

## Immunophenotype of CD146/MCAM-Expressing T Cells in Granulomatous Lung Diseases

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### Abstract

**Background:** Granulomatous lung disorders (sarcoidosis, chronic beryllium disease [CBD]) are T-helper 1 (Th1) cell biased. T cells in the peripheral blood that express the melanoma cell adhesion molecule (MCAM/CD146) bind to laminin-411 expressed on the endothelium to infiltrate target tissue. We investigated MCAM/CD146 expression on bronchoalveolar lavage (BAL) and peripheral blood CD4+ T cells in patients with sarcoidosis and CBD.

**Methods:** Bronchoscopic and blood samples from twelve subjects with granulomatous lung disease and five healthy controls (HCs) were analyzed using fluorescence-activated cell sorter analysis.

**Results:** MCAM was expressed by BAL CD4+ memory T cells (sarcoidosis, 23.3%; CBD, 28.3%; HCs, 21.7%) and a small percentage of interleukin-17+ cells (sarcoidosis, 1.4 ± 1.5%; CBD, 0.7 ± 0.6%; HCs, 0.9 ± 0.9%). There were more MCAM+ T cells in the BAL than in the peripheral blood; this T-cell population may be lung-compartmentalized. More MCAM-expressing memory T cells were Th1 cells in patients than HCs (sarcoidosis, 60.3 ± 6.7%; CBD, 57.4 ± 17.0%; HCs, 36.2 ± 16.2%).

**Conclusions:** In sarcoidosis and CBD, 25% of BAL memory T cells express MCAM, and most are Th1 cells. Only 1% of MCAM expression identified Th17 cells. MCAM is thus not a specific marker of Th17 cells and is predominantly expressed on Th1 cells in patients with sarcoidosis and CBD.

**Keywords:** Sarcoidosis; Berylliosis; Cytokine Biology; Lymphocyte Biology

### Abbreviations

MCAM: Melanoma Cell Adhesion Molecule; CBD: Chronic Beryllium Disease; Th1: T-Helper 1; Th17: T-Helper 17; IL-17A: Interleukin 17A; BAL: Bronchoalveolar Lavage; HC: Healthy Controls; FEV1: Forced Expiratory Volume in the First Second; PBMC: Peripheral Blood Mononuclear Cells; WBC: White Blood Cells

## Introduction

In both sarcoidosis and chronic beryllium disease (CBD), antigen exposure leads to granuloma formation. Sarcoidosis affects multiple organs, predominantly the lungs, and develops because of exposure to an as yet unknown antigen(s) [1]. CBD predominantly affects the lungs and is clinically, radiologically and pathologically similar to sarcoidosis, leading some experts to suggest that CBD is a form of sarcoidosis but with a known antigen, beryllium [2]. Sarcoidosis and CBD are considered to be T-helper 1 (Th1) cell-biased diseases [1,3]; however, recent studies have implicated other CD4<sup>+</sup> T cells, such as T-helper 17 (Th17) cells and T-regulatory cells, in the pathogenesis of sarcoidosis [4-10]. Understanding the immunophenotype of T cells involved in both disorders may reveal novel and more targeted therapeutics to modulate disease without compromising innate immune responses.

The expression of melanoma cell adhesion molecule (MCAM), also known as CD146, on a subset of memory CD4<sup>+</sup>/CD8<sup>+</sup> T cells defines their ability to secrete interleukin 17A (IL-17A), which, along with other cytokines, produces localized inflammation and identifies a population of Th17 cells that is critical in multiple autoimmune diseases [6,11]. MCAM facilitates T-cell infiltration through interaction with laminin-411, which is expressed by the vascular endothelial basement membrane at sites of inflammation [11]. Patients with sarcoidosis have more T cells expressing MCAM in their peripheral blood than do healthy persons [6]. However, the T-cell subset (e.g. Th1 or Th17) expressing MCAM in bronchoalveolar lavage (BAL) fluid has not been fully characterized.

