Peripheral blood leukocyte immune responses in sarcoidosis

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Abstract

Sarcoidosis is a multisystem granulomatous inflammatory disease of unknown aetiology with heterogeneous clinical manifestations. The disease is characterised by elevated Th1-like pro-inflammatory immune responses in tissue, whereas peripheral blood has been described as either having enhanced, diminished or similar responses in patients compared with healthy controls. The aim of this study was to characterise the immune responses from peripheral leukocytes in patients with sarcoidosis, focussing on inflammatory and regulatory features present in the disease.

Immunophenotyping of peripheral blood mononuclear cells (PBMCs) by flow cytometry showed that 86% of patients with sarcoidosis displayed a T-cell lymphocytopenia, with over a 2 fold reduction in cell number compared to healthy controls, confirming the findings of other groups. The proportion of the more pro-inflammatory 'Intermediate' monocyte subset ($CD14^{++}$ $CD16^{+}$) was increased in patients with sarcoidosis, matched by a reduction in the 'Classical' monocyte subset ($CD14^{++}$ $CD16^{-}$).

High concentrations of phytohaemagglutinin (PHA) induced significantly increased IL-6 and TNF-α release from the blood of patients with sarcoidosis compared with controls in *ex vivo* whole blood assays. By contrast, IL-6 release in response to a high concentration of Staphylococcal enterotoxin A was significantly reduced in patients. There was no statistically significant difference in cytokine release from patients compared with controls when whole blood was treated with a range of other ligands and cytokines.

The regulatory CD200 receptor (CD200R) expression was reduced on monocytes from patients with sarcoidosis, whereas there was no significant difference in IL-10 receptor or SIRP-α expression between patients and controls. The $CD200R^{low}$ phenotype on monocytes was associated with elevated PHA induced IL-6 and TNF-α release from whole blood.

Patients with sarcoidosis are capable of robust peripheral blood leukocyte immune responses and display alterations in the regulatory CD200R/CD200L axis that may play a role in the immuopathogenesis of the disease.

Thesis Associated Manuscripts and Conference Abstracts

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FRASER, S., SADOFSKY, L. & HART, S. 2015. Peripheral blood leukocyte immune responses are distinctly altered in sarcoidosis. European Respiratory Journal, 46. – Poster Discussion.

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Abbreviations

1α-hydroxylase	25-Hydroxyvitamin D_3 1-alpha-hydroxylase
18F-FDG PET/CT	F-18 fluorodeoxyglucose positron emission tomography/Computerised Tomography
ACE	Angiotensin Converting Enzyme
AMs	Alveolar Macrophages
ANOVA	Analysis of variance
ANXA11	Annexin A11
APC	Allophycocyanin
β-glucan	1,3 Beta-glucan
BAL	Bronchoalveolar Lavage
BALF	Bronchoalveolar Lavage Fluid
BCG	Bacillus Calmette–Guérin
BS	Blau Syndrome
BSA	Bovine Serum Albumin
CBD	Chronic Beryllium Disease
CCL	C-C Motif Chemokine Ligand
CCR	CC Chemokine Receptor
CD	Cluster of Differentiation/Cluster of Designation
CD200L	CD200 Ligand
CD200R	CD200 Receptor
CLRs	C-type Lectin Receptors
CNS	Central Nervous System
CRP	C-reactive Protein
CT Scan	Computerised Tomography
CTLs	$CD8^+$ cytotoxic T-lymphocytes
CXCL	C-X-C Motif Chemokine Ligand
CXR	Chest X-Ray
DAMP	Damage Associated Molecular Pattern
DCs	Dendritic Cells
dsRNA	Double Stranded RNA
DOK2	Docking protein 2
ELISA	Enzyme Linked Immunosorbent Assay
ELISPOT	Enzyme-Linked ImmunoSpot
EN-RAGE	S100A12 protein
EOS	Early Onset Sarcoidosis
ES-RAGE	Endogenously Secreted RAGE
ESAT-6	*Mycobacterium tuberculosis* antigen ESAT-6
FACs	Flow Cytometry
FBS/FCS	Foetal Bovine/Calf Serum
FEV	Forced Expiratory Volume
FFPE	Formalin Fixed Paraffin Embedded
FITC	Fluorescein isothiocyanate
FL-RAGE	Full Length RAGE
Fc	Fragment crystallisable (region)
FSC/Fsc	Forward Scatter
GM-CSF	Granulocyte macrophage colony-stimulating factor

GMFI	Geometric Mean Fluorescence Intensity
HLA	Human Leukocyte Antigen
HMBG1	High-mobility Group Protein B1
HRP	Horseradish peroxidase
HSP	Heat Shock Protein
IE-DAP	γ-D-Glu-mDAP
IGRA	Interferon Gamma Release Assay
IHC	Immunohistochemistry
IL	Interleukin
IL-10R	IL-10 Receptor
ILD	Interstitial Lung Disease
IFN	Interferon
IFN-γ	Interferon Gamma
IgG	Immunoglobulin G
IPF	Idiopathic Pulmonary Fibrosis
IQR	Interquartile Range
LPS	Lipopolysaccharide
JAK/STAT	Janus Kinase/ Signal Transducer and Activator of Transcription
JS	Juvenile Sarcoidosis
MAPK	Mitogen-activated Protein Kinases
MCP-1	Monocyte Chemoattractant Protein 1
MDMs	Monocyte-derived Macrophages
MDP	Muramyl dipeptide
MDT	Multi-disciplinary team
MHC	Major Histocompatibility Complex
MIP-1α	Macrophage Inflammatory Protein 1 alpha
MKatG	*Mycobacterium tuberculosis* catalase-peroxidase
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
MTB	*Mycobacterium tuberculosis*
MYD88	Myeloid Differentiation Primary Response 88
NF-Kb	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	Natural Killer Cell
NKT cell	Natural Killer T cell
NLRs	NOD-like Receptors
NOD	Nucleotide-binding Oligomerization Domain-containing protein
PAMP	Pathogen Associated Molecular Pattern
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PHA	Phytohaemagglutinin
PMA	Phorbol 12-myristate 13-acetate
Poly I:C	Polyinosinic:polycytidylic acid
PRR	Pattern Recognition Receptor
PTH	Parathyroid hormone
PV	Plasma Viscosity

RA	Rheumatoid Arthritis
RAGE	Receptor for Advanced Glycation End-products
RASGAP	Ras GTPase activating protein
RLRs	RIG-I-like receptors
S100 protein	S100 Calcium-binding protein
sACE	Serum Angiotensin Converting Enzyme
SD	Standard Deviation
SEA	Staphylococcal Enterotoxin A
SEM	Standard Error of the Mean
sIL-2R	IL-2R (soluble or serum)
SIRP-α	Signal regulatory protein alpha
SLE	Systemic Lupus Erythematosus
SSC/Ssc	Side Scatter
TB	Tuberculosis
TCR	T cell receptor
TGF-β	Transforming Growth Factor Beta 1
Th cell	T helper lymphocyte cell (1/2/17)
THP-1	Human acute monocytic leukemia cell line
TLB	Transbronchial Lung Biopsy
TLR	Toll-like Receptor
TNF-α	Tumour Necrosis Factor Alpha
Treg	T regulatory cell
WGA	Wheat Germ Agglutinin

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Author's Declaration

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Chapter 1 Introduction

1.1 Overview of the Disease

Sarcoidosis is a multi-system granulomatous inflammatory disease of unknown aetiology [1] that predominantly affects the pulmonary system, but also commonly appears in the eyes, skin, neural and cardiac systems in a number of patients [1-3]. The heterogeneous nature of the disease, along with the lack of a defined cause and poorly understood pathogenesis, provides a problem for effective diagnosis and management of the condition. Up to two thirds of patients with chronic sarcoidosis will self-resolve, others may require corticosteroid intervention [2, 4, 5]. Diagnosis is currently by exclusion, involving tissue biopsy and subsequent histopathology to identify evidence of the characteristic non-caseating granulomata (granulomas) that cannot be attributed to a known source [3, 6, 7]. The granuloma is a collection of myeloid derived cells (macrophages, epithelioid cells, Langhans' giant multi-nucleated cells) and T-lymphocytes of different types (mainly CD4$^+$ Th1 cells) [8-12]. This type of non-necrotic granulomata are the distinctive feature present that allows the assignment of a disease state in an organ. Superficially, the granulomata in sarcoidosis appear similar to those seen in tuberculosis (TB) infection, though are differentiated by being non-caseating (non-necrotising) in nature [3, 13], more akin to those present in another granulomatous condition, berylliosis [14, 15]. Whilst tissue inflammation has been characterised by elevated inflammatory cytokine release associated with Th1-like responses, peripheral blood has previously been subject to contradictory reports suggesting immune responses in sarcoidosis may be enhanced or specific to an antigen [16-20], diminished [21-25] or similar to healthy populations [26]. It is clear that there is a complexity to the immunology of the disease that merits further study.

1.2 History of Sarcoidosis Research

Sarcoidosis was described over a century ago, during which time many discoveries have contributed to the current understanding of the disease (Figure 1.1). In 1877 the prolific physician Jonathon Hutchinson identified lesions in a patient with gout that he would later describe as "livid papillary psoriasis" [27]. 12 years later, the French Physician Besnier would describe a condition with nasal and finger inflammation as "lupus pernio" [28], though this was in fact a manifestation of sarcoidosis. Little connection was made between these and subsequent cases, as clinical presentation differed significantly enough for them to be considered separate. It would not be until almost the turn of the century for sarcoidosis itself to be identified as a distinct, unified disease.

Boeck was the first to name the now recognisable granuloma containing epitheliod cells as "Sarcoid" from skin biopsy [29, 30]; further researchers would find similar inflammatory masses in other organ systems and later classify them as such. He identified patients of Dr Hutchinson as having suffered the same malady; his own work was also the first to connect these granulomata in various organs to a common disease [29]. The systemic nature of sarcoidosis was further described by Jorgan Schaumann, though neither he nor Boeck were aware of what type of disease it was [31, 32]. The alternate name for sarcoidosis: 'Besnier-Boeck-Schaumann disease', is based on the contribution of these researchers in defining sarcoidosis as a distinct disease in its own right [33]. Löfgren's syndrome was identified by Sven Löfgren, a disease now considered an acute form of sarcoidosis [34, 35].

Originally, Nickerson and Williams identified that a reaction from tissue of a patient with sarcoidosis elicited a response, though they did not follow through with this observation [36]. It was later, in 1941, that Kveim would use the lymphatic tissue of a sarcoidosis patient to diagnose others suffering from the disease [37]. Intradermal injections of the tissue from one patient would lead to papules containing the epithelioid cells in granulomata symptomatic of sarcoidosis in another patient.

This was a crude method, yet the particulates (tissue suspension) were refined by Siltzbach years later to produce a more viable solution from sarcoid spleen [20]. This was

validated by extensive global testing by the physician himself and became known as the 'Kveim-Siltzbach Test', shown to be both specific to the condition and reliable [38, 39].

There were other early major contributions, including the description of sarcoid anergy to tuberculin and other antigens by Boeck and later Friou [21, 40] and the introduction of corticosteroids as a therapy [40-42]. Much of the recent work has advanced the knowledge of the field in specific areas, building upon the initial work of these scientists and physicians; though the main question of aetiology still has yet to be answered.

Sir Jonathan Hutchinson

1877 – First identification: "livid papillary psoriasis"

1889 – Besnier describes a similar condition, "Lupus pernio"

1899 – **Boeck** assigns the name after his paper: "Multiple Benign Sarkoid of the Skin"

1917 - Jorgan Schaumann Identifies the systemic nature of the disease – multiple organ involvement.

1935 – Nickerson and Williams Discovered that the sarcoid tissue of one individual could induce a response in another patient.

Morten Ansgar Kveim
1941
Used processed lymphatic (or spleen) tissue from sarcoid in intradermal injections to induce granulomata in those with the disease: diagnostic. 'Kveim Antigen'

1950's – Use of corticosteroids in treatment for sarcoidosis

1951 – **Boeck and Friou** Discovery of sarcoidosis patient anergy to tuberculin

Louis E. Siltzbach

1961 – Refined the Kveim Antigen, the test became the 'Kveim-Siltzbach Test'

ACCESS study 1996-1999

Baughman, Iannuzzi, Judson, Moller, Newmann et al

Multi-centre U.S. Case controlled study with over 700 sarcoid patients to determine common genetic, environmental and epidemiological factors – a search for an aetiology

Some associations with occupation; insecticides, bacterial bioaerosols and agriculture.

No single aetiological factor was identified in the cohort.

No single gene polymorphisms – multifactorial?

Figure 1.1: Timeline of sarcoidosis discovery

Highlights of the main historical research in sarcoidosis. Images from [43-45].

