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Evidence for M2 macrophages in granulomas from pulmonary sarcoidosis: A new aspect of macrophage heterogeneity

Masoud Shamaei[a], Esmaeil Mortaz[a,b], Mihan Pourabdollah[c], Johan Garssen[b,d], Payam Tabarsi[a], Aliakbar Velayati[e], Ian M. Adcock[f,g,*]

[a] Clinical Tuberculosis and Epidemiology Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran
[b] Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, Utrecht, Netherlands
[c] Chronic Respiratory Diseases Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran
[d] Nutricia Research Centre for Specialized Nutrition, Utrecht, Netherlands
[e] Mycobacteriology Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Masih Daneshvari Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[f] Airways Disease Section, National Heart & Lung Institute, Imperial College London, London, UK
[g] Priority Research Centre for Healthy Lungs, Hunter Medical Research Institute, The University of Newcastle, Newcastle, NSW, Australia

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ABSTRACT

Background: Sarcoidosis is a granulomatous disease of unknown etiology. Macrophages play a key role in granuloma formation with the T cells, having a significant impact on macrophage polarization (M1 and M2) and the cellular composition of the granuloma. This study evaluates macrophage polarization in granulomas in pulmonary sarcoidosis.

Materials and Methods: Tissue specimens from the Department of Pathology biobank at the Masih Daneshvari Hospital were obtained. Paraffin sections from 10 sarcoidosis patients were compared with those from 12 cases of tuberculosis using immunohistochemical staining. These sections consisted of mediastinal lymph nodes and transbronchial lung biopsy (TBLB) for sarcoidosis patients versus pleural tissue, neck, axillary lymph nodes and TBLB for tuberculosis patients. The sections were stained for T-cells (CD4+, CD8+) and mature B lymphocytes (CD22+). CD14+ and CD68+ staining was used as a marker of M1 macrophages and CD163+ as a marker for M2 macrophages.

Results: Immunohistochemical staining revealed a 4/1 ratio of CD4+/CD8+ T-cells in sarcoidosis granuloma sections and a 3/1 ratio in tuberculosis sections. There was no significance difference in single CD4+, CD8+, CD22+, CD14+ and CD68+ staining between sarcoidosis and tuberculosis sections. CD163 expression was significantly increased in sarcoidosis sections compared with those from tuberculosis subjects.

Conclusion: Enhanced CD163+ staining indicates a shift towards M2 macrophage subsets in granulomas from sarcoidosis patients. Further research is required to determine the functional role of M2 macrophages in the immunopathogenesis of sarcoidosis.

1. Introduction

Sarcoidosis is a multiorgan granulomatous disorder that most often affects the lungs. Although sarcoidosis has unknown etiology, the association of sarcoidosis with specific occupations, genetic susceptibility, and various infectious pathogens has been described [1]. Classical sarcoidosis is characterized by well-formed, tightly packed, non-necrotizing granulomas surrounded by lamellar hyaline collagen [2]. Granulomas are a closed, centrally organized collection of macrophages and epithelial cell surrounded by lymphocytes [3]. Chronic cytokine stimulation of macrophages leads to their differentiation into multi-nucleated giant cells over a period of time [4].

Macrophages have been classically defined as M1 and M2 dependent upon their functionality [5]. M1 macrophages activated by lipopolysaccharide (LPS) and interferon γ (IFN-γ) release interleukin 12 (IL-12) and tumor necrosis factor α (TNF-α) [6]. M2 macrophages are produced in the presence of Th2 cytokines (IL-4 and IL-13) and have a suppressive and immunoregulatory function being able to produce both

IL-10 and IL-1ra [7]. In addition to activation by Th2 cytokines, M2 macrophages are also activated by immune complexes [6,8]. Although there are no gold standard means of detecting macrophage subtypes, M1 and M2 macrophages have been detected historically by immunohistochemical (IHC) staining using anti-CD68 for M1, and anti-CD163 antibodies for M2 cells [9–14]. Granulomas are recognized as Th1-mediated and Th2-mediated granulomas according to predominant type of participating T-cell helper cell present [15].