MCAM expression in patients with multiple sclerosis is more robust in Th17 cells than in Th1 cells [12]. Furthermore, MCAM-expressing CD4<sup>+</sup> T cells experience greater production of IL-17, IL-22 and, to a lesser extent, interferon- $\gamma$  [11]. Some of these have been associated with the Th17-cell subset [6,13,14]. It has been proposed that MCAM is a Th17-associated cell surface marker, both alone [11] and in combination with other markers such as CCR6 [15,17]. However, some IL-17-secreting T cells do not express MCAM [6]. Moreover, Wu, *et al.* [16] reported that MCAM did not specifically identify Th17 cells but did identify T cells that produce IL-17 while maintaining the ability to express Th1 cytokines. Thus, T cells expressing MCAM may be poised not only to infiltrate tissue but to be specialized to secrete both Th17 and Th1 cytokines.

With this background, in a cross-sectional study, we sought to characterize the expression of MCAM, IL-17 and T-cell surface markers on T cells from the lungs and peripheral blood of patients with sarcoidosis and CBD to better understand the specific immunophenotype of T cells involved in these granulomatous lung diseases.

## Materials and Methods

### Study design and patient population

We recruited patients with sarcoidosis, CBD and healthy controls (HCs) who met our inclusion and exclusion criteria. Sarcoidosis patients were included if they were 18 years of age or older, had biopsy-proven sarcoidosis from any organ based on the American Thoracic Society/European Respiratory Society criteria [1] and showed evidence of pulmonary parenchymal involvement with evidence of Scadding Stage II, III or IV abnormalities on chest X-ray or evidence of parenchymal involvement by chest computed tomography and were not on immunosuppressive therapy at the time of enrollment and within the past 6 months. Those with CBD were included if they were 18 years of age or older, had evidence of beryllium sensitization with abnormal results of peripheral blood beryllium lymphocyte proliferation tests and biopsy-proven granulomatous lung disease. HCs were included if they were 18 years of age or older, had never smoked and had no known chronic or active disease. Participants from all groups were excluded if they were pregnant; were unable to undergo venipuncture and bronchoscopic procedures, had significant hypoxemia on room air (e.g. pO<sub>2</sub> < 45 mm Hg at Denver altitude 5,280 feet), were on immunosuppressive therapy within 6 months prior to recruitment, had reduced forced expiratory volume in the first second (FEV<sub>1</sub>) < 1 L or other co-morbid conditions affecting their tolerance for bronchoscopy, were older than 80 years of age and had another medical condition precluding bronchoscopy; had positive lung lavage or biopsy cultures for fungi or mycobacterial disease and/or currently or recently ( $\leq$  6 months) used tobacco (smoking or otherwise). Consent was obtained from each subject before enrolment.

Medical charts were reviewed to extract clinical data and to ensure eligibility. The study was approved by the Institutional Review Board at National Jewish Health (HS# 2781).

### **Bronchoscopy with bronchoalveolar lavage**

BAL was performed using standard bronchoscopy procedures, while the patient was under conscious sedation, by instilling four sequential 60-ml aliquots of sterile normal saline into one bronchopulmonary sub-segment for a total of two sub-segments per subject. BAL fluid and cells were processed as detailed below.

### **Peripheral blood and BAL cell isolation and treatment**

Peripheral blood was collected at the time of bronchoscopy to assay peripheral blood mononuclear cells (PBMCs). PBMCs were isolated by Ficoll density gradient centrifugation, and their viability was confirmed by Trypan blue staining. Viable cells were quantified in a Neubauer chamber (Zeiss, Oberkochen, Germany), and remaining cells were cryopreserved in liquid nitrogen. BAL cells were stimulated with Leukocyte Activation Cocktail (BD Biosciences, Franklin Lakes, NJ), with added protein transport inhibitors, for 3 hours in RPMI [Roswell Park Memorial Institute] medium with 5% fetal calf serum at 37°C.