1.3 Clinical Pathology

1.3.1 Summary of Clinical Manifestation and Presentation

As an inflammatory disease, sarcoidosis is characterised by the presence of granulomata in susceptible organs. The most common area of inflammation remains the lungs; approximately 90% of sarcoidosis patients have this involvement [1, 46]. Other organs commonly affected include the skin, heart, kidneys, eyes, spleen and peripheral lymph nodes [3, 46, 47]. Neither is any organ an obligatory site across all cases; one patient may only display cutaneous lesions, others may have widespread systemic granulomatous involvement. Table 1.1, taken from a review by Chen and Moller [2], summarises the organ involvement and the typical percentage of cases displaying these. Lungs (and associated lymph nodes), skin and eyes are most commonly involved, leading some researchers to conclude that the disease is likely to be due to an external environmental factor [4]. The heterogeneity and multi-system nature of the disease, as well as the widespread range of other organs that can be involved, point to a complex disease far removed from other related granulomatous diseases such as TB or berylliosis. Depending on the study and the methodologies employed in sarcoidosis assessment, approximately 50% of patients will present with two or more organs involved (see Table 1.2) [47], with the mean number of organs increasing over time from approximately 2 to 3 with disease progression, more so in Black patients [48]. The involvement of particular organs, like cardiac sarcoidosis, may be underestimated due to the difficulty in diagnosis. For example, a study in Japan suggested 46.9% of deaths were due to sarcoidosis displayed cardiac involvement after autopsy, yet only 26.7% were diagnosed with cardiac involvement prior to death [49]. It must also be acknowledged that various studies may represent a degree of 'referral bias' in the figures for different organ involvement. The multi-organ nature of the disease means recruitment of patients across all the different departments in clinics has tended to favour specific disciplines more than others for these reports, as was the case with pulmonary involvement within the ACCESS study (A Case Control Etiologic Study of Sarcoidosis) [46, 47]. Typically, the disease will be either chronic or remitting; approximately 2 out of 3 cases will belong to the latter and will lead to clearance within the first 10 years of diagnosis, half of these within the first 3 years [4]. The remaining third are usually chronic and persistent in nature, requiring intervention

and with multi-organ involvement [2, 5, 50, 51]. Whilst re-occurrence after remission remains low, less than 5%, sarcoid granulomata can arise in any organ at any age in this subset. Löfgren's syndrome is an acute form, particularly affecting women, which resolves in 70-80% of patients, though will be severe in other cases [52]. Multi-organ and specific types of extrapulmonary involvement are associated with a worse prognosis in patients [47, 48, 53-55]. Race, gender and age can also impact the incidence, severity and organ distribution in the disease (Section 1.5.1 Epidemiology).

Table 1.1: Organ involvement in sarcoidosis
Taken from [2].

Organ/Organ System	Frequency of Occurrence (%)	Common Findings
Lungs and thoracic lymph nodes	>90	Dyspnoea, cough, chest pain, pulmonary hypertension, mixed pulmonary function test abnormalities (obstruction, restriction, diffusion deficits)
Skin	20–30	Nodules, plaques, lupus pernio, erythema nodosum (a nongranulomatous panniculitis)
Eyes	20–25	Uveitis, conjunctivitis, lacrimal gland enlargement, sicca syndrome, optic neuropathy
Liver and/or spleen	10–20	Hepatosplenomegaly, jaundice, elevated liver function tests, cirrhosis, hypersplenism
Cardiovascular system	10–20	Ectopy, heart block, arrhythmias, cardiomyopathy, sudden death
Central nervous system	10–25	Cranial neuropathy, mass lesions, aseptic meningitis and/or encephalitis, myelitis, spinal cord and peripheral neuropathy, small fibre neuropathy, pain, hypothalamic–pituitary involvement
Sinuses and upper respiratory tract	5–10	Chronic sinusitis, laryngeal involvement, parotid gland involvement
Bones, joints, muscle	5–15	Chronic arthritis, dactylitis, lytic bone lesions, myopathy
Haematological system	>50	Peripheral lymphopenia, hypergammaglobulinemia
Renal system (including calcium abnormalities)	5–10	Hypercalcaemia and/or hypercalciuria, nephrocalcinosis, nephrolithiasis
Endocrine system	5–10	Hypothalamic–pituitary involvement, pancreatic mass, Heerfordt syndrome (uveoparotid fever)
Gastrointestinal and reproductive tract	<1	Gastric nodules, ovarian or testicular masses

Table 1.2: Frequency of multiple organ involvement in sarcoidosis.

Adapted from data obtained in the ACCESS study of 736 sarcoidosis patients [47]**.**

Number of Organs Involved	Approximate Percentage (%)
1	50
2	30
3	13
4	5
5 or more	2

Symptoms can vary depending upon the organs involved. Common symptoms include weight loss, fever and fatigue, though cough, dyspnoea (shortness of breath) and chest pain are also more commonly observed, in keeping with the high incidence of pulmonary involvement in patients [1]. The mortality rate is less than 5% [1], the majority of these result from respiratory involvement, typically due to the progression of the disease to pulmonary fibrosis, though cardiac sarcoidosis, neurologic sarcoidosis and hepatic organ involvement are also associated with higher mortality [47, 56-59]. Despite the apparent anergy displayed to tuberculin [21], the condition is not associated with opportunistic infection, thus indicating an immunocompetence exists [1]. Other than the physiological finding, depression has been reported in 46% of sarcoidosis patients compared to 27% of control cases [46]. Chronic cases clearly have this psychological component, as remittance may be harder to achieve. The clinical presentation is highly variable; patients can be relatively asymptomatic or present with multiple symptoms indicative of systemic and/or chronic disease [1, 3]. Although classification differs between reports, usually patients are separated into radiographic subsets (Scadding CXR Stages) depending on their presentation, based upon the criteria established by Professor John Guyett Scadding (see Table 1.3) [60].

Table 1.3: Scadding CXR stages and the proportion of patients within each stage.

Adapted from data obtained in the ACCESS study of 736 sarcoidosis patients [47].

Scadding CXR Stage	Description	Percentage of Patients (%)
0	Lung fields and lymph nodes clear	8.3
I	Lymph nodes only	39.7
II	Lymph nodes and Lung involvement	36.7
III	Lung tissue involvement only	9.8
IV	Fibrotic presentation	5.4

1.3.2 Granulomata in Sarcoidosis

The work of early researchers such as Boeck and Schaumann led to the histological identification of the granuloma in sarcoidosis; since then, the key features as viewed under a microscope have changed little [29-32]. A granuloma is defined as a collection of macrophage and macrophage-derived cell types [8], epithelioid and Langhans' multinucleated giant cells are the cell types present that characterise this type of inflammation [9, 11]. The predominant cells present are macrophage subtypes and lymphocytes, usually Th1 polarised T-lymphocytes [61]. These T-lymphocytes surround the internal region of inflammation; activated mononuclear immune cells are required for stimulation of the inflammatory pathways [12]. Figure 1.2 shows the typical histopathology and a diagrammatic representation of a granuloma, the outer ring contains lymphocytes, fibroblasts and other mononuclear cells surrounding a central core of macrophages and epithelioid cells [10]. The process involved in the formation of a granuloma are described later within the immunopathogenesis of the disease (Section 1.4.2).

Granulomata are considered to be either necrotising or non-necrotising, in sarcoidosis the latter is typically observed and is termed 'non-caseating' [1, 3]. This is the key distinguishing feature of sarcoidosis, separating it from other granulomatous conditions such as TB, which usually presents necrotising granulomata [10]. Despite this, there is sometimes 'overlap' between the two; some cells within a sarcoid granuloma may have necrotising features or might 'mimic' the other [62-64]. The non-necrotic phenotype of the granulomata has also been seen in other diseases, including berylliosis [14], Cryptococcus infections [65, 66]and unusual cases presented in other conditions [67]. However, non-caseating granulomata remain the most common and distinguishing feature of the disease; the main diagnostic indicator in classifying an unknown disease such as sarcoidosis after others causes are excluded [3, 47, 68].

The function of a granuloma is a matter of some debate; though it has been suggested that they are the result of hypersensitivity or the inability of immune cells to process a specific antigen through either dysfunction or deliberate evasion [69, 70]. Without an aetiology the reason for the development of sarcoidosis granulomata can only be

speculated at. In berylliosis, the inability of cells to process the inorganic element is believed to lead to the end granuloma pathology [14, 15, 71]. With this producing a morphologically similar histological pattern to sarcoidosis, the same underlying dysfunction may be at work.
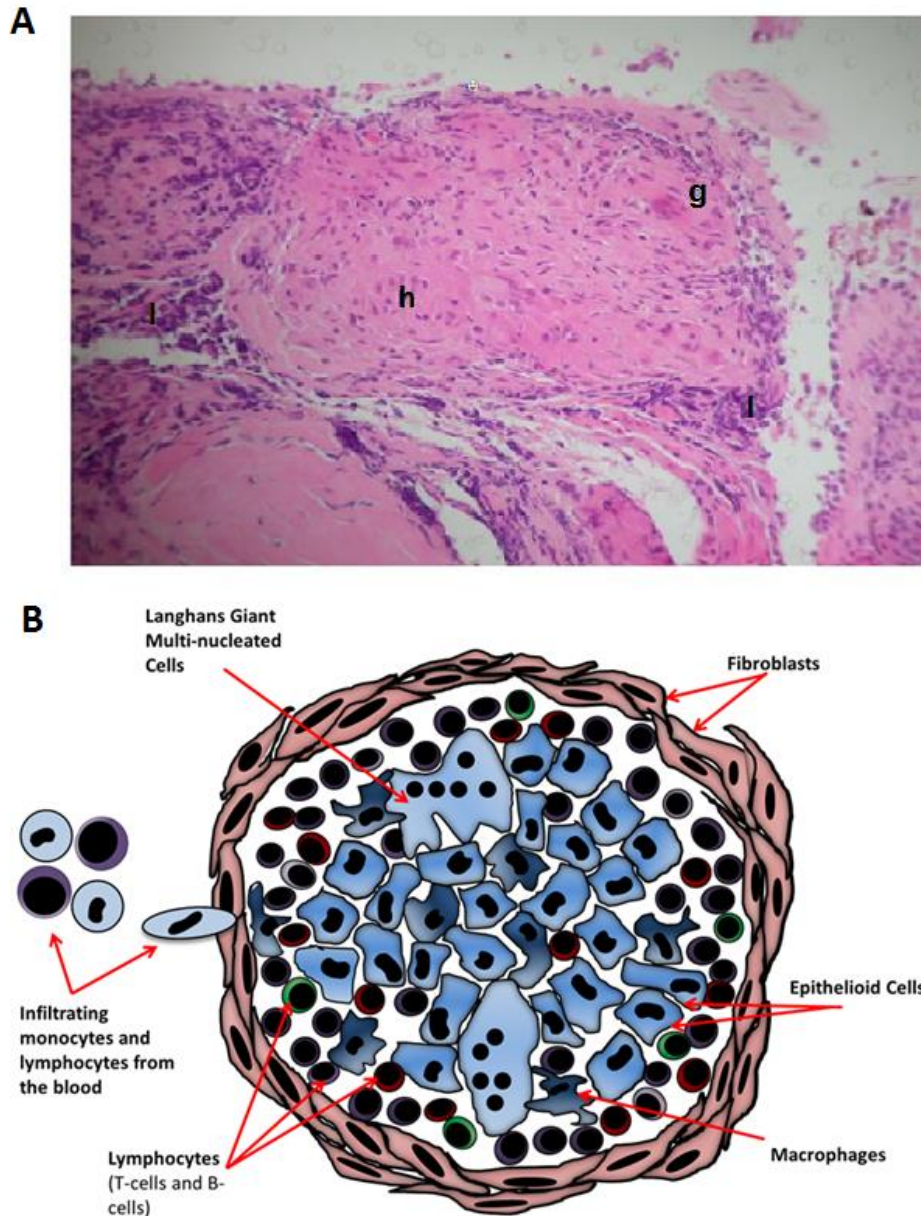
Figure 1.2: The granuloma in sarcoidosis.

A: Histology of transbronchial biopsy from a 43 year old man with pulmonary sarcoidosis. Labelled: 'g'- giant cell, 'l' lymphocytes, 'h' histiocytes. Picture courtesy of Dr Anne Campbell. **B:** Diagramatic representation of a non-caseating sarcoidosis granuloma. The central core of the granuloma consists of macrophage-derived cells (blue) and lymphocyte subsets (red, green and purple). Epithelioid cells and Langhans giant multi-nucleated cells differentiate from macrophages, the periphery of the granuloma is surrounded by fibroblasts. Leukocytes migrate from the peripheral blood and differentiate in the granuloma.

1.3.3 Pulmonary Sarcoidosis

Pulmonary involvement in sarcoidosis, whether it be direct lung or associated lymph node granulomatous inflammation, is the most common organ system to be affected by the disease [1, 46]. The greater incidence of lung symptoms means that the majority of deaths associated with sarcoidosis arise because of problems within the pulmonary system [47, 56]. Under the standardised procedure for diagnosing the disease [1], the requirement for a tissue biopsy for confirmation of a diagnosis of sarcoidosis also means that the lungs and mediastinal lymph nodes are common regions sampled. However, this still remains a disease established through exclusion of other explanatory causes rather than a disease with a diagnostic feature specific only to sarcoidosis [3, 7, 72]. There are a number of features associated with pulmonary sarcoidosis, the principle being in how the disease is staged. Use of chest radiographs and a scoring system, known as the 'Scadding CXR Stage' (see Section 1.8.2, CXR is chest X-ray), has allowed a degree of differentiation between disease cases [60]. The efficacy of this system is questionable however, as using alternate imaging techniques such as CT scans (Computed Tomography Scans) can often reveal differences absent in chest radiographs that are observed using a higher resolving method [73-75]. In addition, radiographic staging can lack consistency in assessment, arising from inter-observer variation [76]. Other reports suggest that traditional radiographs remain an effective tool for determining the state of the disease, when combined with histological and clinical findings [77].

The stages range from 0-IV, where 0 represents those without visible involvement in the X-ray, stage I is lymph adenopathy, stage II is lymph and parenchymal involvement, stage III is parenchymal involvement only and IV represents those with significant fibrotic disease throughout the parenchymal tissue (Figure 1.3 and Table 1.3) [60]. The ACCESS study of 736 patients determined that the majority of patients with sarcoidosis present with stage I and stage II, 39.7% and 36.7% respectively, only 5.4% showed stage IV fibrotic pulmonary disease [47]. It is appreciated that the disease in those with stage III and stage IV disease is less likely to self-resolve than in stages I and II [1, 4, 60]. Patients will not necessarily transition from 0 to I, from I to II and so on in a linear progression in the Scadding stages, suggesting that this may not be an effective predictor of disease progression [1, 78, 79]. This staging should thus be viewed as a temporal 'snapshot'

estimate of the disease activity, rather than a definitive classification for use in predicting outcomes.