Human CD163 is a restricted monocytes-macrophage lineage with high expression in macrophages like as red pulp, bone marrow, liver (kupffer) and lungs [16]. The most well-known stimulants of CD163 expression are glucocorticoids, IL-6, IL-10 and heme/Hb, whereas IL-4, LPS, TNF-α, INF-γ, CXC chemokine ligand 4 (Cxcl4) and granulocyte-macrophage colony-stimulating factor (GM-CSF) downregulate CD163 expression [17–21]. CD163 is a member of the scavenger receptor cysteine-rich family (SRCR) and human CD163 expression is restricted to the monocyte–macrophage lineage [16]. Some studies suggest that CD163 is a pattern recognition receptor and plays a critical role as a part of innate immunity [22,23]. IFN-γ and IL-12 are their predominant cytokines found in Th1-mediated granulomas and a low level of CD163 expression is expected [24].

Tuberculosis (TB) is often considered a Th1 cell-mediated immune response both in active and latent tuberculosis infection [25] although a wide spectrum of alveolar macrophage activation and immune activation in lymph nodes exists [26,27]. Polarization to a Th2 cell-mediated immune response is seen in severe tuberculosis (miliary TB) [28] and active pulmonary tuberculosis (mixed Th1/Th2) [29,30]. In sarcoidosis, after the initial Th1-mediated granuloma formation there is a relative increase in the expression of IL-4 and IL-10 which can induce CCL18 within the granuloma [31]. This may reflect a shift from a Th1-mediated to a Th2-mediated immune response resulting in fibrosis due to the persistence of this inflammatory milieu [32,33].

Macrophage polarization plays a crucial role in chronic inflammatory disease. Overall, the persistence, resolution or progression of granulomas and the conversion to fibrosis is a balance between various inflammatory, regulatory, apoptotic, Th1/Th2 cytokine responses and M1/M2 polarization [34]. It is important to understand this micro-environment in sarcoidosis, to improve our knowledge in the diagnostic and therapeutic aspects of patient management.

To the best of our knowledge, despite the clinical and histopathological similarities between sarcoidosis and tuberculosis [35,36] there are no previous studies evaluating the relativeness of CD163 expression between sarcoidosis and tuberculosis in lung and mediastinal lymph nodes. The aim of the present study is to identify macrophage polarization in pulmonary sarcoidosis in comparison with tuberculosis as a disease with similar clinical, radiological and pathologic patterns.

2. Material and methods

2.1. Patient's selection

Formalin-fixed paraffin-embedded human tissues were obtained from the archives of the department of pathology, Masih Daneshvari Hospital, a pulmonary tertiary referral hospital in Tehran-Iran. Tissue samples consisted of pleura, lymph nodes and transbronchial lung biopsy (TBLB). The medical records of sarcoidosis and TB patients were reviewed based on their clinical, radiological and pathological information by at least two pulmonologists and two pathologists. After review, tissue blocks from 33 sarcoidosis and 27 TB patients with confirmed diagnosis were selected and sections prepared from those with sufficient tissue for immunohistochemical staining. All samples were analysed by PCR for MTB DNA. Samples from 10 sarcoidosis and 12 TB patients were eventually used as providing good quality analysis for all antibodies with the presence of at least three granulomas. TB tissue samples comprised of 4 pleural tissues (33.3%), 6 lymph nodes (50%) and two transbronchial lung biopsy (TBLB) specimens (16.6%)

Table 1
Demographic characteristics and PCR assay of study subjects.

Sarcoidosis (n = 10)	Tuberculosis (n = 12)	Variable
8/2	8/4	Sex (F/M)
42.8 ± 14.3	40.1 ± 16	Age (Mean ± SD)
0	10 (83.3%)	Positive MTB PCR[†]
4 (40%)	6 (50%)	Tissue sample type
6 (60%)	2 (16.6%)	Lymph node
–	4 (33.3%)	TBLB[‡]
		Pleural tissue

[†] Real-time polymerase chain reaction (PCR) assay targeting insertion sequence *IS6110*.
[‡] Transbronchial lung biopsy (TBLB).

while sarcoidosis tissue specimens contained 4 mediastinal lymph nodes (40%) and 6 cases of TBLB (60%) which are addressed in Table 1. Immunohistochemical analysis was performed in two batches of lymph node and non-lymph node tissue for both TB and sarcoidosis patients.