### **Flow cytometry**

Cell surface markers were detected with the following antibodies (BD Biosciences): PerCP mouse anti-human CD3, allophycocyanin (APC)-Cy7 mouse anti-human CD4, APC mouse anti-human CD45RO, phycoerythrin (PE) mouse anti-human CD146 and PE-Cy7 mouse anti-human CD196 (CCR6). Cells were incubated (4°C for 20 minutes), washed twice, fixed and permeabilised using the Cytotfix/Cytoperm Kit (BD Biosciences). Fixed/permeabilised cells were incubated with AlexaFluor 488-IL17A (BD Biosciences) for Th17, FITC-IFN $\gamma$  (BD Biosciences) for Th1, APC-FoxP3 (BD Biosciences) for Treg and PerCP CY5.5-IL4 (eBioscience, San Diego, CA) for Th2 detection at 4°C for 20 minutes. Cells were treated with fluorochrome-matched isotype control antibodies as a control to confirm binding specificity and for baseline corrections. Fluorescence-activated cell sorter analysis was performed using LSRII (BD Biosciences) and FlowJo (FlowJo, LLC, Ashland, OR) software.

### **Statistical analysis**

Variables were summarized using descriptive statistics (frequency, mean, standard deviation). Differences between groups were compared using Student's t test or non-parametric test, as appropriate. Normalizing transformations were made if the data were non-Gaussian. When data transformations were unsuccessful, suitable non-parametric tests were used. Statistical significance was defined a priori at a two-tailed  $\alpha = 0.05$ .

## **Results**

### **Patient characteristics**

Baseline patient demographics and clinical characteristics are shown in table 1. Twelve subjects with granulomatous lung disease (n = 8 with sarcoidosis and n = 4 with CBD) and five HCs were recruited (Table 1). HCs were significantly younger than patients with sarcoidosis or CBD and most were female, whereas 50% of sarcoidosis patients and none of the CBD patients were female. All sarcoidosis cases were diagnosed by lung biopsy. Five of eight sarcoidosis subjects had evidence of worsening chest radiography in a pattern consistent with sarcoidosis at the time of bronchoscopy and three had stable chest radiography. None had other underlying pulmonary or inflammatory disease. Extra-pulmonary organ involvement included hypercalcemia (n = 1), hepatic (n = 1), nephrolithiasis (n = 2) and ophthalmic (n = 1). BAL white blood cell count differential in sarcoidosis and CBD showed increased total WBC (mean  $\pm$  standard deviation; sarcoidosis  $49.6 \pm 23.8$ , CBD  $41.0 \pm 16.5$  and HC  $25.6 \pm 11.6$ ; p = ns) with lymphocytic predominance in sarcoidosis ( $15.2 \pm 10.9$ ) and CBD ( $13.0 \pm 7.7$ ) and no evidence of neutrophilia to suggest an infectious process.

	HC n = 5	CBD n = 4	Sarcoidosis n = 8
Age, years, mean ± SD	42.4 ± 14	62.3 ± 9.3*	58.4 ± 8.6*
Male, %	20	100	50
CXR Scadding stage (0, 1, 2, 3, 4) <sup>†</sup>	—	—	(1, 1, 4, 1, 1)
FVC, %, mean ± SD	—	93.8 ± 20.8	85.6 ± 13
FEV1, %, mean ± SD	—	94.5 ± 15.9	85.5 ± 12.8
DLCO, %, mean ± SD	—	95.3 ± 25.3	94.6 ± 12.6
DLCO/VA, %, mean ± SD	—	103.3 ± 28.8	108.8 ± 20.8

**Table 1:** Demographic and clinical characteristics.

\**p* < 0.05.

<sup>†</sup>Subjects with Scadding stages 0 and 1 had evidence of parenchymal nodularity compatible with parenchymal sarcoidosis on chest tomography at the time of enrolment.

CBD: Chronic Beryllium Disease; CXR: Chest X-Ray; DLCO: Diffusion Capacity of the Lung for Carbon Monoxide; FVC: Forced Vital Capacity; FEV1: Forced Expiratory Volume in the First Second, DLCO/VA: DLCO Correct for Alveolar Volume; HC: Healthy Control.

