients in group A (n = 29, mean age 40 ± 10.1 years) were compared with group B (n = 18, 43 ± 12.2 years). T-cell subsets: the CD4/CD8 ratio was abnormal in two groups but had no significant difference (p = 0.63). Ratios of Th1/Treg, Th2/Treg and Th17/Treg were significantly increased in group A as compared to group B [p <0.001] indicating polarisation of T-cell subsets. CD8 T-cells in group A had higher granzyme B expression (p = 0.03). B cells were increased in group A [p = 0.04]. Ratio of IFN-γ /IL10, IL-4/IL10, IL-17/IL10 in sera as well as culture supernatant were significantly higher in group A as compared to group B.

Conclusion: The T-cell axis was skewed towards Th1, Th2 and Th17 in sarcoid arthritis when compared to non-articular patients.

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Keywords: musculoskeletal, joint, granulomatous disease, non-tubercular, T-cell phenotypes, flow cytometry, cytokines, T-regulatory cells

Financial and Competing Interests: No conflict of interests declared

Introduction

Sarcoidosis is a multisystem disorder with diverse manifestations and complex pathogenesis characterised by non-caseating granulomas in many organ systems. Diagnosis and management of sarcoidosis continues to be challenging.[1] Clinical presentation and prognosis may vary from being totally asymptomatic to a short self-limiting course to a more relapsing or persistent chronic course.[2–4] Differences in genotype and corresponding phenotype are being defined for different subsets.[5]

Compartmentalisation of T-cells has been described previously.[6] Sarcoid granulomas have accumulation of CD4+ T-cells and macrophages at sites of inflammation.[7] These granulomas comprise epithelioid cells as well as mononuclear cells and CD4+ T-cells with a limited CD8+ T-cells around the periphery.[8] In patients with sarcoidosis the proportion of T-cells is increased in the bronchoalveolar fluid and typically comprises 20–60% of the total cell count. CD4+ T-cells dominate, with a CD4+:CD8+ T-cell ratio typically >3–5:1 compared with a ratio of 2:1 in healthy controls.

Despite advances in our knowledge of the immunopathogenesis of sarcoidosis, the cause of varied presentations of this disease remains unclear. Studies focussing on the pathogenesis have largely been confined to understanding of disease process in the lung and have highlighted the role of Th1, Th2, Th17, Treg cells and CD8 T cells.[9–12] Rheumatologic manifestations, such as arthritis, tenosynovitis and dactylitis, are reported in 4–35% of the cases, making these the most frequent extrapulmonary features reported with sarcoidosis.[13–15] There are data, albeit scarce, linking human leukocyte antigen (HLA) to disease phenotype. HLA-DRB1*1101 is associated with cardiac sarcoidosis, hypercalcaemia and environmental exposure to insecticide.[16]

Similarly, HLA DRB1*0301 are associated with excellent prognosis and recovery unlike HLA DRB1*14 and HLA DRB1*15 which are strongly associated with a chronic disease course and a high risk of developing pulmonary fibrosis.[17] T-cell subtypes have been shown to determine disease phenotypes in other diseases like systemic lupus erythematosus and are being considered as therapeutic targets.[18–20] However, the same is lacking when it comes to sarcoidosis and its varied manifestations. Most of

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the literature has focussed on pulmonary sarcoidosis or compared Löfgren to non-Löfgren without details on articular manifestations.[21,22]

Therefore, we hypothesised that different manifestations of sarcoidosis may be associated with their characteristic T-cell phenotype. In the present study, we evaluated the peripheral blood T-cell signature in patients with sarcoidosis, and assessed whether it differs in those with and without articular manifestations.