Stage I
(lymphadenopathy)

Stage II
(lymphadenopathy and infiltrates)

Stage III
(infiltrates only)
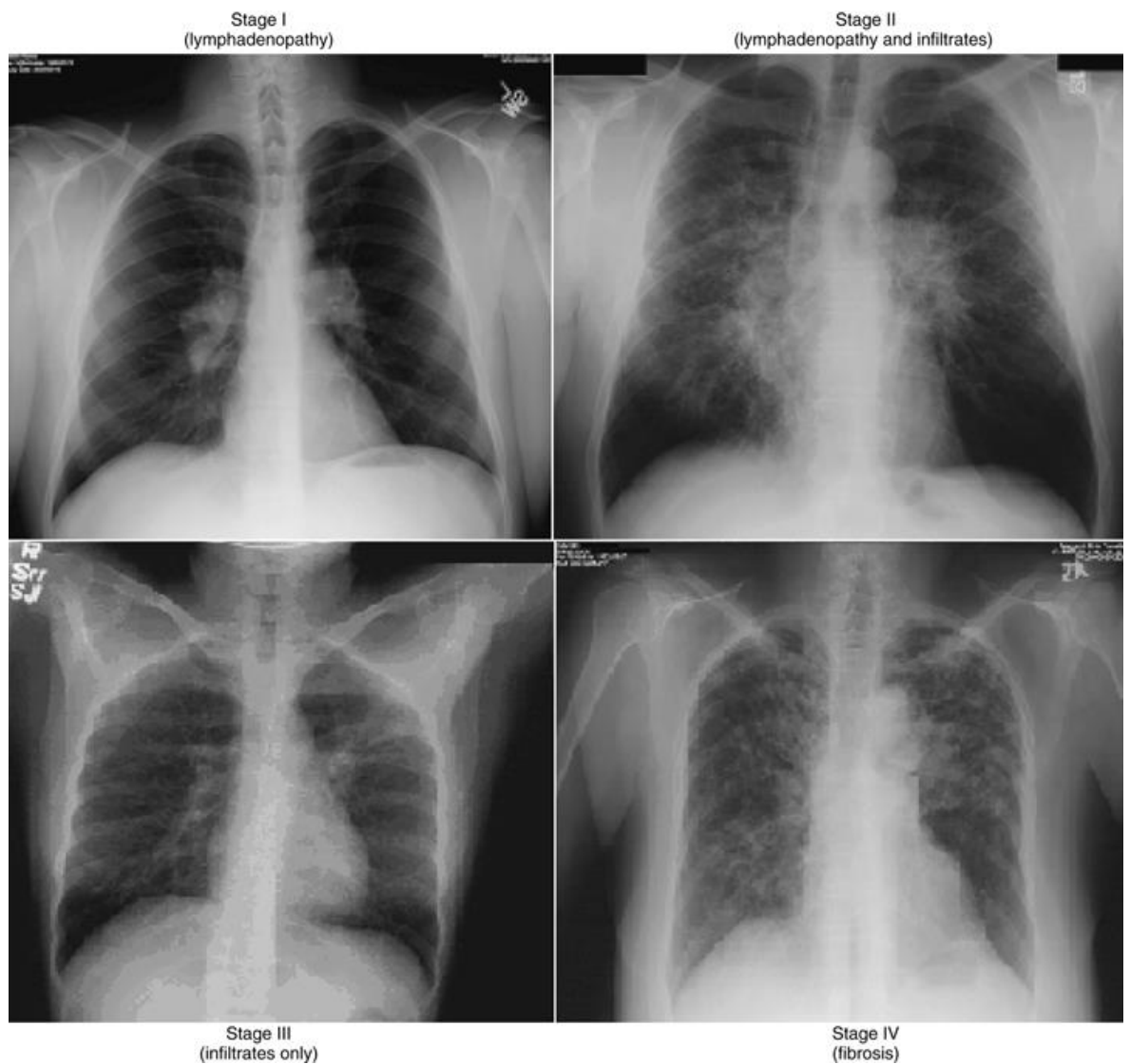
Stage IV
(fibrosis)

Figure 1.3: Scadding CXR stages in pulmonary sarcoidosis.

Stages I-IV in the Scadding CXR Staging system in radiographic assessment. Top left – Stage I, lymph node restricted. Top right – Stage II, lymph node and lung involvement. Bottom left – Stage III, paranchymal lung and no lymph node involvement. Bottom right – Stage IV, fibrotic disease. Taken from [80].

Lung function does appear to be compromised in patients with pulmonary sarcoidosis, but the specific manifestations can be varied, in keeping with the heterogeneous nature of the disease [1]. When comparing active and inactive disease, based on lung presentation in a chest X-ray, those with an active disease profile generally displayed

reduced lung function, such as diffusion and vital capacity [81]. The same group displayed that patients with more expansive or fibrotic lung involvement also tended to display reduced lung function over those with minimal lung perturbation, such as lymph node restricted stage I patients [81, 82]. Similar results for impaired lung function have been observed in a number of other studies looking at features such as the FVC (Forced Vital Capacity), FEV_1 (Forced Expiratory Volume), FVC_1/FEV (FVC and FEV ratio) and other pulmonary function test criteria (such as Forced Expiratory Flow, Peak Expiratory Flow, Carbon Monoxide Diffusion Capacity etc) [46, 47, 83, 84]. Whilst the majority of patients will show improved or stable pulmonary function after a 2 year period [78], some research suggests that within a 20 year period the disease is associated with a higher mortality rate amongst patients, particularly those who previously presented with impaired pulmonary function and stage III radiographic assessment [85].

Airway involvement is a common feature in sarcoidosis patients displaying pulmonary symptoms [1], though the proportion of those with airway obstruction can vary significantly depending on the cohort under investigation. Racial differences can be significant, with Black patients showing a tendency for more severe pulmonary functional impairment (63% of patients had reduced FEV FVC ratio) and 2 out of 3 patients displayed some form of airway obstruction [86]. The proportion of Caucasian patients displaying pulmonary involvement has been reported to be similar to Black patients [47] or lower [48], though there is evidence that lung function and associated symptoms are less severe in Caucasian and Asian groups compared with Black patients [46, 87]. Gender differences also appear to play a role, with male patients tending to present more advanced disease, as defined by the Scadding CXR stage [48].

Airway obstruction has been observed in all Scadding stages of the disease [84, 88], though the degree of obstruction appears to increase at the higher stages [46]. Additionally, airway obstruction has been variously associated with reduced pulmonary function in patients [46, 84], which increases proportionally with the amount of obstruction [46], and increased reported dyspnoea in higher stages [46]. Lower stage sarcoidosis (I and II) will display small airway distortions without the large detrimental effects on lung function observed in higher stages [89]. It should be noted however, that other groups have found no direct correlation between the staging of the disease and pulmonary function test results [86].

For those exhibiting fibrotic pulmonary sarcoidosis (stage IV), CT scans identified that bronchial distortion was the most common visible observation (47%), whereas honeycombing and linear displays appeared in 29% and 24% of observations [90]. Honeycombing has been related to the greatest degree of pulmonary function impairment, with linear displays associated with the least severe functional restriction [90].

Unusually for an interstitial lung disease, there is a lower incidence of sarcoidosis amongst smokers compared with non-smokers [91-94], though whether smoking confers a true protective aspect in sarcoidosis is unknown. Given the increased risks of developing other diseases associated with smoking, there is little research engaged with identifying whether tobacco might reduce the risk of developing sarcoidosis.

Overall, the incidence of pulmonary sarcoidosis tends to be higher in Black populations and the associated mortality is also greater than any other racial group [48, 86, 87, 95]. In particular, pulmonary hypertension appears to be significantly higher in Black populations, though all patients with sarcoidosis will variably have increased risk of different concomitant pulmonary conditions, such as pulmonary fibrosis and acute respiratory failure, when compared with healthy controls [48, 87]. An increased rate of incidence of sarcoidosis in females has also been observed, impacting the mortality statistics in women [87], despite typically presenting with a less severe pulmonary manifestation than male patients [48].

1.3.4 Skin Involvement in Sarcoidosis

Cutaneous manifestations of sarcoidosis are not uncommon, various studies have suggested anywhere between 15- 35% of sarcoidosis patients may have skin involvement [47, 48, 96-98]. Cutaneous sarcoidosis is more common in Black patients compared with Caucasians [47, 48, 95, 98] and is also more common in female patients than male [48, 98], the extent of the difference depending greatly on the study cohort. There are a variety of cutaneous manifestations present with the disease (Figure 1.4, Table 1.4), which have been separated into 'Specific' and 'Non-Specific', where 'Specific' applies to

those with granulomatous involvement [96, 97]. Described as "The Great Imitator" [99], whereby clinical presentation may be confused for other morphologically similar conditions, cutaneous sarcoidosis poses a particular problem for accurate diagnosis. Despite this, it has been shown that cutaneous sarcoidosis is an early manifestation of systemic sarcoidosis and would be useful as a diagnostic and prognostic indicator [100]. The most common skin presentation is erythema nodosum [96, 98], a non-specific lesion displaying as a raised red or pink nodules [96, 101]. Löfgren's syndrome is a specific subset of acute sarcoidosis presenting with erythema nodosum, bilateral lymphadenopathy, arthralgia (joint pain) and possibly other symptoms such as uveitis [35, 102, 103]. Erythema nodosum can be a differential diagnostic tool separating Löfgren's syndrome from other forms of sarcoidosis, which tend to be chronic in nature [47, 100]. Additionally, erythema nodosum has a degree of use in determining the prognostic outcome of sarcoidosis [54, 100]; most cases identified with erythema nodosum are classified as Löfgren's syndrome in which the majority self-resolve within 2 years [54, 102, 104]. Erythema nodosum can arise from infections and other non-sarcoidosis conditions such as a response to medicines [101]. Other non-specific cutaneous symptoms like erythema multiforme are rarely observed [98, 105]. Non-specific cutaneous manifestations are indicative of less severe symptoms, as they tend to be more common in Scadding Stage 0, I and II and patients are also more likely to have pulmonary symptoms self-resolve [96, 106].

There are a range of specific cutaneous sarcoidosis presentations, the more common being a macropapular rash, subcutaneous nodules and skin plaques [96, 98]. Lupus pernio occurs in 2.7-4% of sarcoidosis patients and is considered a positive indicator of the presence of the disease [96, 98]. It can present with purple/violet, hardened papules or plaques that can centre around the face [97]. Unlike erythema nodosum, this presentation tends to be associated more with progressive disease and occurs with greater frequency in those with extensive pulmonary symptoms [96, 100, 106]. Lupus pernio is also associated with a worse prognosis than other cutaneous manifestations [100].

Table 1.4: Skin manifestations in sarcoidosis.

Adapted from a 2 studies of 145 and 170 sarcoidosis patients [96, 98].

Cutaneous Manifestation	Specific or Non-Specific	Percentage of Total Sarcoidosis Cases (Range %)
Erythema Nodosum	Non-Specific	10.0 - 20.5
Maculopapular rash	Specific	3.7 – 7
Skin Plaques	Specific	4.3 – 6
Subcutaneous nodules	Specific	4.3
Lupus pernio	Specific	2.7 – 4
Scar Lesions	Specific	1 - 2.9
Psoriasiform lesions	Specific	0.9
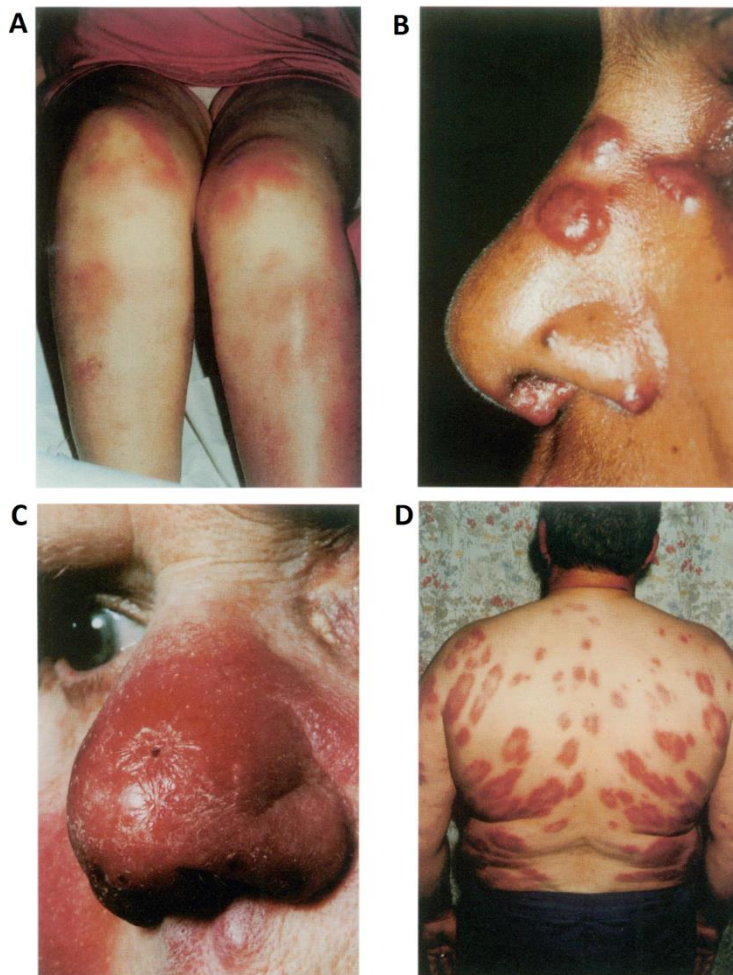
Figure 1.4: Cutaneous manifestations in sarcoidosis.

Examples of skin presentation of sarcoidosis. **A**: Erythema nodosum. **B**: Nodular sarcoidosis. **C**: Lupus pernio. **D**: Plaque sarcoidosis. Images taken and figure adapted from [107].