**MCAM cells were more predominant in BAL cells than blood cells of patients and HCs**

Total white blood cell (WBC) counts and the percentages of lymphocytes and memory T cells were compared between BAL and PBMC (Table 2). As expected of patients with granulomatous lung disease, total BAL WBC counts were higher in patients than in HCs. There was a non-significant increase in the percentage of lymphocytes and memory T cells in subjects with sarcoidosis and CBD compared with HCs. Most BAL lymphocytes were memory cells (CD3<sup>+</sup>/CD4<sup>+</sup>/CD45RO<sup>+</sup>) in patients and HCs (Table 2). However, patients with sarcoidosis and CBD had a slightly higher percentage of BAL memory T cells that expressed MCAM than did HCs. Specifically, CBD patients had a higher percentage than sarcoidosis patients, and sarcoidosis patients had a higher percentage than HCs (*p* > 0.05; Table 2). There were substantially fewer MCAM-expressing T cells in the peripheral blood than in BAL in sarcoidosis patients (*p* < 0.001) and HCs (*p* < 0.01), suggesting that MCAM-expressing cells are compartmentalized in the lungs (Table 2). There were no statistically significant differences in the CBD group, which may be due to the limited sample number in the CBD group (*n* = 4). Nonetheless, numbers of MCAM+ cells in the blood of patients with granulomatous lung disease were 2.6-fold to 2.8-fold higher than in the blood of HCs (Table 2).

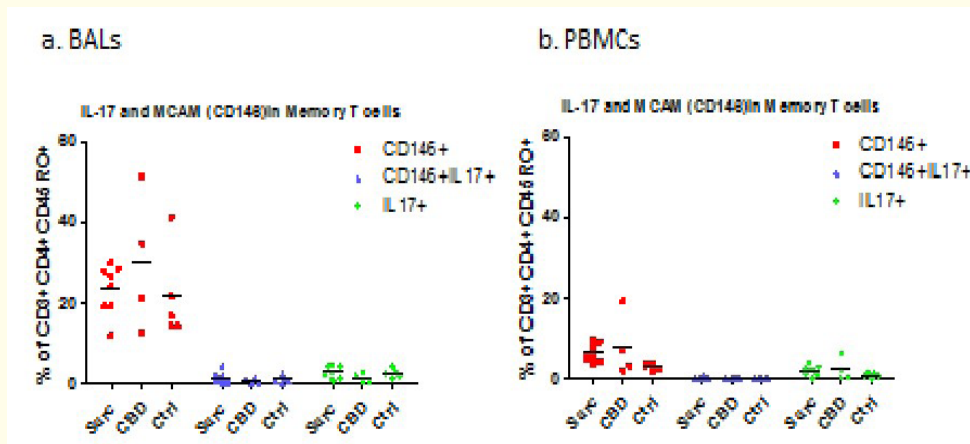
	HC n = 5		CBD n = 4		Sarcoidosis n = 8	
	BAL cells	PBMCs	BAL cells	PBMCs	BAL cells	PBMCs
Total WBCs (×10 <sup>6</sup> )	25.4 ± 11.5	25.5 ± 11.2	49.5 ± 28.4	28.4 ± 10.9	40.9 ± 16.5	35.3 ± 7.8
WBCs, %, mean ± SD						
Lymphocytes	9.2 ± 4.7	-	15.2 ± 10.9	-	13.0 ± 7.7	-
Memory cells	84.8 ± 8.7	45.2 ± 8.0	92.1 ± 5.2	43.9 ± 18.6	92.1 ± 4.4	29.2 ± 8.8
MCAM <sup>+</sup> memory cells	21.7 ± 11.4**	2.9 ± 0.8**	28.3 ± 19.3	7.9 ± 7.9	23.3 ± 6.1***	6.5 ± 2.4***

**Table 2:** Comparison of BAL lymphocytes and blood samples in patients with granulomatous lung disease and healthy controls.

BAL: Bronchoalveolar Lavage; CBD: Chronic Beryllium Disease; HC: Healthy Controls; MCAM<sup>+</sup>: Melanoma Cell Adhesion Molecule-Positively Labeled; PBMCs: Peripheral Blood Mononuclear Cells; WBCs: White Blood Cells. \*\**p* < 0.01, \*\*\**p* < 0.001 [6].