Methods

Study subjects, peripheral blood

For the purpose of this cross sectional study, patients with sarcoidosis attending the outpatient department at Sanjay Gandhi Postgraduate Institute of Medical Sciences, a tertiary care hospital in North India, were recruited from June 2016 to May 2018. Due to heterogeneity in disease manifestations, diagnosis of sarcoidosis was based on a combination of compatible clinical profile plus demonstration of non-caseating granulomas in involved tissues and exclusion of other causes of granulomatous inflammation. In the present study, the following diagnostic criteria for articular sarcoidosis were used:[14,22]

a) Arthritis + chest X-ray showing non-necrotic and symmetrical hilar / mediastinal lymph node enlargement (LNE) (Mantoux negative) AND
b) Bilateral ankle arthritis with duration < 2 months, age < 40, erythema nosodum (EN) (3/4) OR
c) Tissue diagnosis showing non-caseating granulomas negative for acid-fast bacilli, fungal elements or foreign bodies.

Patients with non-articular sarcoidosis were diagnosed on the basis of the presence of either one or a combination of any of these manifestations: constitutional symptoms with/without dry cough, dyspnoea on exertion or rest, infiltrative skin lesions, generalised lymphadenopathy or hepatosplenomegaly, renal failure with/without hypercalcaemia or hypercalciuria, granulomatous uveitis, lacrimal or parotid-gland enlargement and other symptoms suggestive of sarcoidosis, along with demonstration of hilar and/or mediastinal lymphadenopathy or features of interstitial lung disease on imaging and presence of non-caseating granulomas negative for acid-fast bacilli, fungal elements or foreign bodies.

Clinical details and available laboratory/ imaging investigations were recorded. All patients were treatment-naïve. Patients were divided into two groups according to articular involvement: group A had articular involvement while group B did not. Articular involvement was defined as having one or more of the following: arthralgia/arthritis of one or more joints, tenosynovitis, enthesitis, inflammatory backache, sacroiliitis assessed clinically or radiologically. Oligoarthritis was defined as involvement of less than five joints whereas polyarticular was defined as involvement of five or more joints. Chronic polyarthritis was defined as more than six months' duration of joint symptoms.

T-cell staining

T-cell immunophenotype was assessed in peripheral blood after diluting in a ratio of 1:1 in all patients by flow cytometry with appropriate gating using CD4, CD8, IFN-γ, IL-2, IL-17, and CD25, FOXP3 Th1, Th2, Th17 and Treg cells, respectively (supplementary figure 1).[23] Briefly, whole blood was diluted 1:1 with RPMI 1640 culture media (Sigma Aldrich, St. Louis) and stimulated with phorbol 12-myristate 13-acetate (20 ng/ml; Sigma Aldrich, St. Louis) and ionomycin (1 lg/ml; Sigma Aldrich, St. Louis) for 5 h. Monensin (2 lM; BD Biosciences, San Diego, CA) was also added for the final 2 h of activation as a protein-transport inhibitor. After surface staining, red blood cells were lysed with BD FACSTM lysing solution. Cells were washed, fixed and then permeabilised with Cytofix/Cytoperm™ kit (BD Pharmingen) according to the manufacturer's instructions. At least 10,000 cells in 'lymphocyte gate' were acquired on BD FACS Canto™ II (Becton Dickinson, Mount View, CA, USA). Isotype-matched antibodies were used as controls. All the stained cells were analysed using FlowJo software v10.0.7 (Tree Star, USA). CD8 T-cells expression was assessed using antibodies against CD8 and granzyme by flow cytometry.

Cytokine analysis

Collected venous blood was diluted 1:1 with RPMI 1640 (Sigma Aldrich, 3050 Spruce Street, Saint Louis, MO 63103, USA), containing Hepes (25 mM), gentamicin (50 pg/ml) and 10% heat-inactivated fetal calf serum (FCS). Cells were resuspended in RPMI 1640 supplemented with 2 mM of extra glutamine and 10% FCS. In vitro culture of the PBMCs was performed with mitogen phorbol 12-myristate 13-acetate (50 ng/ml; Sigma Aldrich, St. Louis, MO 63103, USA) and ionomycin (1 µlg/ ml; Sigma Aldrich, St. Louis, MO 63103, USA) in a flat-bottom six-well culture plate at 37˚C, 5% CO2, 100% humidity. Culture supernatants were harvested after 24 h and stored at –80˚C until analysis.