1.3.5 Ocular Sarcoidosis

Eye involvement in sarcoidosis has been reported with a large degree of variation in occurrence depending on the study and geographical region [108-110]. Generally, it has been stated that between 10-50% of patients will display some form of ocular symptom [47, 109-111]. The figure has been reported as higher in Japanese studies, as high as approximately 80% of patients displaying ocular involvement [112, 113]. Despite this, ocular symptoms, much like cutaneous, appear to be a more common manifestation in Black individuals than Caucasian [47, 48]. Eye involvement is also more common in female patients compared with males [47, 48].

Uveitis, inflammation of the uvea (which contains the iris, choroid and ciliary body), is the most common symptom displayed by those with ocular sarcoidosis (Figure 1.5), up to 84% [111, 114-117]. This can be anterior, posterior or both, which has previously been described to present more commonly in the anterior portion [115]. Differences between Caucasian and Black patients suggests that, whilst the latter does tend to display this presentation [114, 115, 117], there is a suggestion that in some studies, Caucasians will present commonly in the posterior region [114, 118]. Sarcoidosis is noted for the presence of specific features in the uveitis inflammation, such as mutton fat keratic precipitates (white cellular deposits resulting from inflammation) or small granulomatous keratic precipitates (cellular deposits, usually smaller, resulting from inflammation), which aid in diagnostic determination of the disease in eyes [119]. As with any inflammatory condition within the eye, blindness or visual impairment is a concern in uveitis, approximately 10% develop some form of blindness in either one or both eyes [112, 120, 121]. Macular oedema is particularly a problem in progressive ocular uveitis, and may be associated with the loss of ocular function in sarcoidosis patients [112, 120, 121]. Other symptoms occur in varying degrees depending on the cohort in the study, but conjunctival and lacrimal involvement (including keratoconjunctivitis sicca), adnexal granulomata and cataracts or glaucoma resulting from uveitis are other symptoms observed in eye involvement in sarcoidosis [114, 116, 118, 122].
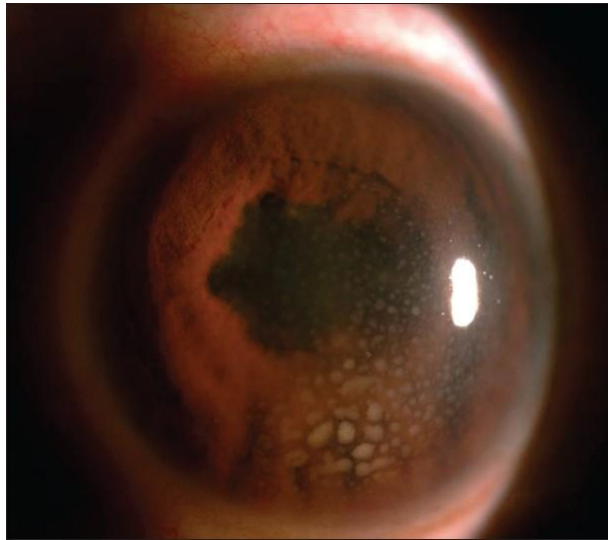
Figure 1.5: Anterior uveitis in sarcoidosis.

Taken from [123].

1.3.6 Cardiac Sarcoidosis

The often cited rate of incidence of cardiac involvement in sarcoidosis has been considered low, making up as little as 2.3% of the ACCESS study patient group [47] to approximately 5% [1, 124, 125]. This may be a large underestimate however, as a much higher percentage of patients with sarcoidosis have been identified with cardiac involvement after autopsy in a number of studies [49, 57, 124] , ranging from 13% [126] to 58% [58] in Japanese patients. Cardiac sarcoidosis is the primary cause of mortality in Japanese patients [49, 58, 59], where the incidence of heart involvement is greater [124, 127]. This contrasts with the principle cause of death in Caucasian and Black individuals being pulmonary complications, such as pulmonary hypertension and pulmonary fibrosis, followed by cardiac and neurologic causes [54, 56, 87, 128-130]. It is understood that cardiac sarcoidosis and pulmonary associated pathology with the heart, like cor pulmonale (right side heart enlargement), are associated with a worse prognosis in sarcoidosis in general [54]. Whilst the incidence of cardiac sarcoidosis (in terms of proportion within the cohort) is greater in Japanese patients, the lower number of sarcoidosis patients in this cohort, as few as 1 per 100,000 people [131], means that more Black and Caucasian patients suffer from cardiac complications. There is no reported significant difference in the incidence of cardiac sarcoidosis between Caucasian and Black cohorts and there are conflicting reports of gender differences [47, 48, 131].

Whilst cardiac sarcoidosis often occurs in unison with other organ systems, identifying heart restricted sarcoidosis when symptoms presenting could be indicative of other, more common, underlying causes is difficult [132]. Part of the difficulty in assessing cardiac sarcoidosis relates to issues in properly diagnosing the disease involvement in this organ, where a biopsy is not a useful tool and ECG (electrocardiogram) cannot always detect it [132]. As a significant proportion of those with cardiac involvement are asymptomatic [57], it is unsurprising that clinical diagnosis of the disease is greater post-mortem. Depending upon the study, sudden death and congestive heart failure are the most common symptoms of the disease [54, 58, 87, 128, 129], though some of these are only determined after death. Ante-mortem symptomatic cardiac sarcoidosis can present with ventricular/supraventricular tachycardia/arrythmyias, heart block, pulmonary hypertension and associated cor pulmonale and other more uncommon symptoms, such as ventricular aneurysms, pericardial disease and heart valve involvement [54, 57, 58, 133-136]. The association with sudden death and the revelation that a large number of patients with cardiac symptoms go undiagnosed suggests that this is a particularly difficult manifestation to manage in patients.

1.3.7 Neurosarcoidosis

The term 'neurosarcoidosis' applies to the inflammatory symptomatic involvement of the nervous system, including the brain and cranial nerves, as well as the peripheral nervous system [137]. The reported incidence of neurosarcoidosis amongst patients ranges between 4.6 – 12.8% depending on the study, with the majority closer to the lower end of the range (approximately 5%) [47, 48, 138-142]. Whilst this figure presents as a minority of sarcoidosis cases, post-mortem reports suggest that only up to 50% of those with neurologic involvement who die are diagnosed prior to autopsy [49, 143], suggesting that the numbers of patients with neurosarcoidosis may be higher due to subclinical presentation. This is further complicated by the difficulty in establishing a positive diagnosis of neurological involvement, due to the misdiagnosis or 'mimicry' of symptoms and clinical pathology with other neurological diseases [144]. As with a number of other symptoms, the incidence of neurosarcoidosis tends to be greater in Black patients compared with Caucasian patients [47, 48]. The impact of gender on risk of developing

neurosarcoidosis is more complicated though, with some studies suggesting that women have a greater risk of developing neurologic symptoms [47, 138, 145], other studies find a small skew towards male involvement [146-148] and some reporting there is no significant difference between males and females [48]. It may be that different regional variations impact the results or that the effect of different racial demographics within cohorts may be significant in gender associated risk.

Neurological features were the presenting symptom in anywhere between 48 - 70% of neurosarcoidosis patients, the remaining would develop the neurologic symptoms after sarcoidosis was confirmed in other organ systems [138, 142, 145-151]. Neurosarcoidosis is exclusive in a proportion of cases, variably ranging from 5.9% (in the ACCESS study) [47] up to 17% of neurosarcoidosis patients (in other series assessments) [149, 152]. Part of this variation may be the result of differences in diagnostic assessment or the length of assessment of patients, though the ACCESS study acknowledges a bias towards pulmonary symptoms, whereas other studies target and select those with neurologic symptoms specifically. Despite the difficulties in diagnosing central nervous system (CNS) sarcoidosis involvement, there have been attempts to separate the cases into 'Definite' (or 'Confirmed'), 'Probable' and 'Possible' based on suggestions proposed by Zajicek *et al*. [147]. Alternative criteria for each classification have been suggested by Judson *et al.* [137], or the system by Zajicek *et al* has been modified by other individual studies [146, 153], or the classification is used unaltered [148], which still means that there is a great deal of variation in how neurosarcoidosis is defined.

The symptoms of neurosarcoidosis can be significant and varied, though will usually appear within 2 years of the diagnosis [138, 151] and can be an early onset symptom associated with severe disease [55]. Neurological symptoms (with the exception of the seventh cranial nerve) tend to be associated with chronic disease rather than acute [53, 54]; 75% of patients with CNS involvement will have a protracted course lasting more than 2 years [54]. The most common symptoms are cranial neuropathies (damage to the cranial nerves) and facial palsy is usually the most frequently observed of these, followed by optic nerve involvement [138, 140, 141, 148, 154]. A large spectrum of other manifestations and symptoms have been observed however, including headaches, seizures, peripheral neuropathies, meningitis, spinal cord inflammation and myopathy

(skeletal muscle nerve involvement) amongst others (reviewed in [155-157]). Development of psychiatric pathologies like psychosis and schizophrenia have been shown to occur in neurosarcoidosis [158-160], though these tend to be a rare manifestation. Overall, psychiatric symptoms may occur in as few as 20% of neurosarcoidosis patients, covering mood changes, depression, delirium, memory loss and other psychological disturbances [46, 154, 161].

1.3.7 Sarcoidosis affecting the Liver

Hepatic sarcoidosis has been described as being present in 5-79% of different patient cohorts, which represents a range incorporating those with significant symptomatic liver involvement at the lowest end [162] to the reporting of granulomatous inflammation within biopsy samples at the top [163, 164]. As with other organs discussed, the definition of what constitutes hepatic sarcoidosis involvement therefore requires clarity. It could be concluded that hepatic sarcoidosis may be subacute in a large number of patients, though liver dysfunction of some form, when tested, is described in approximately 35 -50% of patients with hepatic involvement [162, 165, 166]. Often classified as being independent of gender and age [47], other studies have reported that the liver is more frequently involved in female patients [48]. Hepatic sarcoidosis is significantly more common in Black patients compared with Caucasian patients [48, 165].

Despite being relatively common, most patients with hepatic involvement on biopsy are asymptomatic [165, 167]. Of those who are symptomatic, hepatomegaly (enlarged liver), abdominal pain, fever, cholestatic pruritus (skin itch due to liver disease) and jaundice may be observed in a minority of cases (reviewed in [168, 169]). The extent of granulomatous inflammation within the liver also determines the degree of liver dysfunction present [166]. It should be noted that significant hepatic involvement has been associated with a worse prognosis in patients [1, 54, 56]. However, the length of time for the liver symptoms to severely impact a patient is greater than other extrapulmonary organ systems, indicative of an insidious but progressive manifestation [55].

1.3.8 Other organ involvement

Given the role of the spleen, it is unsurprising that this organ commonly features granulomatous inflammation. Much like the liver, spleen involvement is not often detected by standard examination and is typically asymptomatic, leading to estimates as low as 5.6% involvement [47, 170, 171]. However, the proportion of patients showing indicators of disease in the spleen increases when using non-invasive CT scans or X-rays (approximately 15-53% [172, 173]) and tissue biopsy (approximately 24-59% [174-176]). Unlike other intra and extrathoracic organ involvement, the spleen has been reported to be more commonly affected in Caucasian patients compared with Black patients [48]. Symptoms associated with hypersplenism (overactive spleen) and the presence of splenomegaly (enlarged spleen) are uncommon [170, 171, 177], massive/giant splenomegaly is very rare [177-179]. A model suggests that splenomegaly in sarcoidosis patients may be associated with worse clinical outcomes for patients [180]. Peripheral lymph nodes display granulomatous inflammation in approximately 12-23% of patients [47, 48, 54, 181, 182], though one study did present a much higher proportion of patients with peripheral lymph node involvement (73%) [125]. Care must be taken in associating peripheral lymphadenopathy with sarcoidosis, rather than granulomata arising from other inflammatory activity [181]. As previously mentioned, mediastinal bilateral lymphadenopathy is characteristic of sarcoidosis and present in a much larger proportion of patients compared with peripheral lymph nodes [1, 47].

The bones and joints are not commonly affected by sarcoidosis in patients, ranging from 0.5-14% [47, 48, 125]. Arthritis presents in anywhere between 15-25% of patients [183-185], either as acute, early onset arthritis or a chronic form [183], the former usually resolving well with a good outcome [186] and the latter being a rare but progressive manifestation [187]. Rheumatoid factor in the serum has been reported in as many as 38% of patients, though this may not be a specific indicator of arthritis [188]. Osseous lesions occur in fewer patients, less than 13% [189], and other bone manifestations present are uncommon amongst patients [184].

Patients with sarcoidosis are often fatigued and complain of muscle weakness that impacts the quality of life and overall health [190, 191]. Whilst symptomatic muscle

involvement, in terms of myopathy and other muscle pathologies, is stated to rarely occur [47, 191-194], biopsies of tissue suggest granulomata are present in 50-80% of patients and would indicate that the impact may be more profound [193].

The gastrointestinal and reproductive systems are rarely involved in sarcoidosis; the former usually affects the stomach, is present in less than 1% of patients and can be mistaken for other granulomatous diseases such as Crohn's disease [195, 196]. Organ systems like the reproductive systems, pancreas, thyroid and other glands are variably affected by sarcoidosis, but are usually rarely observed, occurring in less than 5% of patients [1, 47, 48, 197]. The ear, nose and throat (ENT) is affected in 5-10% of cases [47, 48], though this may be greater depending on the definition of what constitutes an ENT symptom.

1.3.9 Calcium Metabolism and the Kidney

Renal involvement is a complicated issue in sarcoidosis. Granulomatous presentation on kidneys (as determined by biopsy) has been demonstrably rare in patients, ranging from 0.7% to 6.6% in various studies [47, 48, 54, 125, 198-200]. However, altered kidney function and kidney dysfunction occurs in a much larger proportion of patients, in particular 40-62% of patients display some degree of hypercalciuria (elevated levels of calcium in the urine) [54, 200] and as many as 14% of patients present with calcium deposition (nephrocalcinosis) or kidney stones (nephrolithiasis) [198, 200], although other studies suggest lower levels of the latter [54, 199]. Interestingly, in one study, nephrolithiasis was associated with a chronic phenotype, but absent in acute presentation [54].