**MCAM was expressed only on a minority of IL-17 cells**

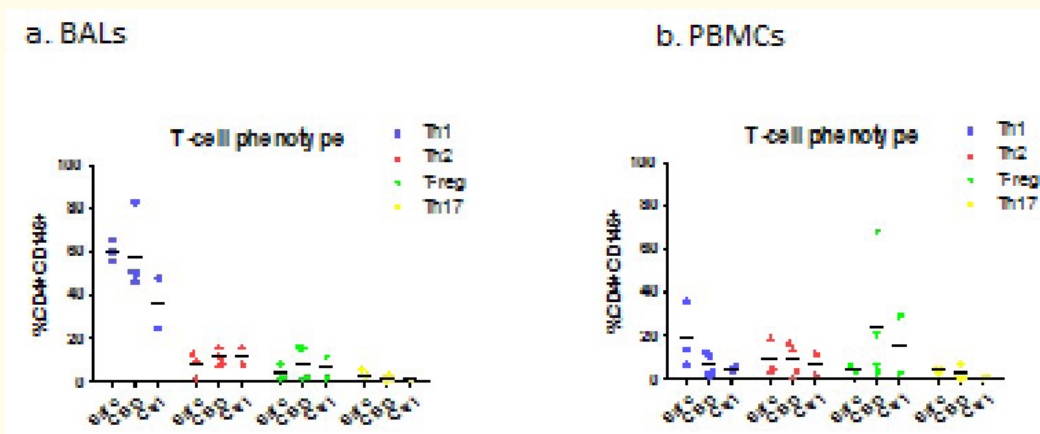
Only a minority of the 21.7% to 30.0% BAL T cells that expressed MCAM co-expressed IL-17 (sarcoidosis, 1.4 ± 1.5%; CBD, 0.7 ± 0.6%; HCs, 0.9 ± 0.9%; p > 0.05) (Figure 1A). Similarly, few peripheral blood MCAM-expressing T cells co-expressed IL-17 (sarcoidosis, 0.2 ± 0.3%; CBD, 0.2 ± 0.2%; HCs, 0.1 ± 0.1%; p > 0.05) (Figure 1B).



**Figure 1:** Comparison of memory T cells by their expression of MCAM and IL-17 in (a) BAL and (b) PBMC samples. Memory T cells were identified by their co-expression of cell surface markers CD3, CD4 and CD45RO. BAL: Bronchoalveolar Lavage; CBD: Chronic Beryllium Disease; HC: Healthy Controls; IL-17: Interleukin 17; MCAM: Melanoma Cell Adhesion Molecule (CD146); PBMCs: Peripheral Blood Mononuclear Cells; Sarc: Sarcoidosis.

**MCAM and CCR6 co-expression differed between cases and controls**

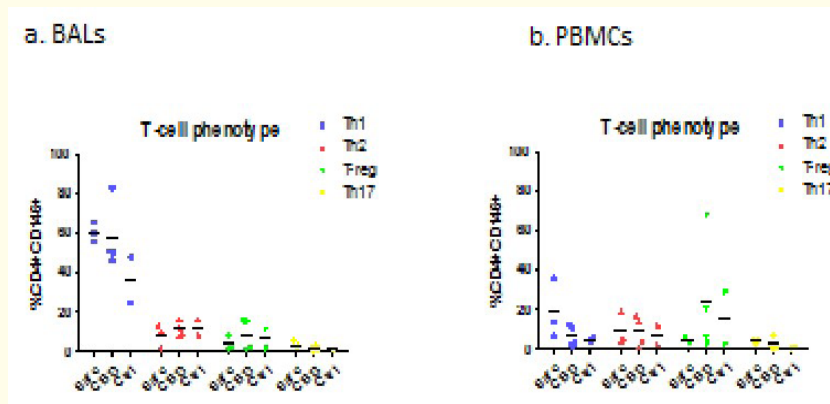
CCR6 expression has been associated with memory T cells that selectively express IL-17 [17]. Patients with sarcoidosis had the highest numbers of CCR6-expressing T cells in the BAL (sarcoidosis, 64.6%; CBD, 31.7%; HCs, 39.8%; sarcoidosis vs HCs, p < 0.05; sarcoidosis vs CBD, p < 0.01; CBD vs HCs, p > 0.05) and PBMC (sarcoidosis, 44.6%; CBD, 42.2%; HCs, 34.5%; p > 0.05) (Figure 2). A smaller proportion of CCR6-expressing cells co-expressed MCAM; these were more prevalent in BAL than in blood, supporting that these cells are compartmentalized in patients' lungs. Moreover, levels of MCAM/CCR6 co-expressing cells were marginally elevated in patients with sarcoidosis compared with other groups (BAL: sarcoidosis, 16.6 ± 3.6%; CBD, 10.8 ± 4.0%; 13.6 ± 13.7%; sarcoidosis vs HCs, p > 0.05; CBD vs HCs, p > 0.05), though sarcoidosis patients demonstrated significantly more than CBD (p < 0.05). In PBMCs, MCAM and CCR6 co-expressing cells were more prevalent in sarcoidosis patients, but not CBD patients, than in HCs (sarcoidosis, 5.0 ± 2.6%; CBD, 4.6 ± 5.6%; HCs, 2.0 ± 1.0%; sarcoidosis vs HCs, p < 0.05; sarcoidosis vs CBD, p > 0.05; CBD vs HCs, p > 0.05).