The ratio of effector T-cell subsets to regulatory T-cells, B-cells and the ratio of inflammatory cytokines to IL-10 were calculated and compared in the two groups to understand the impact of these cells and cytokines in disease pathogenesis.

Statistical analysis

Statistical analysis was performed using graph prism version 5.01 and SPSS version 20 (IBM Corp, Armonk, NY). The data are represented as median with range (minimum–maximum) unless otherwise specified. The Mann-Whitney U-test was used to compare the median in the two groups and an independent T-test for comparing the mean. For all comparisons a p value less than 0.05 was considered significant.

Ethical approval

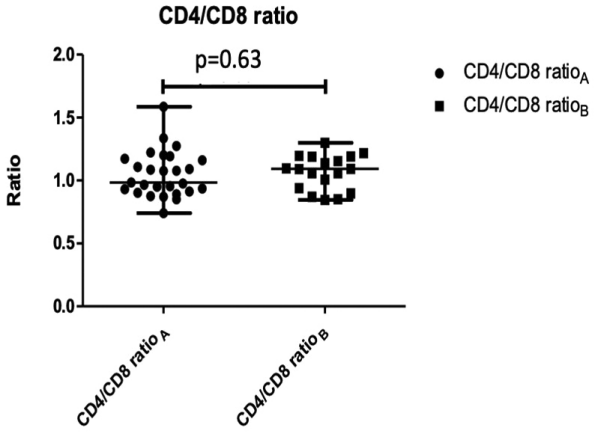
Approval was obtained from the Institutional Ethics Committee (IEC code no. 2016-29-DM-EXP). Written informed consent were obtained from all patients included in the study.

Table 1 Clinical characteristics of sarcoidosis patients

	Group A (n = 29)	Group B (n = 18)	p
Age (mean ± SD, in years)	40 ± 10.1	43 ± 12.2	0.36
Male : female	9 : 20	11 : 7	
Total duration of illness (Mean ± SD, in years)	1 ± 2.2	2 ± 3.3	0.22
EN	11	2	0.19
Hilar/mediastinal	26	16	0.09
ILD	12	13	0.07
Uveitis	4	0	0.28
Constitutional			
Fever	13	5	0.35
Weight loss	4	7	0.07
Cutaneous*	9	7	0.75

*other than EN.
EN: erythema nodosum; ILD: interstitial lung disease

Figure 1 CD4/CD8 ratio in whole blood compared between group A (CD4/CD8 ratioA) to group B (CD4/CD8 ratioB). Middle horizontal bar represents the median and horizontal bar above and below refers to the range.



Results

Patient characteristics

Forty-seven patients were enrolled in the study, 29 with articular manifestations (group A) and 18 without articular manifestations in group B (Table 1). There was no difference in baseline characteristics and extra-articular manifestations in the two groups. All patients were treatment-naïve and had active disease. The most common finding on chest imaging was hilar/mediastinal nodes followed by interstitial lung disease (ILD) (Table 1).

Group A with articular manifestations

Of the 29 patients, acute polyarthritis (n = 16) was the most common manifestation followed by acute oligoarthritis (n = 9), chronic polyarthritis (n = 3) and chronic oligoarthritis (n = 1). The ankle was most commonly involved joint (n = 26) followed by the knee (n = 17), the wrist (n = 14) and one patient had sacroiliitis.

Group B with no articular manifestations

Pulmonary sarcoidosis was the most common phenotype (10/18) with no other organ system involvement followed by renal (4/18 patients) and 4 others (2 gastrointestinal, 1 glandular, 1 multi-system sarcoidosis).

T-cell phenotyping

Absolute percentages of CD4 T, CD8 T-cells, T-cells subsets, B-cells and cytokine levels are presented in supplementary Table 1.