Inclusion criteria for pulmonary sarcoidosis patients were clinical data matching lung involvement with radiology findings (i.e. hilar adenopathy, reticulonodular infiltration or pulmonary fibrosis). Histopathologic findings compatible with the ATS definition of sarcoidosis were considered as the presence of tight and well-formed perilymphatic and interstitial granulomas with a rim of lymphocytes and fibroblasts in the outer margin of the granuloma [37]. All microbial factors were excluded for sarcoidosis cases and confirmed by H & E staining in addition to routine tests for bacteria, fungi, and culture for acid-fast bacilli (AFB) and auramine O staining. TB patients entered in the study had a positive sputum smear or culture for AFB, positive Ziehl–Neelsen staining or a positive molecular test for MTB DNA (IS 6110) along with a positive response to standard anti-TB therapy. This study was conducted with the approval of Masih Daneshvari Hospital ethic committee and utilizing protocols approved by the respective institutional review boards (SBMU1.REC.1393.60).

2.2. Real time PCR

DNA was extracted using the Qiagen QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. *Mycobacterium Tuberculosis* detection was performed using a *Mycobacterium tuberculosis* PCR Kit (GeneProof, Brno, Czech Republic) based on amplification of the specific multi-copy insertion sequence (IS6110) according to the manufacturer's instructions. All PCRs were performed using StepOne™ Real-Time PCR Systems (Roche Diagnostics Deutschland GmbH, Sandhofer Straβe, Mannheim, Germany). The kit specifically detects *M. tuberculosis, M. bovis, M. africanum* and *M. microti* strains in addition to vaccination strains (e.g. BCG).

2.3. IHC analysis

Ten sarcoidosis patients with appropriate tissue blocks with 12 TB patients were entered into the study. Five-μm tissue sections were deparaffinized and stained with monoclonal antibodies for CD4, CD8, CD22, CD14, CD68, and CD163 (Leica Microsystems Newcastle Ltd, STANSFIELD, Suffolk, UK). The antibody clone, dilutions, and incubation times are shown in Table 2. After deparaffinization and fixation with 70–99% alcohol, specimens were rinsed with distilled water and PBS and treated with methanol and 3% H_2O_2 for 10 min to remove endogenous peroxidase. After antigen retrieval and cooling, the tissues were rinsed with distilled water and PBS and specimens blocked with control autologous antiserum. Antibodies were prepared in Tween 20 + Tris. The primary antibodies were incubated with issue sections in a water saturated environment at room temperature for 30 min and, after rinsing the specimens with PBS, were incubated with secondary antibody provided in kits (DAKO EnVision™ Detection Kit, Peroxidase/

Table 2
Antibody clones, dilution and incubation conditions used for staining.

Antibody	Clone	Dilution	Incubation	Positive control
CD4	4B12	1:20	60 min	Tonsil
CD8	1A5	1:20	60 min	Tonsil
CD22	FPC1	1:20	60 min	Tonsil
CD14	7	1:20	60 min	Placenta
CD68	514H12	As provided	60 min	Tonsil
CD163	10D6	1:1200	30 min	Placenta

DAB) for 2 h. After staining with chromogen (provided in kits), hematoxylin was used as the contrast dye. For each Ab, one negative and one positive control section was also used. The intensity of staining was assessed semi-quantitatively under light microscopy (200x Magnification) on a 0–4 scale: 0 – no immunoreactivity, 1 – mild immunopositivity in scattered cells, 2 – immunopositivity in up to a third of cells, 3 – immunopositivity in up to a half of cells and 4 – strong immunopositivity in the majority or all cells [38]. Any staining at grade 1 or above (mild/scattered cells to strong/all cells) was considered as positive. Histological and immunohistochemical analysis was performed blindly by two independent pathologists. The Fisher's exact test was employed to compare frequency of variables between two groups. A P value < .05 was considered statistically significant. Statistical analysis was carried out using SPSS version 16 (SPSS Inc., Chicago, IL, USA).