**Figure 2:** Comparison of memory T cells by their expression of MCAM and CCR6 in (a) BAL and (b) PBMC samples. BAL: Bronchoalveolar Lavage; CBD: Chronic Beryllium Disease; CCR6: Chemokine (C-C motif) Receptor 6; HC: Healthy Controls; MCAM: Melanoma Cell Adhesion Molecule (CD146); PBMCs: Peripheral Blood Mononuclear Cells; Sarc: Sarcoidosis. \*p < 0.05, \*\*p < 0.01.

**MCAM-expressing memory T cells were more likely to be Th1 in sarcoidosis patients than in CBD patients**

Cell surface markers expressed by MCAM-positive T cells were examined to determine the memory T-cell phenotypes involved because IL-17 was not significantly co-expressed in these cells. Most MCAM-expressing memory T cells were Th1 cells in the BAL of granulomatous lung disease (sarcoidosis, 60.3 ± 6.7%; CBD, 57.35 ± 17.0%; HCs, 36.2 ± 16.2%; sarcoidosis vs CBD, p > 0.05). The statistical differences in T-cell MCAM expression between either sarcoidosis or CBD cases and HCs could not be determined because of the small sample number of HCs (n = 2), the limited cell numbers from the other three HCs and the lower percentage of Th1 MCAM+ cells in HCs. There were no significant differences in trends for PBMC T-cell phenotypes with regard to MCAM expression.



**Figure 3:** Comparison of the percentage of Th1, Th2, T-regulatory and Th17 T cells that co-express MCAM and CD4 in (a) BAL and (b) PBMC samples. BAL: Bronchoalveolar Lavage; CBD: Chronic Beryllium Disease; CD4: Cluster of Differentiation 4; HC: Healthy Controls; MCAM: Melanoma Cell Adhesion Molecule (CD146); PBMCs: Peripheral Blood Mononuclear Cells; Sarc: Sarcoidosis; Th: T-Helper Cell; T-reg: T-Regulatory Cell. \*p < 0.05.

**Discussion**

Our study is the first to examine the expression of MCAM on immune cells in two granulomatous lung diseases, sarcoidosis and CBD, which share many similarities in clinical features and pathogenesis because both disorders feature Th1 cells as predominant effector cells [2] and Th17 cells potentially play a role in sarcoidosis [4,5,7,18,19]. We show that both sarcoidosis and CBD are associated with increases in MCAM-expressing memory T cells in the lungs. However, MCAM expression was expressed predominantly by Th1 and not by Th17 cells in both diseases. Although MCAM has been reported to specifically mark a subpopulation of Th17 cells that have the capacity to produce IL-17 [6,11], MCAM may mark cells with the ability to produce a variety of cytokines, not limited to Th1 or Th17 types, in a context-dependent fashion.

The co-expression of CCR6 and MCAM marks a subpopulation of T cells in the circulation that contribute substantially to IL-17 secretion [17]. In our study patients with sarcoidosis tended to have higher levels of this dual-labeled T-cell population than did HCs, but these T cells were not more prevalent in patients with CBD. Future studies should determine whether this T-cell subtype plays different roles in these disorders. These data are consistent with our previous findings that CBD cells do not have significant Th17 gene expression [20].