Absolute lymphocyte count and CD4/CD8 ratio

Total leucocyte count (TLC) and absolute lymphocyte count (ALC) were similar in the two groups but the CD4/CD8 ratio was altered in the two subsets. Group A had median TLC, ALC 8350 (7195–9035) cells/mm^3, 2141 (1824–2588) cells/mm^3 respectively and group B had median TLC 7400 (6000–8960) cells/mm^3 and median ALC 2220 (1748–2350) cells/mm^3 with no difference in the two groups (p >0.05)

CD4/CD8 ratio was abnormal in Group A [1.0 (range 0.74–1.59)] as well as Group B 1.1 (range 0.84–1.3) with no difference between two groups (p = 0.63) (Figure 1).

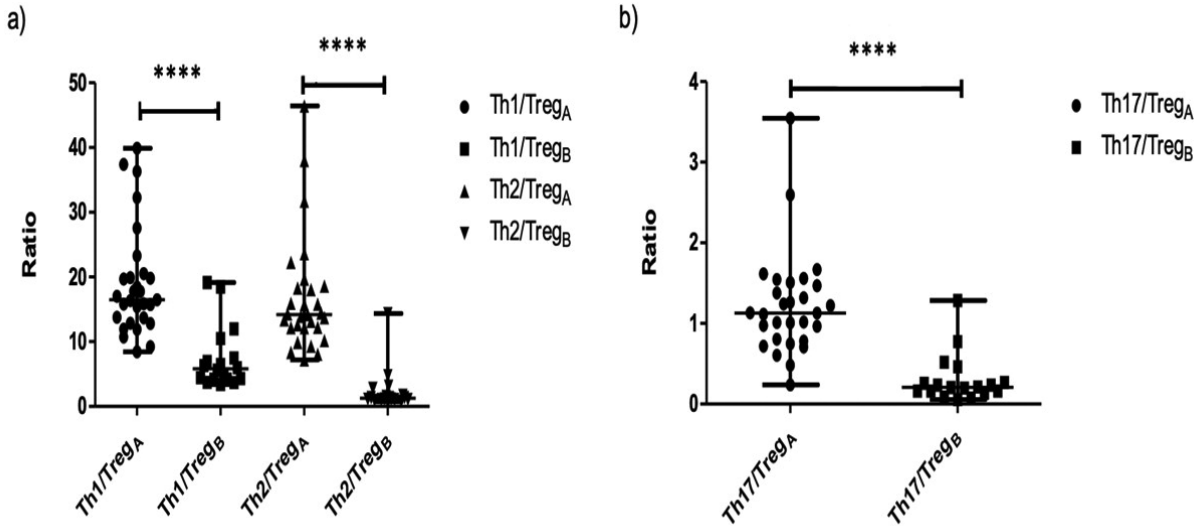
Imbalance of Th1/Treg, Th2/Treg, Th17/Treg and Th1/Th17

The T-cell axis was assessed calculating the ratio of various T-cell subsets (Th1, Th2, Th17) to Treg in the peripheral blood expressed as median with range (minimum–maximum) (Figure 2). Th1/Treg, Th2/Treg and Th17/Treg ratio were significantly higher in group A (p <0.0001). Th1/Treg ratio was 16.5 (8.4–39.9) in group A as compared to group B 5.8 (3.4–19.14). Th2/Treg and Th17/Treg showed the same trend and were found to be increased in group A 14.2 (7.21–46.45) and 1.1 (0.24–3.54), respectively when compared to Th2/Treg and Th17/Treg ratio in group B 1.3 (0.71–14.36) and 0.2 (0.05–1.29), respectively (Figure 2a and 2b). The Th1/Th17 ratio was significantly lower in group A, 14.93 (9.06–50.33) when compared to group B, 28.05 (13.86–76.33), p <0.0001 (Figure 3). The findings were confirmed when the Th1, Th2, Th17, Treg cytokines ratio was assessed in sera and culture supernatant. A significantly higher ratio of IFN-γ/IL10, IL4/IL10 and IL17/IL10 was found in sera and culture supernatant of patients in group A (p <0.05) (Figure 4).

CD8 T-cell and B cells in sarcoidosis

CD8 T-cells in group A had higher granzyme B expression when compared to group B expressed as median with IQR25-75 [12.5 (10.4–14.6) and 10.5 (8.6–12.6) respectively, p 0.03] (Figure 5a). B cells were also significantly increased (p = 0.04) in the articular group in the peripheral blood with 8.1 % (IQR25-75, 6.7–9.1) vs 6.9% (IQR25-75, 6.1–7.3) in the non-articular group (Figure 5b).