3. Results

The 10 patients with sarcoidosis had a mean age of 42.8 ± 14.3 years and consisted of 8 females and 2 male subjects (20%). Patient demographics and clinical characteristics of the patients are summarized in Table 3. The 12 patients with TB had a mean age of 40.1 ± 16 and 4 cases were male (30%) 10/12 TB specimens (83.3%) were positive by PCR for MTB DNA while all the sarcoidosis tissues were negative (Table 1). Four sarcoidosis cases had lymph nodes showing numerous well-formed non-caseating granulomas with partial obliteration of the lymph node structure by H & E staining.

As T-helper cells have been associated with granuloma formation in sarcoidosis, we confirmed the presence of CD4+ (T helper) cells surrounding the granulomas with few weakly staining cells within the granulomas (Fig. 1, Table 4). This was selective as there were few scattered weakly staining CD8+ and CD22+ cells inside and outside the granulomas (Fig. 1, Table 4). There was no significant difference between sarcoidosis and tuberculosis samples in CD4+ and CD8+ distribution (Fig. 1). The summary results of all the immunohistochemical analysis are shown in Table 4.

CD68+ immunoreactive cells included macrophages (alveolar, interstitial and epithelioid) and giant cells in both the lung and lymph node sections from TB and sarcoidosis patients. CD68+ immunoreactivity of interstitial and epithelioid macrophages, as well as of giant cells, was as strong as that seen in alveolar

macrophages (Fig. 2). Neither alveolar epithelial cell type II cells nor endothelial cells expressed CD68 (data not shown). CD163 was expressed weakly in alveolar macrophages and alveolar epithelial type II cells surrounding the granulomatous lesions but was highly expressed on cells forming the granulomatous lesions.

CD163 + staining was significantly increased in intensity and in the number of immunoreactive cells included macrophages and giant cells in the lung and lymph node sections of sarcoidosis compared with tuberculosis patients (P < .05). In addition, there was a significant correlation between radiologic stage and CD163 expression score in sarcoidosis patients (p < .0001, r = 0.94 Spearman's correlation) (Table 3). There was no effect of corticosteroid use on the positive staining score for CD163 (P = .11, Fisher's exact test).

The number of CD163 + macrophages increased in presence and intensity of staining inside the granuloma (IG) and outside the granuloma (OG) in both lymph node and non-lymph node tissues. This suggests that CD163 + macrophages increased homogeneously in sarcoid patients as compared to TB patients.

4. Discussion

This study shows that there are a greater presence and intensity of CD163 + staining of M2-like macrophages in lymph node and non-lymph node tissues in pulmonary sarcoidosis compared with tuberculosis which reflects a shift towards M2 macrophages in chronic cases of pulmonary sarcoidosis. In addition, the CD4+/CD8 + ratio and the distribution of other macrophage subset markers (CD14 and CD68) were not different between the two diseases.

Granuloma formation in sarcoidosis is believed to result from an immunologic response to a persistent or poorly degradable antigen by multiple immune cells over a period of days to weeks [39–41]. This immunogenic amplification can be associated with either Th1 or Th2 cells [31,42]. T-cells can effectively regulate the induction, functional differentiation, and the survival of macrophages [43,44]. Although the polarization of monocytes into M1 or M2 cells can occur in the absence of T cells [45], in most instances it is likely that Th1/Th2 cells usually push monocytes toward M1/M2 macrophages [46]. This Th2 immune response may be of clinical relevance especially in fibrosis [47]. Prasse et al. have demonstrated increased release of profibrotic chemokine CCL18 by alveolar macrophages (AMs) in sarcoidosis-associated pulmonary fibrosis [48]. Interestingly, this chemokine is also released by M2 macrophages [49].