This represents a larger issue in sarcoidosis related to calcium metabolism. Hypercalcaemia (increased calcium levels in the blood) is less common than hypercalciuria, presenting in between 3.7-17% of patients depending on the study in question [47, 48, 125, 200]. There has been a great deal of interest in this, as some patients produce a greater level of circulating Vitamin D and are required to avoid excessive sunlight that would make the situation worse [201, 202]. This relates to the

increase in active sarcoidosis of the bioactive form of Vitamin D, known as calcitriol (1,25-dihydroxyvitamin D_3 or 1,25-$(OH)_2D_3$) [203]. Calcitriol is the active form of vitamin D, converted in the kidney from the inactive form, calcifediol, by the action of the enzyme 25-hydroxyvitamin D_3 1-alpha-hydroxylase (1α-hydroxylase), also known as cytochrome p450 27B1 (CYP27B1) [204, 205]. In healthy individuals, calcitriol is predominantly expressed within the kidney, though 1α-hydroxylase has been shown to be present in a large variety of other tissues including the skin and lymph nodes [206]. In sarcoidosis, some patients have shown increased levels of serum calcitriol related to hypercalcaemia [201-204, 207]. Further study has highlighted that macrophages can express the 1α-hydroxylase enzyme in granulomas, which is overexpressed leading to the increased conversion of inactive calcifediol to calcitriol [204, 205, 208-210]. In addition, this expression has been shown to be altered (in some studies) by interferon gamma (IFN-γ), an immune modulator highly expressed in granulomas, independent of the usual activator parathyroid hormone (PTH) [211-213]. It is often suggested that vitamin D has a large number of immunological roles, which are the focus of much research [214], though the exact mechanism of CYP27B1 regulation in sarcoidosis has yet to be fully elucidated. Of particular note, the increased activity of 1α-hydroxylase, increased calcitriol production and subsequent hypercalcaemia has been associated with a chronic disease course and the need for treatment intervention in patients [207]. However, for some this hypercalcaemia can be resolved with the removal of vitamin D supplements, reduced exposure to sunlight or low vitamin D diets [201, 215]. This increase in calcitriol is also not associated with the relative severity of the disease or Scadding Staging [207], suggesting that different clinical features of the disease may be better associated with specific clinical indicators.

1.3.10 Quality of Life, Depression and Anxiety

Chronic disease can often have a profound impact on the mental health of patients [216-218]; in that respect sarcoidosis is no different. Interestingly, a study reported that stress may be a trigger for the disease [219], suggesting that psychological factors may even influence onset as well as be symptoms of sarcoidosis. A number of studies have looked at psychological factors in sarcoidosis, usually concluding that the overall rates of

depression and anxiety are higher in patients compared to healthy controls [46, 220-224]. The rates of depression amongst patients differs depending on the study, due in part to the method used to assess the depression and the severity of the disease in the selected cohort [220, 223, 225]. This depression and anxiety is associated mostly with physical symptoms, such as reduced lung function, sleep problems and fatigue/lethargy, and tends to be greatest amongst those with more severe symptomatic sarcoidosis [46, 220-223]. Inevitably perhaps, patients will report a reduced quality of life associated with the disease [221, 223, 224, 226]. As these psychological conditions can require a great deal of resources from healthcare providers, this aspect of sarcoidosis should not be overlooked when assessing patient wellbeing [225, 227].

1.3.11 Serum Angiotensin Converting Enzyme (sACE/serum ACE)

Serum Angiotensin Converting Enzyme (sACE/serum ACE) has long been associated with sarcoidosis [228] and is considered the prototypical indicator of the presence of the disease. This is contentious however, as whilst increased serum ACE levels can be indicative of sarcoidosis, not all patients will present with elevation of this protein and not all individuals with elevated ACE will have the disease [229-232]. The utility of this protein for diagnostics, defining the state of the disease or tracking disease progression is a matter of some debate [10].

ACE converts angiotensin I to angiotensin II, the latter acting as a vasoconstrictor, hence the targeting of ACE with 'ACE inhibitors' in the management of those with increased blood pressure [233-235]. The enzyme also breaks down bradykinin, which is a vasodilator, thus aiding in overall constriction of the blood vessels [233, 236]. Depending on the cohort, approximately 50-60% of patients present with elevated levels of ACE [231, 232, 237], though this appears to not necessarily correlate well with lung radiographic staging or disease progression [237-239]. Granulomata are the purported source of this ACE [240, 241], as epithelioid cells have been shown to be the cell producing the protein [241] and the serum ACE is associated with the degree of granulomatous burden systemically and locally [232, 242-244]. It is also suggested that this relates to extrapulmonary involvement, whereby sACE is elevated more when there are additional

organs involved in addition to the lungs [244]. Black patients tend to have a higher sACE level than Caucasian patients [231]. There are issues using the serum protein levels for diagnosis, as the specificity and sensitivity of ACE levels are not enough to exclude other diseases, such as tuberculosis or hypothyroidism, without confirmation by histopathology [230, 231, 245]. This sensitivity and specificity increases if only active disease is included [231, 246], or if the gene insertion polymorphism (I/D), which accounts for a great deal of variance in ACE levels, is applied to the test [230, 247].

The insertion/deletion genotype has been associated with elevated serum ACE, wherein homozygous II will produce less sACE than heterozygous ID and DD homozygous genotypes produce the most sACE [248, 249]. The distribution of the genotypes do differ between racial groups, but generally studies have found no significant difference in the distribution of these genotypes between healthy controls and patients within each racial group [248-251]. The I/D polymorphisms also do not appear have prognostic value [249, 250], though 1 report found that ID or DD conferred a worse prognosis than II in their cohort in Finland [251] and another found that Black patients specifically had a greater risk of progression if the II genotype was present [252].

1.3.12 Haematological Symptoms

Reported sarcoidosis involvement in the bone marrow is considered relatively rare as a manifestation, between approximately 4-10% of patients display this [47, 48, 253]. Despite this, a higher proportion of patients will present with anaemia (approximately 13-31%) [125, 253-255], though anaemia is more likely if the patient has bone marrow involvement [253]. A range of other haematological changes can occur in sarcoidosis, though these are not specific or consistent enough for diagnostic use [1]. Conditions such as leukopenia (reduced leukocyte number), lymphopenia (reduced lymphocyte number), thrombocytopenia (reduced platelets), changes in serum globulin levels and eosinophilia (increased eosinophilia counts) are some of the changes observed [125]. Of these, leukopenia and lymphopenia tend to be present in up to 55% of patients [254-256], typically associated more with a lymphocyte depletion rather than other leukocyte types

[257, 258]. Work also suggests that this reduction in lymphocytes is more pronounced in patients with active disease than asymptomatic conditions [259].

1.4 Immunopathogenesis

1.4.1 Summary of the Immunopathogenesis

A key issue associated with research into sarcoidosis stems from the lack of a known aetiology and the poorly understood immunopathogenesis of the disease (Section 1.6 Aetiology) [1]. This is a self-reinforcing issue; without an aetiology the initiation processes cannot be placed in context, a lack of understanding about the pathogenesis prevents an elusive cause being established. As such, various proposed mechanisms have been suggested for the immunopathogenesis of the disease, though a predominant general model is considered the prevailing paradigm of disease/granuloma progression [3, 260, 261]. This consists of 4 broad phases (summarised in Table 1.5): Initiation, Accumulation, Effector and Resolution [3, 260]. There is some variation in the specifics for all phases depending on the reviewer, though a general outline for sarcoidosis is provided in Figures 1.6, 1.7, 1.8 and 1.9. The lack of an animal model of sarcoidosis itself means that other granuloma causing infectious models, such as those observed in tuberculosis and leprosy, are used to best understand the development of granulomata *in vivo*. It should be acknowledged that there may be significant differences between these infectious models and non-infectious sarcoidosis granuloma development.

Table 1.5: Summary of the phases involved in the immunopathogenesis of sarcoidosis.

Phase	Overview
Initiation	Tissue macrophage activation – possible processing of an unknown antigen Interaction of macrophages with T-lymphocytes T-lymphocyte priming.
Accumulation	T-lymphocytes and macrophages release early pro-inflammatory cytokines and chemokines. Additional leukocytes are recruited to the tissue. Initial formation of granulomata structures. Initial differentiation of macrophages to histiocytes. TNF-α release - granuloma dependent on the cytokine.
Effector	Granulomata formed of epithelioid cells, giant cells and histiocytes and lymphocytes. Dynamic turnover of leukocytes in the granuloma, reinforcing inflammatory process. Release of significant quantities of TNF-α, IFN-γ, IL-6 and other pro-inflammatory mediators. Generation of new granulomata.
Resolution/Fibrosis	Reduction in pro-inflammatory cytokine release. Reduction in granulomata. Resolution – IL-10 and Th2 cytokines mediate. Fibrosis – TGB-β and Th2 cytokines mediate.
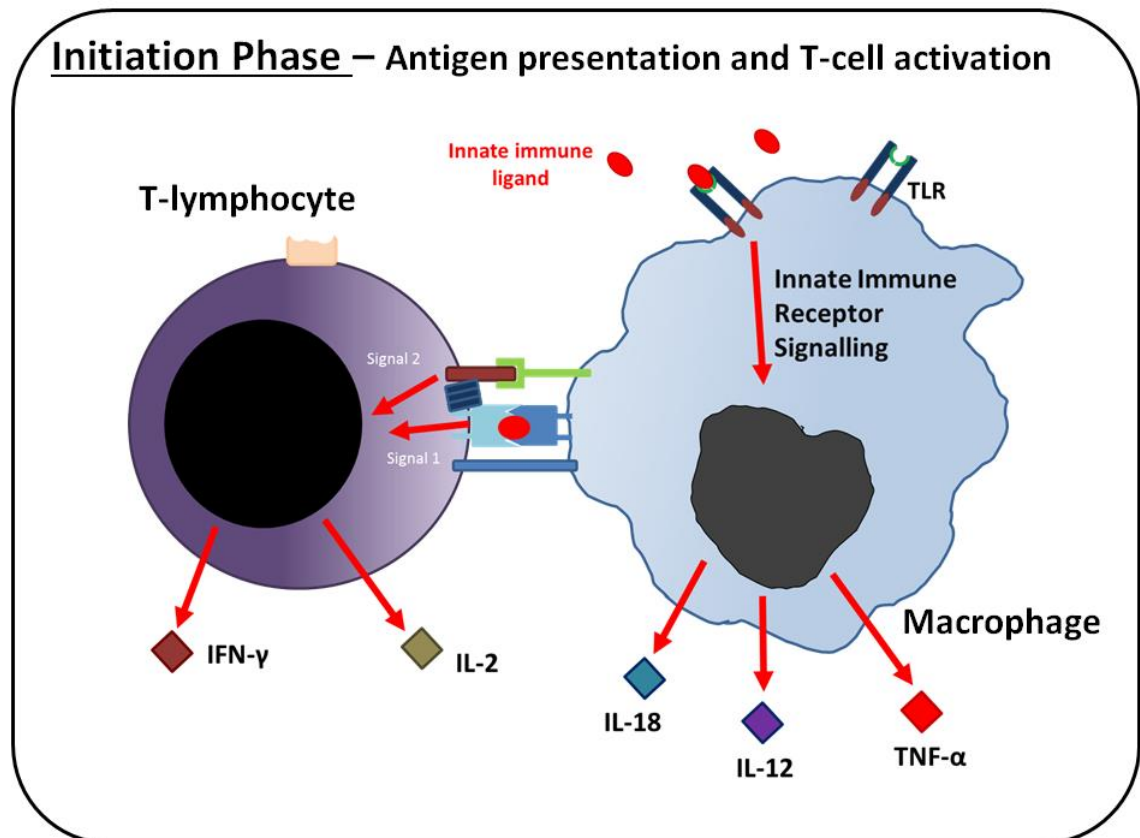
Figure 1.6: Summary of the initiation phase in the immunpathogenesis of sarcoidosis.