Figure 2 Flow cytometry analysis of T-cell subset ratio in peripheral blood in two groups. Higher Th1/Treg, Th2/Treg and Th17/Treg in group A meant skewing of axis to Th1, Th2 and Th17 axis. Ratio are expressed as median with range, Th1/TregA, Th2/TregA, Th17/TregA refers to the ratio in group A and Th1/TregB, Th2/TregB, Th17/TregB in group B. **** $p < 0.0001$.

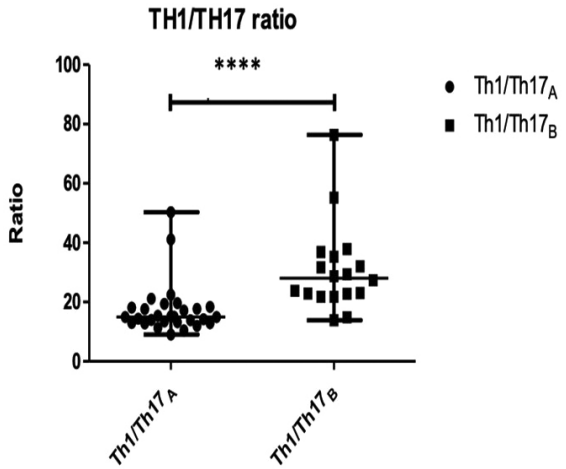


Discussion

There was skewing of the T helper-cell axis towards Th1, Th2 and Th17, with a reduction in Treg cells along with their respective cytokines in the peripheral blood of patients with articular involvement compared with non-articular sarcoidosis. Additionally, there was higher granzyme expressing CD8 T-cells and higher frequency of B cells in the peripheral blood of patients with articular sarcoidosis, giving a distinct cell signature to these patients.

IFN-γ is central to the formation of granuloma in sarcoidosis and traditionally is believed to be secreted by Th1 cells, however, there is accumulating evidence for the role of Th17 cells in secretion of IFN-γ especially by a subset of Th17 cells, Th17.1 (CCR6+ CXCR3+) found predominantly in bronchoalveolar lavage (BAL) fluid.[24] Th17 cells have been reported to play an important role in granulomatous inflammation of the gut in Crohn's disease.[25] It has been reported that there is increased frequency of IFN-γ-producing Th1, Th17.1 and 1L-17A-producing Th17 cells and deficient Tregs numbers and function in BAL as well as mediastinal lymph nodes in non-Löfgren's syndrome sarcoidosis. Upregulation of Th1 transcription factors Tbet, STAT1 and IFN-γ inducible chemokines and chemokine receptors have been reported in sarcoidosis.[26,27] In a German study identifying genetic risk factors of sarcoidosis it was predicted that IL23/Th17 signalling pathway predisposes to sarcoidosis.[28] Facco et al. reported higher frequency of Th17 cells in peripheral blood and BAL fluid in pulmonary sarcoidosis and linked the role of these cells to the granulomatous and fibrotic phase of the disease.[6] Th17 cells are believed to play an important role in inflammatory arthritis of various aetiologies as has been reported in rheumatoid arthritis, seronegative spondyloarthropathy, inflammatory bowel disease, juvenile idiopathic arthritis, Lyme arthritis, multiple sclerosis and psoriasis.[29,30] Increased Th17 cells in the synovial fluid compartment have been reported in severe forms of arthritis (extended oligoarticular juvenile idiopathic arthritis) as compared to milder form of arthritis (persistent oligoarticular JIA) in children.[31] The higher Th1, and Th17 frequency and increased IFN-γ/IL10, IL-17A/IL-10, IL-4/IL-10 in sarcoid arthritis as compared to non-articular sarcoidosis in the present study is reflective of a higher inflammatory microenvironment and a possible role of these cells and pro-inflammatory cytokines in the pathogenesis of arthritis.