MCAM is a cell adhesion molecule located on the cell surface. It is involved in cell-cell and cell-matrix adhesion that was initially defined as a specific marker for malignant melanoma but that subsequently has been associated with a variety of carcinomas [21]. As



discussed, MCAM acts as a receptor for laminin-411, which facilitates cell infiltration [11]. In addition, increases in MCAM<sup>+</sup> T cells have been identified in some autoimmune disorders, including Behçet's syndrome, Crohn's disease and, as in our study, sarcoidosis [6]. MCAM expression may also mark tumor cells with metastatic potential [21], suggesting that the processes of metastasis and T-cell infiltration co-opt a common pathway to cross vasculature and invade tissue. The importance of MCAM–laminin interactions in inflammation is supported by reductions in the severity of experimental autoimmune encephalitis in laminin-411-deficient mice [16] and in mice treated with anti-MCAM antibodies [11]. This latter study also demonstrated a reduced number of T cells infiltrating the brains of mice treated with anti-MCAM antibodies [11].

In their study, Dagur, *et al.* [6] demonstrated that the frequency of CD4<sup>+</sup> T cells that co-express MCAM in peripheral blood was more than twofold higher in sarcoidosis patients than in HCs (sarcoidosis, 6.5 ± 2.4%; HCs, 2.9 ± 0.8%). However, they did not explore the expression of MCAM on T cells in the lung or its co-expression on Th1 cells in the lung or peripheral blood [6]. Our findings reveal that MCAM-expressing T cells in the peripheral blood of sarcoidosis patients are comparable with those in HCs but that the lungs of sarcoidosis patients demonstrate a greater frequency of these cells, suggesting compartmentalization of MCAM-expressing cells in the lungs. This compartmentalization of MCAM<sup>+</sup> memory cells and their predominant expression by Th1 cells, the main effector cells in granulomatous lung disease, may play a mechanistic role in sarcoidosis, as noted. In addition, the expression of MCAM on a large percentage of pathogenic Th1 cells in sarcoidosis patients could be considered a potential therapeutic target; agents that block MCAM–laminin-411 binding may substantially reduce the infiltration of this effector cell type into the lungs.

Our study is limited by the small sample size and cross-sectional design of the study. This limitation limits our ability to draw any conclusions between the expression of MCAM on T-cells and disease course and/or severity. Our study sought to initially characterise the expression of MCAM on T cells in sarcoidosis and CBD to better understand the specific immunophenotype of T cells present in these granulomatous lung diseases. In the future, a larger longitudinal study is needed to investigate the association between MCAM expression on T-cells and disease course and/or severity and the impact of therapy on MCAM expression.

## Conclusion

Our study supports the results of previous studies in other autoimmune diseases [6] that MCAM/CD146 is not limited to IL-17-producing T cells and that most MCAM<sup>+</sup> T cells in the lungs of patients with sarcoidosis and CBD are Th1 cells. It is possible that these MCAM<sup>+</sup> T cells play a role in T-cell infiltration in the lung, though this was beyond the scope of the current study. Future studies should address the role of these cells in granulomatous lung disease and whether they promote granulomatous inflammation. Regardless, the presence of a significant percentage of these cells in lungs supports the development and trial of novel therapeutic agents that can interrupt MCAM–laminin-411 interactions and potentially block effector T-cell infiltration into target tissues. If MCAM impacts lung infiltration in patients with granulomatous disease, a therapeutic agent that blocks these cells might attenuate both inflammation and disease progression and should be assessed in future interventional studies.

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## Contributors and Conflict of Interest

LL contributed to the study design, sample and data analysis and drafting of the manuscript. KF and LAM contributed to the study design, data analysis and drafting of the manuscript. NYH contributed to the study design, patient recruitment, performance of procedures, data analysis and drafting of the manuscript. All authors revised the manuscript critically for important intellectual content, approved the final version to be submitted and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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KF is an employee of Prothena Biosciences Inc and a stockholder of Prothena Corporation plc.

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