Interactions between macrophages and T cells are critical in the link between innate and adaptive immunity. The activation and recruitment of monocytes-macrophages are regulated by Th1 and Th2 cells resulting in the promotion of cellular and fibrotic processes respectively [50]. After granuloma formation, T-cell activation is either downregulated or there is a shift from a Th1- to a Th2-dominated phenotype [31]. Downregulation of T-cell activation whilst macrophages are still activated may cause the shift from M1 to M2. Although this event could occur in tuberculosis, our data suggest that this is more specific for
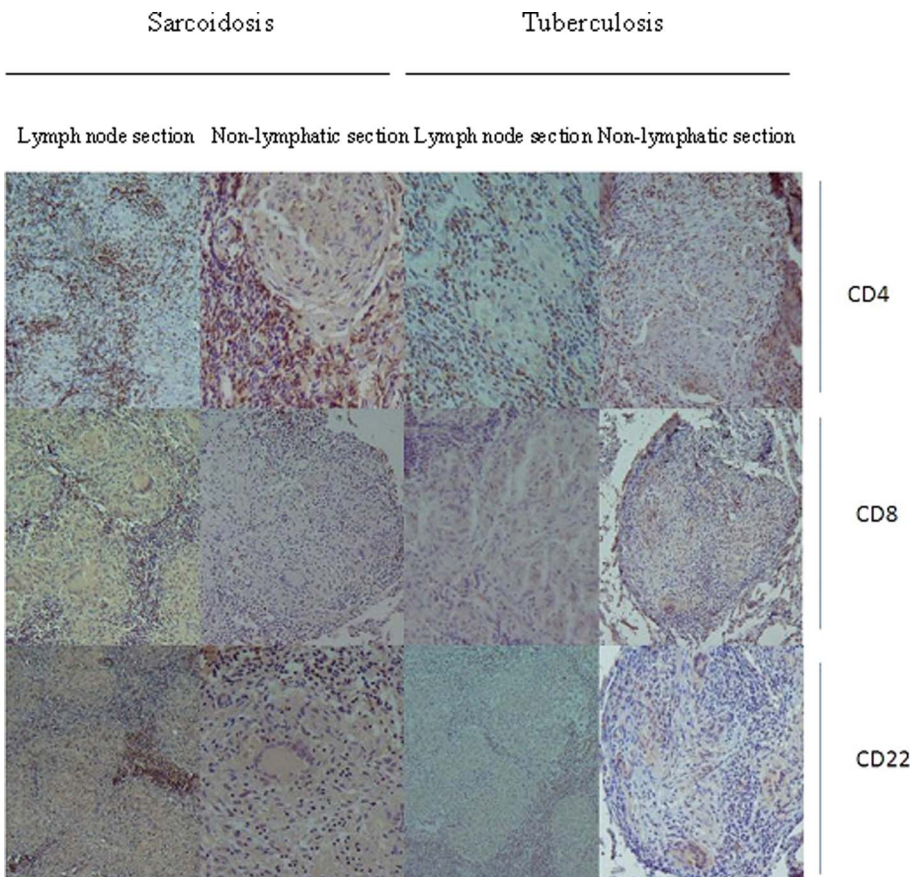
Table 3
Clinical characteristics and CD163 expression of patients with pulmonary sarcoidosis.

Patient No	Sex	Age	Sample type	Duration of Disease	Radiologic stage	Steroid use	CD163 expression scale
1	F	56	TBLB	10 months	III	No	3
2	F	43	Lymph Node	2.5 years	III	Yes	3
3	F	16	TBLB	5 months	II	No	1
4	M	47	Lymph Node	2.3 years	IV	Yes	4
5	F	52	Lymph Node	1.7 years	IV	Yes	4
6	M	30	TBLB	6 months	III	No	2
7	F	59	TBLB	7 months	III	No	3
8	F	46	TBLB	1.2 years	III	Yes	3
9	F	23	TBLB	3 months	II	No	1
10	F	48	Lymph Node	2 years	III	Yes	3

Fig. 1. T-cell (CD4 + & CD8 +) and B lymphocyte (CD22 +) staining in lymph nodes and non-lymphatic tissue from patients with sarcoidosis and tuberculosis. Representative photomicropgraphs of CD4, CD8 and CD22 staining in lymph node and non-lymphatic sections. There was no significant difference in the distribution of T-cells and B-cells between pulmonary sarcoidosis and tuberculosis. Sections were analysed from 10 sarcoidosis (4 lymph nodes and 6 non-lymphatic samples) and 12 tuberculosis (6 lymph nodes and 6 non-lymphatic samples) patients.

sarcoidosis and this may reflect differences in specific mediators released by T-cells in the two diseases.