Figure 3 Th1/Th17 ratio was significantly lower in group A since the T cell expressing IFN gamma in CD4 positive T cells were lower when compared to non-articular group B). Ratio is expressed as median with range. Th1/Th17A refers to ratio in articular group A whereas Th1/Th17B refers to non- articular group B. **** $p < 0.0001$.



Treg cells have an important role in immune regulation and various mechanisms of impaired regulation by Treg cells have been described.[32] Frequency of the Treg cell population

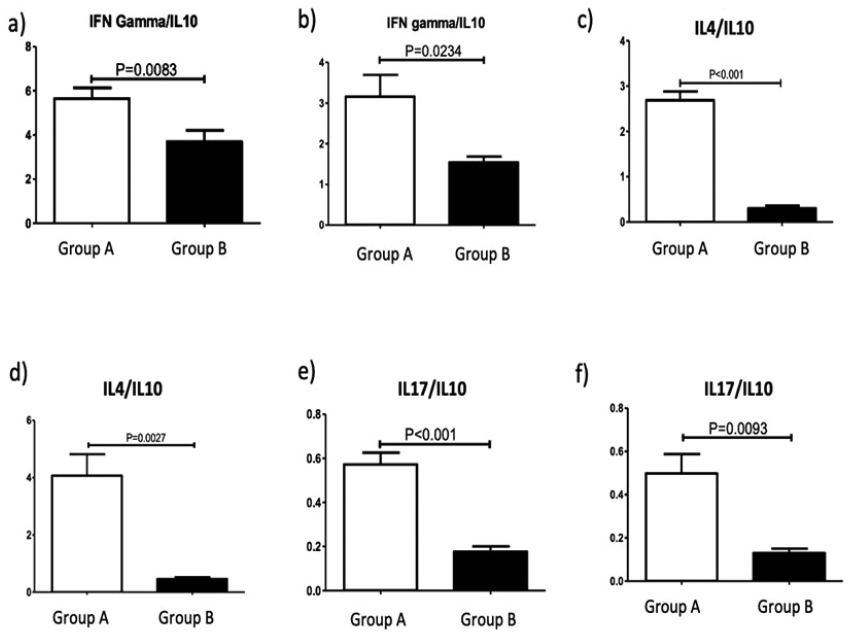
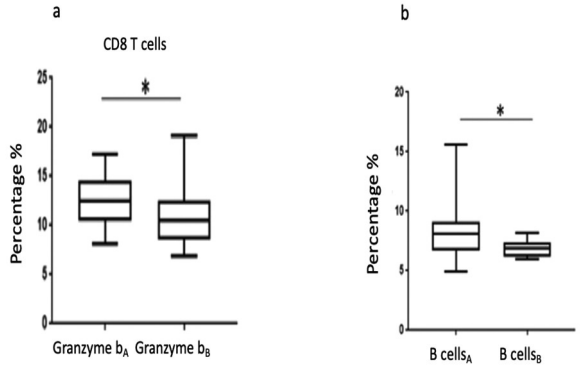


Figure 4 ELISA of Th1, Th2, Th17 cytokines in serum sample and culture supernatant of the patients: Ratio of IFN Gamma/IL10, IL4/IL10 and IL17/IL10 were significantly increased in group A as compared to group B in both culture supernatant (4a, 4c, 4e) as well as serum samples (4b, 4d, 4f).

has been reported to be inconsistent in peripheral blood and BAL fluid and is affected by organ involvement.[33–35] There are studies which have reported a higher frequency of Treg cells in peripheral blood in sarcoidosis but with a poor regulatory mechanism; Tregs are prone to apoptosis [9,36,37] thus questioning the regulatory action of these cells. In the present study, we observed higher Treg frequency in the peripheral blood in the non-articular group and despite having an increased number of Th1 cells as compared to the articular group, both serum as well as culture supernatant had decreased levels of IFN-γ and IL-17 A and increased levels of IL-10 cytokines. Thus, Treg cells in our cohort of non-articular sarcoidosis were likely to be functionally active. As observed by us, an imbalance between Th17 and Treg cells in sarcoidosis has been reported previously.[35]