The initiation phase is believed to involve antigen presentation by macrophages to T-helper cells, with activation of pattern recognition receptors in innate immune responses. This leads to macrophage IL-12, IL-18 and early TNF-α release and T-lymphocyte IFN-γ and IL-2 release.
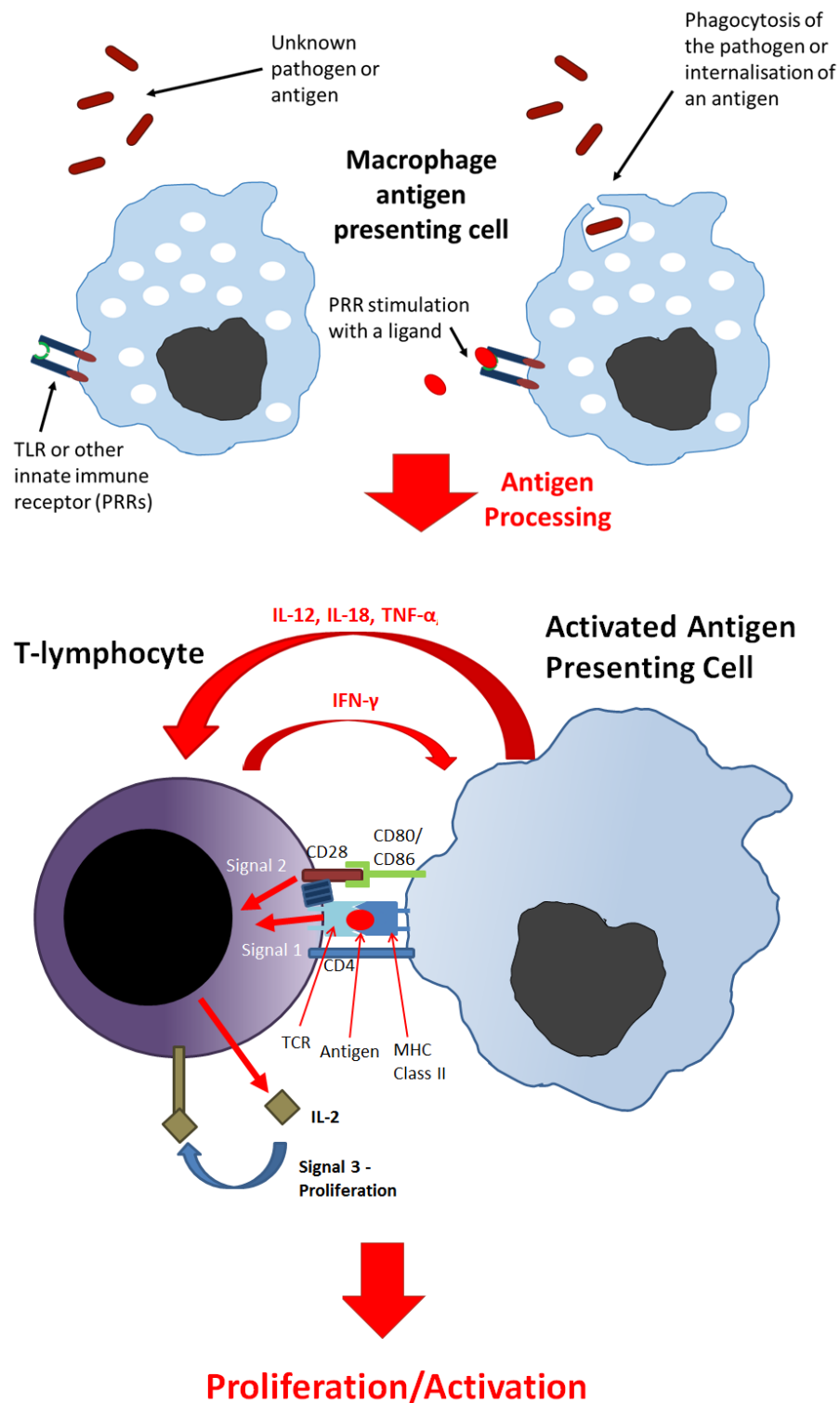
Figure 1.7: Initiation of granuloma development through antigen presentation.

In tissue, macrophages phagocytose an unknown antigen, with possible additional activation of the innate immune receptors (pattern recognition receptors – PRRs). These macrophages present processed antigen to CD4+ T-lymphocytes via the MHC Class II (HLA Class II). Macrophages release T-lymphocyte stimulating factors IL-12 and IL-18, which in turn leads to release of IFN-γ and IL-2 in T-lymphocytes that lead to macrophage activation and T-cell mitotic expansion respectively. The inflammatory signal is dominated by TNF-α, which aids in the development of granuloma.

1.4.2 Initiation

The current concept for initiation suggests that an unknown antigen stimulates an initial immunological response from resident tissue macrophages, which phagocytose, process and present the antigen on MHC (major histocompatibility complex) class II molecules (Figures 1.6, 1.7) [3, 260]. Macrophages also release T-lymphocyte stimulating inflammatory cytokines, such as IL-12 and IL-18 [262], and express co-stimulatory molecules necessary for T-lymphocyte activation [263-265]. In some models, this activation of macrophages is reinforced by stimulation of the 'Pattern Recognition Receptors' (PRRs) [266, 267], such as the 'Toll-like Receptors' (TLRs) or 'NOD-like Receptors' (NLRs – NOD receptors). These PRRs recognise either pathogenic or damage molecules that can be recognised as foreign/aberrant, known as 'Pathogen Associated Molecular Patterns' (PAMPs) or 'Damage Associated Molecular Patterns' (DAMPs), and initiate an innate immune response accordingly [267]. These PAMPs and DAMPs are present and have a role to play on both macrophages and T-lymphocytes [266]. TLR2 is considered important in sarcoidosis, as it is highly expressed by monocytes and macrophages; stimulation of the receptor leading to enhanced inflammatory responses in the disease [266, 268, 269]. Under existing models, it is suggested that macrophages (or dendritic cells) presenting an antigen activates the T-lymphocytes, polarising them and sparking the initiation of inflammation [270]. This antigen-dependent mechanism is consistent with the findings that preparations of nonviable autologous bronchoalveolar lavage (BAL) cells from sarcoidosis are capable of initiating a specific granulomatous response in patients, but not healthy controls [271]. A similar patient response has been observed using the Kveim-Siltzbach reagent (Section 1.6.6) [20, 37], suggesting that an antigen presentation pathway is possibly involved in the pathogenesis. Alveolar macrophages in sarcoidosis display elevated CD72, CD80 and CD86 costimulatory/accessory molecules compared with controls and sarcoidosis BAL T cells show an increase in expression of the matching molecules (CD5 and CD28) necessary for T cell Receptor (TCR) mediated activation [272]. Blocking CD86, one of the accessory molecules necessary for TCR signalling, prevents granuloma formation in a pathogen-induced mouse model [273], which may indicate that a similar dependence on T-lymphocytes exists in human sarcoidosis development. Additionally, alveolar macrophages from sarcoidosis have an enhanced ability to present antigen compared

with healthy controls, which usually have a poor capacity for antigen presentation [274, 275]. Despite the antigen in question being unknown, it is thought that the initiation of granulomata arises as a result of poor degradation of the antigen, whether that be the result of an intrinsic deficiency in macrophage processing or the antigen itself being difficult to process [1].

Work on early stages of the disease indicates that interstitial pneumonitis precedes the formation of granulomata [276]. Histiocytosis has been observed prior to development of the granuloma, suggesting that macrophage-like cells are initial early activators of subsequent activity [277, 278]. After this, granulomata develop from this diffuse inflammatory pattern, with macrophages adopting epithelioid morphology and granuloma structures [276-278].
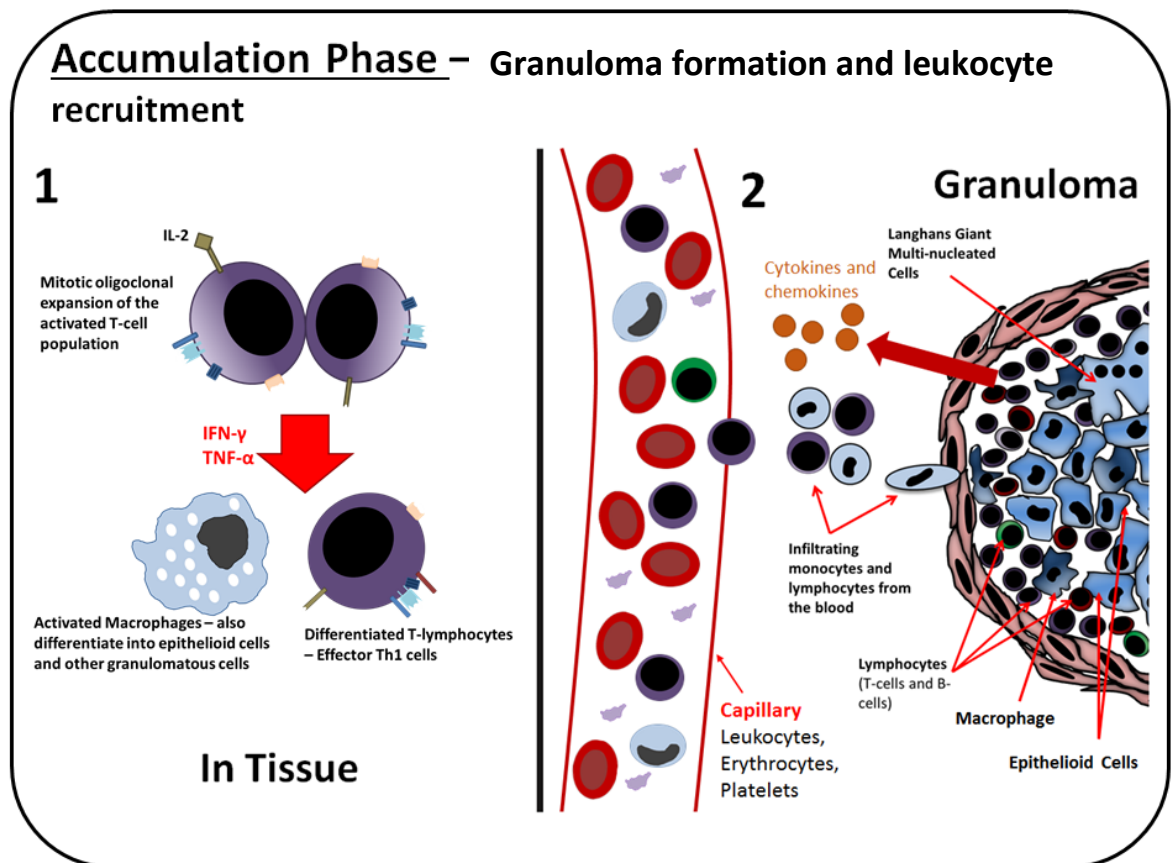
Figure 1.8: Summary of the accumulation phase in the immunopathogenesis of sarcoidosis.

1. IL-2 release leads to oligoclonal expansion of the lymphocytes and activation of resident macrophages. Subsequent TNF-α and IFN-γ release in tissue differentiates T cells to activated Th1-like T-lymphocytes, whilst macrophages also become active and also differentiate into the epithelioid, giant cells and other histiocytes. **2.** During this phase, there is chemotactic recruitment of leukocytes, some arriving from the blood, and the development of the granulomata. Adapted from [3].

1.4.3 Accumulation and Granuloma Formation

The accumulation phase comprises the T-lymphocyte and macrophage mediated recruitment of other leukocytes, the generation of the cellular components of the granuloma and maintaining the granulomatous form (Figure 1.9) [3, 260]. Pulmonary T-lymphocytes in sarcoidosis consist of large numbers of CD4^{+} T-lymphocytes [279], polarised towards the Th1 phenotype [280, 281], which helps drive forward the inflammation and granuloma development in the disease [1, 3, 282]. The ablation of T cell activity in murine models using radiation, knock-outs or athymic mice leads to a reduction in the integrity of granulomata, reduced formation of new granulomata and more extended infective cycles [283-285], suggesting that T-lymphocytes are a necessity for effective granuloma initiation and maintenance. Whilst the classic cell types within a granuloma (epithelioid, giant cells and macrophages) have been generated from blood derived monocytes experimentally [286-288], it is still not fully established whether these cells *in vivo* derive from resident macrophages or are reinforced by peripheral blood leukocytes. A schistosomal model in mice suggests monocytes are a reinforcing cell type that differentiate into macrophages in granulomata for that disease [289], a concept in keeping with the model of alveolar macrophage development from monocytes [290, 291]. In Bacillus Calmette-Guérin (BCG) induced granulomata in animal modelling, monocytes do translocate from the blood to the site of inflammation and undergo mitotic division [292]. The monocyte derived cells are relatively short lived and mitotic division is limited unless there is a persistence in an active stimuli, such as a pathogen [292]. However, fate mapping suggests that resident macrophage replication is not associated with circulating monocytes [293], though this does not necessarily mean that infiltrating leukocytes in infection are necessarily homogeneous in origin.

TNF-α (tumour necrosis factor alpha) is considered vital in the development of granulomata. In bacterial induced granuloma, antibody blocking of TNF-α disrupts granulomatous formations and leads to reduction in the number of granulomata [294]. Similar interference with the process of granuloma formation and integrity is observed using TNF-α blockade in a tuberculosis model, where lung damage as a result of mycobacterium increases [295]. Tissue staining of granulomata in sarcoidosis reveals that the principle source of this TNF-α to be macrophages (CD68^{+} cells in tissue) [296]. A large

number of other studies have also highlighted the importance of TNF-α in the formation of the granulomata, revealing that in its absence the granuloma structures are poorly formed, monocyte/macrophage cells do not differentiate into epithelioid or giant cells and inflammation/infection tends to be diffuse across tissue [297-301]. It also appears that TNF-α is important in initiating chemotactic leukocyte cell recruitment to the site of inflammation, where mice deficient in the cytokine suffer from delays in lymphocyte response [300]. Overall, the importance of macrophage activity, initial T-lymphocyte priming and TNF-α in granulomata cannot be understated, as these appear to be absolutely vital to granuloma development.

1.4.4 Effector Phases and Sustaining the Granuloma

During the effector stage (Figure 1.9), polarised T-lymphocytes continue to promote chemotactic recruitment of leukocytes, whilst both they and the macrophage-derived cells (histiocytes) within the granuloma release inflammatory cytokines, which promote the ongoing disease and maintain the granulomata [3]. Epithelioid cells and giant cells differentiate from the macrophages, stimulated by the inflammatory environment [302]. The term "high turnover" has been applied to sarcoidosis granulomata [302], defined by a dynamic system of high cell death/apoptosis/lymphatic drainage matched by high mitotic division and cell recruitment to the granuloma. This is supported when comparing the rate of apoptosis compared with mitosis of resident cells in the granulomata in sarcoidosis, the former being higher than the latter, suggesting that granulomata require cellular reinforcement by peripheral leukocyte populations [303].

Just as TNF-α is vital in the formation of the granuloma, the cytokine is also necessary for maintaining the integrity of these inflammatory cell structures [295, 298-301]. In mouse models, TNF-α deficiency leads to reduced viability of animals to infection and poorly formed granulomata, with inflammation being disseminated throughout the tissue [297-301]. Additionally, IFN-γ appears to be important in the maintenance of the inflammation in granulomata. In BCG murine models there is a synergistic impact when TNF-α and IFN-γ are present that is diminished in the absence of one or both of these cytokines, though the absence of IFN-γ seems to be less deleterious than the absence of TNF-α [298].