The local cytokine environment is particularly complex in sarcoidosis patients since both proinflammatory and profibrotic cytokine patterns have been described [48]. The Th2 response has both pro- and anti-inflammatory characteristics and it is possible that the proinflammatory activity against the persistent or poorly degradable antigen associated is down-regulated by the host after the initial recognition phase [51,52]. Activated Th2 cells produce IL-10 which inhibits proinflammatory cytokine and chemokine production as well as blocking T-cell responses to specific antigens [53]. Polarized macrophages differentiate in a defined cytokine context and M2 macrophages differentiate in the presence of IL-4, IL-13, and IL-21 and are characterized by the expression of non-opsonizing receptors such as CD163 and the mannose receptor (CD206) [54,55]. Macrophages are a heterogeneous and very plastic group of monocyte-derived phagocytic cells that are either tissue resident, often for many years, or rapidly

recruited according to the patient's need. However, the specific role of different macrophage subtypes in human diseases is poorly understood [54,56].

There are limited studies in evaluation macrophages polarization in human sarcoidosis [3,11,48,57,58]. Strebel and colleagues analysed CD163 as an index marker for Th2-mediated granulomas in schistosomiasis, tuberculosis and foreign body granuloma. The study demonstrated 73% and 85% CD163 + granulomas in schistosomiasis and foreign body-induced disease but only 21% CD163 + granulomas in TB. They concluded that the Th2 response has anti-inflammatory actions and that the proinflammatory activity against the non-degradable foreign body is down regulated by the host after antigen recognition [24]. The results of our study are in accordance with limited previous studies and provide a rational consequence for the down-regulation of T cell activation after chronic antigen stimulation.

There is no gold standard means of detecting M2 macrophages in tissue sections and CD163 alone may not be optimal for measuring M2

Table 4
Distribution and immunoreactivity of antibodies according to whether inside (IG) or outside (OG) the granuloma in sarcoidosis and tuberculosis samples.

Tissue sample (number)		CD4		CD8		CD22		CD14		CD68		CD163[†]	
		IG	OG	IG	OG	IG	OG	IG	OG	IG	OG	IG	OG
Sarcoidosis	Lymph node (4)	1	4	0	1	1	2	4	1	4	1	3	1
	Non-lymph node (6)	1	3	0	0	1	1	4	1	3	1	2	2
Tuberculosis	Lymph node (6)	1	3	0	1	1	2	4	1	3	1	1	0
	Non-lymph node (6)	1	2	0	0	0	1	3	1	2	2	1	0

Abbreviations: IG: inside of granuloma, OG: outside of granuloma (in lymph node tissue, outside of granuloma consists of both *perigranulomatous and* perisinusoidal areas), immunoreactivity was scored according to: 0 – no immunoreactivity, 1 – mild immunopositivity in scattered cells, 2 – immunopositivity in up to a third of cells, 3 – immunopositivity in up to a half of cells and 4 – strong immunopositivity in the majority or all cells. All samples scored with a value ≥1 were considered positive.