Enhanced cytolytic activity of CD8 T-cells expressing higher granzyme was seen in our patients with articular sarcoidosis. Parasa et al. demonstrated enhanced peripheral CD8+ T-cell responses in sarcoidosis when comparted to healthy controls.[38] Their study however revealed a higher response in non-Löfgren patients, contrary to our findings. But our cohort with articular manifestations included patients with both acute and chronic arthritis and it is not clear whether their cohort of non-Löfgren patients had any articular involvement. Higher Th2 and functional CD8 T-cells may also point towards more chances of chronicity, as Th2 and CD8 T-cells have been implicated in granuloma and fibrosis besides M2 macrophages.[12] It has been reported that B cells may enhance the ability of dendritic cells to induce differentiation of Th2 cells.[39]

Figure 5a CD8 T-cells in group A had higher granzyme B expression when compared to group B (p=0.03)
Figure 5b B cells were also significantly increased (p 0.04) in the articular group as compared to non-articular group (p=0.04).



The relevance of B cells in disease pathogenesis has been published previously with increased BAFF[40] and B cells in the periphery of granuloma. Higher B cells in patients with articular involvement could be a potential therapeutic target in these patients. Antibodies to mutated citrullinated vimentin (MCV) have been reported in few genetically susceptible patients of sarcoidosis and Löfgren's syndrome.[41] Since the majority of patients in the articular group had Löfgren's syndrome, it may be speculated that these patients could have had an autoimmune phenotype of sarcoidosis. In such a scenario, Th2 cells could have provided help to B cells to produce autoantibodies, though we have not estimated anti-MCV antibodies in our cohort of patients. Additionally, induction of Th17 cells by B cells (naïve B1-cells or activated B-2 cells) may be a possible explanation for increased Th17 cells in the articular group.[42]

We observed that the CD4:CD8 ratio in the peripheral blood was similar in the two groups. The CD4:CD8 ratio in bronchoalveolar fluid is characteristically more than 3.5 and 4.0 in bronchoalveolar fluid for asymptomatic and symptomatic patients, respectively, with a reversal of ratio in peripheral blood.[43,44] However, recent studies and meta-analysis mention a high variability in the ratio varying from 0.5 to 37.3 thus questioning its consistency and clinical utility.[25,26,45,46] It is possible that the ratio changes according to the stage of disease and the organs involved.

A strength of the present study is that all our patients were treatment naïve, thus precluding the possibility of immunosuppressive drugs affecting T-cell subsets. Patients with articular disease had shorter, but insignificant disease duration, compared to the other group, as articular symptoms often tend to bring the patient to a clinician early.

Limitations of the study include a small sample size and lack of assessment of T-cell subsets in other body fluids such as synovial fluid and BAL, which would have further strengthened the compartmentalisation hypothesis. We also did not assess the functional activity of Treg cells which could have further delineated their relevance in the two groups. The high prevalence of articular involvement in the present study probably owes to recruitment bias as ours is a rheumatology unit. In one study from the pulmonary medicine unit of our institute, none of the 120 patients were reported to have musculoskeletal manifestations.[47]

This study opens the possibility of differing pathogenic mechanisms in diverse manifestations of sarcoidosis, reflected in the differences in T and B cell subsets and pro- and anti-inflammatory cytokines. A deeper characterisation of pathogenically activated axes may give us potential cells and cytokines to target in sarcoidosis in general, and sarcoid arthritis in particular. A longitudinal follow-up study may provide better clues to understanding the differences between articular vs non-articular sarcoidosis.

In conclusion, the T helper-cell axis was skewed towards Th1, Th2 and Th17 and away from Treg cells in sarcoid arthritis when compared to patients without articular involvement. Differences in T-cell subsets may explain to an extent the diversity in the disease manifestations and may have implications for management.

Acknowledgement

For this study Harshit Singh was granted with Senior Research Fellowship by Indian Council of Medical Research with sanction I.D 67/24/2018-Imm//BMS.

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