Comparing the roles of IFN-γ to TNF-α in granulomata, TNF-α appears necessary for granuloma formation and maintenance, as well as controlled inflammation, though it does not appear to exclusively lead to mycobacterial killing [301]. Conversely, IFN-γ is an absolute requirement for potent mycobactericidal activity within granulomata, its absence leads to increased mycobacterial load, though the core structure of a granuloma can form without the presence of this cytokine [301]. The main conclusion would be that both are required for granuloma maintenance, though TNF-α would appear to be the principle cytokine responsible for the granuloma itself. Whether or not this applies in sarcoidosis is difficult to ascertain without a direct comparable model, though the current concept is that it is similar to those models for TB and BCG.

This stage is characterised by the continuation of recruitment of leukocytes to the tissue and reinforcement of inflammatory cytokine release. Key cytokine mediators are released, such as TNF-α, IFN-γ, IL-12, IL-2, IL-6 and GM-CSF (granulocyte-macrophage colony-stimulating factor) [3, 304, 305], as well as Th17 related cytokines IL-17 and IL-23 [306, 307]. It is not known whether the persistence of this inflammation is the result of a continual stimuli, aberrant inflammatory immune receptor-ligand interaction or uncontrolled/deregulated immune response, nor is the process by which resolution occurs fully understood [1, 308]. In other granulomatous infective diseases, the clearance or persistence of the aetiological agent defines the immune response; this may not be comparable in sarcoidosis however.

1.4.5 Resolution or Fibrosis

Classically, the resolution phase is stratified into two alternative outcomes: Resolution/Remission and Fibrosis (Figure 1.9) [3]. The exact underlying mechanisms defining how the immune response elects the particular path are not fully understood [68]; although there has been a focus on TGF-β (transforming growth factor beta) and its role in late stage disease. TGF-β is an immunosuppressive, wound healing and pro-fibrotic cytokine that has a rather complex relationship with sarcoidosis, especially when compared with a disease such as idiopathic pulmonary fibrosis (IPF) where the cytokine is demonstrably increased in patients [309]. One study correlated resolution with higher

TGF-β levels, whereas active disease without this cytokine was associated with a progressing disease phenotype in patients [310]. Another report found no significant difference in the increase of the cytokine from patient alveolar macrophages compared with controls, though patients with compromised lung function did show increased TGF-β relative to those with limited lung damage, suggesting that the factor may contribute to fibrosis in sarcoidosis [311].

Another immunosuppressive cytokine with a role in sarcoidosis pathology is IL-10, released in large quantities by specific activated T regulatory lymphocytes (Tregs), as well as Th2 cells and B-lymphocytes, to control Th1 immune responses [312]. There has been conflicting data regarding this cytokine, with tissue mRNA expression of IL-10 shown to be comparable between control and patients with active sarcoidosis in the lungs [280], but BAL alveolar macrophages spontaneously release more IL-10 in the disease [313]. This is important, as the cytokine and Tregs have been variously associated with sarcoidosis pathology, suggested to be a possible source of dysfunction allowing the chronic course to continue [23, 314] and possibly be necessary for immunosuppression and resolution of the inflammation [315].

Based on existing evidence, the current paradigm suggests that TGF-β may be vital to the remission of the disease for those retaining a Th1 disease profile, whereas progressive fibrotic disease occurs when there is a shift from Th1 to Th2 in patients, supplemented by TGF-β which then leads to the fibrotic phenotype [1, 316]. Noor and Knox [261] present a hypothetical model in which earlier events, particularly in Th1 suppression by TGF-β and IL-10 will resolve the disease, whereas the inability to achieve this will lead to elevated Th1 responses, followed by a chronic course with eventual fibrosis after Th2 class switch. However, what cannot be fully explained is why some patients will undergo a chronic course, sometimes for years, without subsequent fibrosis, whereas others may develop this early or late in the disease. The consensus is that this area requires a great deal of further investigation [1].
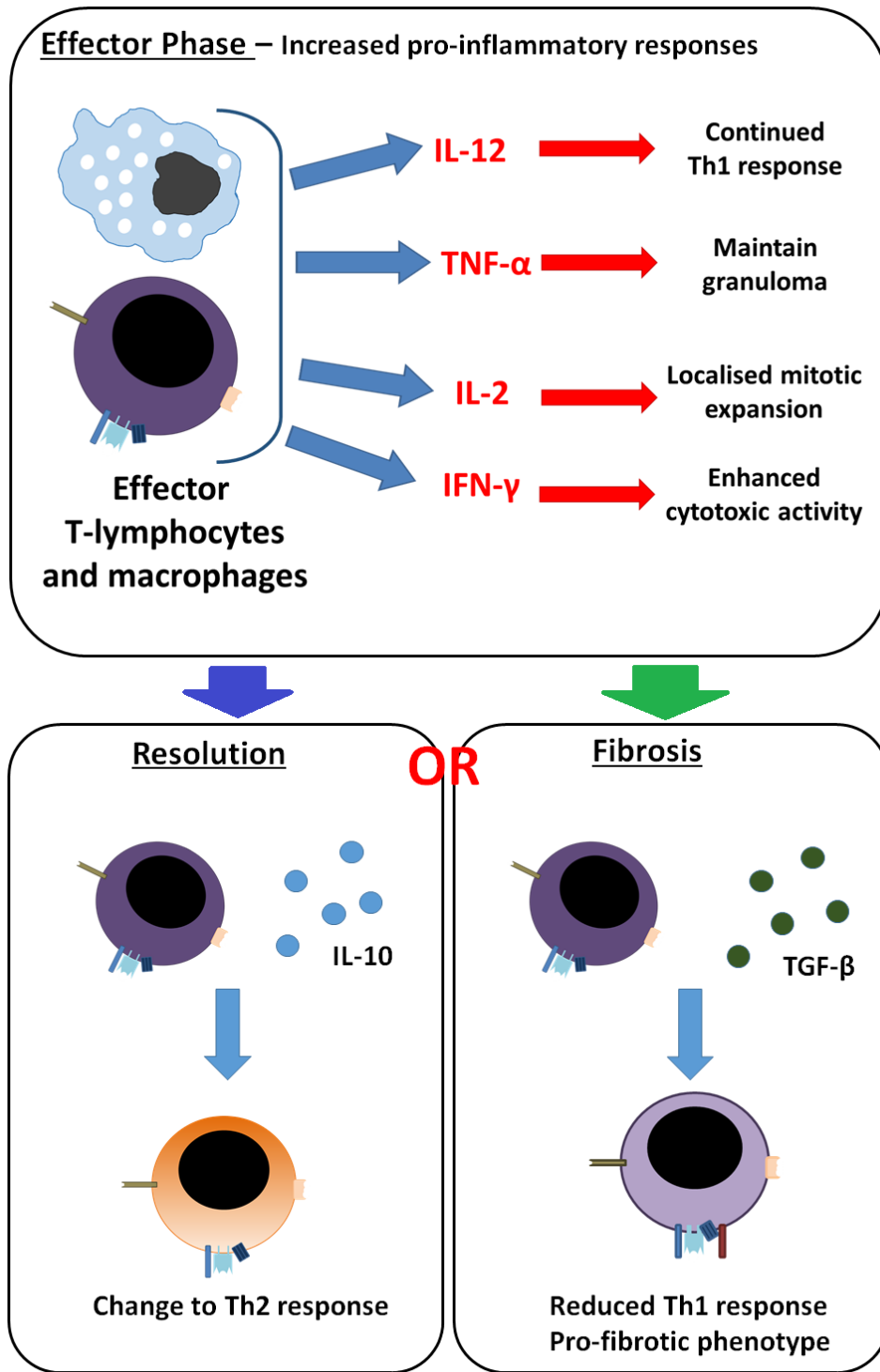
Figure 1.9: Summary of the effector and resolution/fibrosis phases in the immunopathogenesis of sarcoidosis.

The effector stage is defined by high pro-inflammatory cytokine release and promotion of the Th1 immune response which can lead to chronic disease in some cases. The combination of IFN-γ, IL-12, IL-2 and TNF-α drives the inflammation, expands the population and sustains the granulomata. The disease can undergo resolution (IL-10 mediated) or fibrosis (TGF-β mediated); the mechanism by which an outcome is still enacted is not fully understood. Adapted from [3].

1.4.6 The Cytokine Profile in Sarcoidosis

Part of the difficulty in fully beginning to treat and deal with this disease stems from the apparently contradictory evidence and features it displays. This is particularly evident when identifying the immune processes and cytokine profile. What has been described is that, at the site of granulomatous inflammation, numerous cytokines and other immune modulators are overexpressed [304, 305]. Generally speaking, sarcoidosis has been considered a Th1 centred immune disease [280, 281, 317]. IL-1, IL-6, IL-12, TNF-α and Interferon gamma (IFN-γ) are hallmarks of this, overexpressed within granulomata and responsible for the chemotactic infiltration of Th1 T-lymphocytes to the site of inflammation [12].

The terms 'lymphokine' and 'monokine' are a little outdated now, but had previously been descriptors of cytokines released from lymphocytes and monocyte/macrophages respectively, before 'cytokines' became the generalised term; many cytokines were found to be released by multiple cell types [318, 319]. Despite this, some cytokines are associated more with specific cell types than others, thus TNF-α, IL-6 and IL-1 could be termed monokines, as they are predominantly released by monocyte/macrophages [318]. By contrast, IL-2 and IFN-γ are most associated with T-lymphocytes [318].

Whilst analysis of BAL tends to struggle with consistency, it has shown, for example, that IL-2 expression is different in granuloma cells and peripheral blood [320, 321]. BAL from patients have tended to show elevated cytokine concentrations compared with controls, including IL-1, IL-2, IFN-γ, TNF-α, IL-6, GM-CSF and also IL-8 [16, 280, 317, 322]. Isolated BAL cells have also been shown to spontaneously release greater concentrations of IL-1β, IL-6, TNF-α, IFN-γ, GM-CSF and neopterin *in vitro* for sarcoidosis patients [18].

One group reported that serum IFN-γ was higher in sarcoidosis patients than healthy controls [16, 17]. However, when peripheral blood mononuclear cells (PBMCs) were isolated and cultured, there was not a significant spontaneous release from leukocytes in either controls or patients and stimulation with phytohaemagglutinin (PHA), described classically as a T cell activator, showed a reduced response in sarcoidosis patients [17]. Similar results were obtained by another group with PBMCs and PHA, supporting the

finding that PHA induced activation of IFN-γ from PBMCs is lower in sarcoidosis, though this group attributes the reduction to macrophages [25]. Sarcoidosis patients in one cohort had higher concentrations of IL6, TNF-α, IFN-γ and IL-12 in plasma compared with healthy controls, though the mean IL-10 level was identical between the two groups [24]. In another study, isolated and untreated PBMCs were capable of spontaneous release of IL-1β, IL-6, TNF-α, and GM-CSF, all higher in sarcoidosis patients compared with controls, though neopterin release was lower from patient PBMCs [18]. This contrasts greatly with another study, which found serum IL-1β, IL-8, TNF-α, IFN-γ and GM-CSF were not significantly different between patients and controls, but IL-10 and soluble CD23 were elevated in sarcoidosis serum [26]. The majority of the studies mentioned here recruited patients who were not initially undertaking corticosteroid therapy [16-18, 24, 25]. The one study which included a group of patients on therapy reported no significant difference between cytokine release in patients taking steroids and those not taking an immunosuppressive therapy [26]. However, patients with sarcoidosis clearly showed a reduction serum IFN-γ levels with corticosteroid treatment in the study by Prior and Haslam [16]. The differences between the reports may result from patient cohorts under study or methodological strategies. The often contradictory reports and differences between serum and PBMC cytokine release profiles have been problematic in developing a clear picture of peripheral leukocyte immunity.

Serum levels of soluble IL-2 receptor (sIL-2R) appear to be a good prognostic marker defining patients at risk of progression, being elevated in specific patients [24, 239]. Whether this is as a result of the elevated IL-2 observed in tissue is unclear, though both this and serum Th1 associated cytokines shown to be elevated in sarcoidosis suggest that there are systemic peripheral blood effects related to the disease. Th2 cytokines, such as IL-4 and IL-5, have been demonstrably low in BAL fluid in sarcoidosis [322], in keeping with the Th1 profile usually assigned to the inflammation. There has been deviation from the conventional Th1 sarcoidosis paradigm, with research suggesting that a more accurate description would be to describe the disease as a Th1/Th17 disorder [306]. This is far from comprehensive, as different stages of the disease present differently, as do different groups and individuals, thus the full cytokine network is a complex feature of the disease (for more in depth description of cytokines in sarcoidosis, see reviews [304, 305])

A large number of chemokines are proposed to be involved/elevated in sarcoidosis, including IL-8, macrophage inflammatory protein 1 alpha (MIP-1α), CCL5/RANTES and monocyte chemoattractant protein-1 (MCP-1) related to neutrophil and myeloid cells [323, 324], as well as IFN-inducible protein 10 (IP-10) and thymus-and-activation-regulated chemokine (TARC) which relate to T-lymphocytes [325] (for a review, see [304]). In keeping with a running theme, it has been found that the Th1 T-lymphocyte chemoattractant IP-10 is elevated in BAL in sarcoidosis, but not peripheral blood, whereas the Th2 T-lymphocyte chemoattractant TARC is elevated in blood but not BAL [325]. This is consistent with the previous cytokine analyses presented, of local elevation in inflammatory immune response with limited peripheral inclusion detected. Given the large array of chemokines and chemokine receptors [326], this may be an area that requires greater attention in sarcoidosis, where the cytokines have been the principle focus of research.