[†] Fisher exact test demonstrated significant difference between Sarcoidosis and Tuberculosis CD163 IHC marker (P < .05), statistical analysis didn't show significant difference for other CD marker between Tuberculosis and Sarcoidosis.
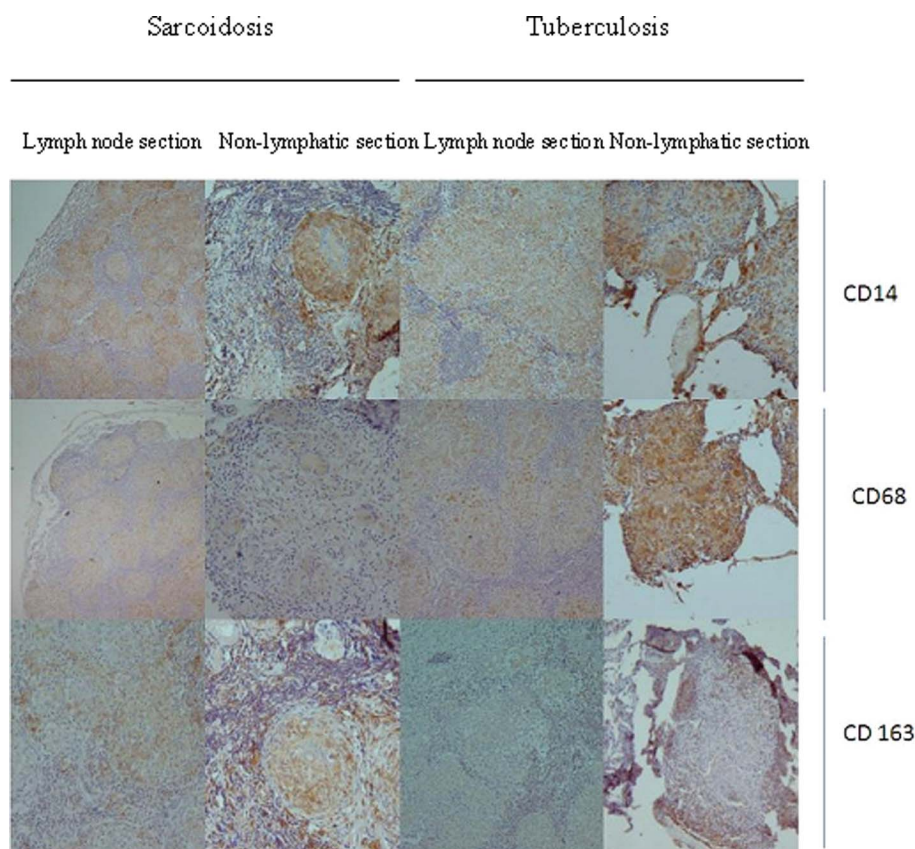
macrophages [34,59]. However, the presence of CD163 + cells is indicative of macrophages and although it may not capture all M2 macrophages it probably reflects distinct subsets of M2 macrophages stimulated by haeme products [60] and/or subsets of atypical M2, tissue resident M2, tumour associated M2, M2a and M2c macrophages [59]. Indeed, although some studies have used CD163 as M2 macrophage marker [9–14], dual staining with c-Maf may provide greater selectivity as pan M2 markers [34]. Gene expression markers [61] and functional properties [59] are increasingly used to define macrophage subsets. Both *ex vivo* and *in vitro* transcriptomic and proteomic studies in human and murine macrophages demonstrate the great complexity and plasticity of macrophage subtypes [61–64] at baseline and in response to various stimuli or drugs. These data also emphasise the differences between human and murine macrophages.

Most of our knowledge concerning M1 and M2 nomenclature and function is derived from animal studies. The studies of Schultze and colleagues [61] emphasizes the limitations of this simple concept in human monocyte-derived macrophages. In these studies, many distinct types of macrophages beyond the standard M1/M2 distinction could be identified dependent upon the exact stimulatory environment and time when studied. These subtypes could also be detected as varying in human disease (chronic obstructive pulmonary disease or COPD).

The present study has some limitations: we had limited amounts of appropriate tissue to provide sections since many of samples were TBLB which were not suitable for slide preparation or didn't contain enough granuloma for our study. Although it is expected that this number will reflect the tissue response and will not change with increasing sample size. Sample size may also affect the lack of correlation seen here between corticosteroid use and CD163 staining which may be addressed in a larger cohort analysis. Secondly, there was a sample selection bias as more chronic sarcoidosis patients or individuals with minimal therapeutic responses have undergone biopsy. This prevents us extending these results to all pulmonary sarcoidosis particularly as there are significantly higher levels of CCL18 in stage IV sarcoidosis [48]. Finally,

for ethical reasons, there is no healthy control group for comparison.

In conclusion, IHC analysis of tissue sample (lung and lymph node) from sarcoidosis versus tuberculosis patients revealed M2 polarization in sarcoidosis despite both these diseases generally eliciting a Th1-mediated immune response. This finding demonstrates new aspects of immunopathology in sarcoidosis which may result in therapeutic approaches directed toward interrupting alternative activation of macrophages to improve treatment of complications and clinical outcomes.

5. Declaration

5.1. Ethic approval and consent to participate

This study was conducted with the approval of Masih Daneshvari Hospital ethic committee and utilizing protocols approved by the respective institutional review boards. (SBMU1.REC.1393.60)

All the patients have the written consent form for FFPE blocks in the medical records for research purpose. Also, informed oral consent for this study was obtained from the owner of FFPE blocks by phone call.

5.2. Consent for publication

There is no individual person's data in this publication.

5.3. Availability of data and material

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interest

The authors declare that they have no competing interest for this study.

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