1.4.7 Monocytes/Macrophages

Monocytes and macrophages are vital components in both innate and adaptive immunity, capable of a wide range of activity, including phagocytosis of pathogens, pro-inflammatory cytokine release, antigen presentation, immune surveillance, cell proliferative activity, wound healing and immune regulation [327, 328]. Macrophages are a heterogeneous group [328]; previously considered to consist (in some work) of two broad subsets, 'classical' and alternative' macrophages, polarised into M1/M2 phenotypes equivalent to Th1 and Th2 T-lymphocytes respectively [329-331]. However, this is based more upon the polarisation of monocyte derived macrophages (MDMs) to specific stimuli, cytokine release of the macrophages and associations with the Th1/Th2 paradigm, itself an oversimplification of T-lymphocyte heterogeneity [332, 333]. Macrophages tend to be more heterogeneous and less binary when the full milieu of tissue macrophage subsets are considered and their differential phenotypes explored more comprehensively [327, 328, 332, 334]. Therefore, macrophages will be considered without the M1/M2 designation, but individual tissue macrophage types, such as interstitial macrophages and alveolar macrophages, will be addressed specifically.

Monocytes can be divided into classical CD14++/CD16-, non-classical CD14+/CD16++, and intermediate CD14++/CD16+ cell populations [335]. In humans the vast majority of monocytes in blood are classical, up to 90% of the monocyte population, with intermediate being the smallest population [336]. The precise role of each subset has not been fully elucidated, though non-classical populations have been shown to be increased in inflammatory conditions [337]. There has been some evidence that the intermediate population may be expanded in sarcoidosis [336, 338], though the significance of this and the role of intermediate monocytes requires further study.

As sarcoidosis is generally considered a disease of elevated local tissue immune response combined with peripheral immune anergy [1, 3, 10, 23, 308], the majority of work has concentrated on macrophages rather than monocytes, typically alveolar macrophages rather than interstitial or other types. Alveolar macrophages (AMs) appear to be capable of spontaneous release of TNF-α, greater in sarcoidosis patients than healthy controls and greater in active disease than inactive disease [239, 272, 296, 339-342]. Patients who are under treatment with corticosteroids show reduced TNF-α release from AMs [339] and there is a correlation between high TNF-α release from AMs and progressive disease [239]. Granuloma Immunohistochemisty (IHC) staining reveals that TNF-α is mainly associated with macrophages in sarcoidosis, not other cell types (Figure 1.9) [296]. Other cytokines are also released/produced to a greater extent from/inside alveolar macrophages in sarcoidosis, including IL-1 [340-342], IL-6 [341], IL-12 [280] and IL-15 [272]. What is also interesting to note is that IL-15, a cytokine with overlapping roles in immune activation as IL-2, is released significantly from sarcoidosis alveolar macrophages and can induce CD28 upregulation and IFN-γ production in sarcoidosis T-lymphocytes, indicating this cytokine may play an important role in the pathogenesis of the disease [272].

Depending on the stimulant used to activate macrophages, enhanced cytokine release has been observed in sarcoidosis AMs. Lipopolysaccharide (LPS) is capable of increasing the release of TNF-α, IL-6 and IL-1, which tends to be greater from sarcoidosis AMs than healthy controls [340, 341]. Other activators may display different results, depending upon the mode of action of the ligand. Alveolar macrophages from active disease also display increased intracellular β-galactosidase activity and adhesion properties, though

these are not correlated with serum ACE levels [343]. Spontaneous release of the anti-inflammatory IL-10 is detectable in alveolar macrophages, and is elevated more in sarcoidosis patients than healthy controls in LPS induced activation, though it should be noted that monocytes released more IL-10 than alveolar macrophages [315]. Monocytes from peripheral blood do not release significant concentrations of TNF-α spontaneously [340], stimulation with LPS has previously been shown to not generate significantly greater concentrations of TNF-α from sarcoidosis cells [340]. The conclusion would appear to be that peripheral monocytes appear to not be 'primed', unlike tissue macrophages which are inflammatory or dysregulated in nature.

1.4.8 T-lymphocytes

T-lymphocytes are broadly separated into a variety of subsets, including CD4+ T-helper cells, CD8+ cytotoxic T lymphocytes (CTLs) and T regulatory cells (Tregs). They can also be naïve or memory T cells. CD4+ cells are also polarised upon activation to Tregs (Th0 or innate), Th1 (cell mediated immunity), Th2 (humoral immunity), Th17 (inflammation and pathogen immunity) and a range of other possible phenotypes [344]. The previous assignment of sarcoidosis as a Th1 immune disease may be an oversimplification [333], as while the disease displays a strong Th1 polarisation [280, 281, 317], Th17 and Tregs appear to also play a critical role in the disease pathogenesis [23, 306].

Much the same as macrophages, T-lymphocytes have been described as more responsive or hyperactive in sarcoidosis than in healthy controls in specific conditions. BAL T-lymphocytes have been reported to spontaneously release cytokines such as IL-2 and IFN-γ to a greater extent in sarcoidosis, though peripheral blood T cells are not associated with similar responses [272, 345, 346]. T-lymphocytes from BAL stimulated with phorbol myristate acetate (PMA) and a calcium ionophore (for stimulation) have been shown to produce greater concentrations of IFN-γ, TNF-α and IL-2 compared to healthy controls, both CD4+ and CD8+, whereas there was no significant difference in peripheral blood T-lymphocyte cytokine production [281]. Whilst it is usually typical for TNF-α to be associated with the macrophages in sarcoidosis [239, 340], T-lymphocytes are clearly capable of releasing the cytokine upon stimulation [281], though PMA/calcium ionophore

treatment may not be physiologically appropriate for this determination, as tissue appears to attribute TNF-α release to macrophages [296].

The ratio of CD4$^+$ to CD8$^+$ T-lymphocytes in BAL has been considered a defining feature of sarcoidosis, separating it from other interstitial lung diseases such as idiopathic pulmonary fibrosis [1, 322]. Generally, sarcoidosis BAL lymphocytes display a high ratio of CD4:CD8, above 4 in many cases (four CD4$^+$ cells for every CD8$^+$ cell) [322, 347]. This can be variable however, and not all patients will display such a distinguishing feature in BAL [348]. Acute patients tend to have a large percentage of T-lymphocytes in BAL and a high CD4:CD8 ratio in one study [349], whereas a different report suggests that patients with improving pulmonary symptoms have larger CD4:CD8 ratios in sarcoidosis [350]. However, patients will tend to have more T-lymphocytes locally in the disease as a percentage of the BAL populations than healthy individuals [68, 347]. This all contrasts greatly with peripheral lymphocyte populations, where sarcoidosis patients are commonly lymphocytopenic [257].

T regulatory cells (Treg) are expanded as a population both locally (in BAL) and in peripheral blood in patients with active sarcoidosis and are capable of anti-proliferative activity [23, 351]. They are not able to completely prevent TNF-α and IFN-γ release, whereas healthy control Treg cells are [23], though the cells appear to have a reduced survival capacity compared with controls [351]. The Tregs have been shown to not directly correlate with disease activity, but may be a preventative mechanism in early disease, supported by the evidence that depletion of Tregs in active sarcoidosis did not influence *in vitro* granuloma formation, whereas it did increase the speed of granuloma generation in inactive sarcoidosis patients [352]. There is a suggestion that Tregs play a role defining whether sarcoidosis develops a fibrotic phenotype or resolves [352].

1.4.9 Other Leukocytes

T-lymphocytes and macrophages are the principle cells of interest in sarcoidosis, as these make up the vast majority of local tissue granulomata. However, other leukocytes such as dendritic cells, natural killer cells, neutrophils and B-lymphocytes have also been studied

in the disease, suggesting that these leukocytes may play important roles in the pathogenesis [353-357].

Neutrophils have previously been considered acute inflammatory cells with ephemeral activity early in the process, though it is apparent that they may be longer lasting participants in inflammation [358]. Neutrophils have been shown to be elevated in the BAL of patients with sarcoidosis, with higher neutrophil counts associated with a worse prognosis [356]. Löfgren's syndrome or limited lung involvement presents with fewer BAL neutrophils, whereas more progressive or extensive disease will have a higher neutrophil count [355]. It is also a feature of progressive disease that patients will display a higher neutrophil to lymphocyte ratio in serum relative to less severe disease presentation [359, 360], suggesting that neutrophils may feature in continuing inflammation. It should be noted that the lymphopenia may explain the increased neutrophil to lymphocyte ratio values in sarcoidosis, as increased raw granulocyte/neutrophil cell numbers in blood are not considered a feature of the disease [257, 258].

Natural killer cells (NK cells) are best known for their anti-tumour activity, though the cells are potent cell-mediated innate immune cells with a variety of roles [361]. Some reports have identified that NK cells are either reduced or not significantly different in number in sarcoidosis patients compared with healthy controls in both BAL and peripheral blood [354, 362]. It is notable that a population of NK cells with enhanced pro-inflammatory activity is associated with sarcoidosis in tissue [354].

Dendritic cells (DCs) are the professional 'antigen presenting cells', a group of diverse subsets with immune functions overlapping with those of macrophages [363]. Whilst peripheral blood DCs have been described as anergic and display a reduced number in sarcoidosis patients [24, 353], tissue dendritic cells may be elevated in number with a greater capacity for activity [353, 364]. Given the number of different subsets of dendritic cells, it is clear that there is a great deal more to this cell type that might be revealed in sarcoidosis.

B-lymphocytes (B-cells) are the principle cell involved in humoral immune response and release immunoglobulins/antibodies (as plasma cells), but also modulate immune

response, present and recognise antigens and release cytokines [365, 366]. B-lymphocytes have previously been assumed to be limited in number based on BAL studies in the lung in sarcoidosis [367, 368], though direct tissue staining suggests there are a large number present in the disease [369]. Peripheral blood B-cells in sarcoidosis tend to be regulatory, IL-10 producing cells [357] and naïve populations are more prominent in the disease [370]. Chronic patients also appear to have compromised peripheral B-lymphocyte activity, with cells less responsive to polyclonal stimulation [371]. B-lymphocytes in sarcoidosis have not been as intensively researched as T-lymphocytes, perhaps in part due to the overwhelming display of cell-mediated Th1 response and numerical predominance of T cells and macrophages/monocytes. This is another cell type that requires further investigation to ascertain whether B-cells play a greater role in immune responses in sarcoidosis.

1.5 Epidemiology and Genetic Factors

1.5.1 Incidence

As with other elements of the disease profile, the incidence, prevalence, distribution and prognosis of patients with sarcoidosis varies depending on a number of factors. Geographical regions, race, gender, age and genetics seem to all play a pivotal role. A number of extensive studies have been performed, including the ACCESS study which characterised 736 cases across the U.S., involving different regions of diverse patient types in a multicentre analysis [93]. Whilst the principle goal was to find a common set of factors responsible for the disease, be they aetiology or genetic predisposition, the data would also give an insight into the distribution amongst the general population [372, 373]. Other studies have large clinical data sets from a single region over a long period of time or retrospective analysis of clinical data, allowing longitudinal tracking of patient progression over time [48, 54, 95, 125, 374]. Defining the epidemiological features of sarcoidosis is problematic, as the variability in diagnostic assessment and variability in disease presentation leads to inconsistent analyses [375].

Due to the nature of the data collected in the ACCESS study, incidence rates could not be accurately assessed [373]; Rybicki *et al* estimated adjusted incidence rates range from 10.9 to 35.5 per 100,000 in white and black populations respectively in their report [95]. Another U.S. study placed the incidence at 6.1 per 100,000 people, though this was an entirely Caucasian population [376]. European cohorts vary significantly; a Swedish study found a mean incidence of 19 per 100,000 [377] and Finnish patients have been reported at 15 per 100,000 [378], whereas 7 per 100,000 is the incidence of sarcoidosis in Switzerland [379]. By contrast, the incidence in Spain is reported to be very low, 1.36 per 100,000 [380], whereas there is an abnormally high incidence on the Isle of Man in the United Kingdom of 14.7 per 100,000 [381], especially considering that the incidence on mainland Britain is estimated to be 5 per 100,000 [382]. This may be related to the screening of patients in a smaller population or more active health monitoring; the incidence prior to 1977 was 3.5 per 100,000, closer to mainland Britain [381]. This may indicate that the incidence might be higher in the UK but subclinical cases are not being detected, an issue which might explain some of the variable results being observed in European studies [375, 381].

In other countries other than those in Europe and the US, the incidence can vary dramatically. East Asian countries tend to report a very low incidence rate, such as Korea (0.125 per 100,000) [383] and China (0.027 per 100,000). Japanese cohorts are unusual in displaying much higher rates of sarcoidosis (1.01 per 100,000) and specific manifestations, such as a predilection for cardiac symptoms [131]. There are issues in establishing the incidence in countries with higher TB rates, as the symptoms may be masked by the condition or misdiagnosed, it is possible that the rate of sarcoidosis may be very high in countries like India [384]. Other countries from Africa and Asia report low incidence rates based on detection of the disease [385], except for South Africa which has defined 3.7 to 23.2 per 100,000 in Caucasian and Black patients respectively [386]. Most other countries tend to have an incidence higher than East Asian populations, but below Black populations [1].

A report from Gribbin *et al* looked at the incidence of sarcoidosis patients in the UK between 1991 and 2003, based on over 1000 patients [382]. The crude (non-adjusted) incidence was 5 per 100,000, with only a modest gender divide of 53%/47% of female to

male patients and a mean age of 47 years. Interestingly, the report identifies an increased rate within London, the West Midlands and Northern Ireland when stratified by region and the patients tend to have a good prognosis (7% death after 5 years), albeit worse than a comparison control cohort (4% death after 5 years) [382]. The regional variation is indicative of racial demographics of the region and is something the authors discuss, which would be in keeping with the numerous other reports of higher rates in certain racial groups [48, 95]. The lack of any change in incidence over time was also notable, contrast with increasing idiopathic pulmonary fibrosis rates in their UK patients [382]. The ACCESS study [1, 47] completed a comprehensive breakdown of the demographics of their cohort, showing that peak incidence occurred between 30-49 years of age, with reasonably high incidence also at 25-29 and 50-54 years of age. They also identified both gender and race specific patterns in the disease. The proportion of the cohort who were Black (44.2%) was higher than the representation of the population as a whole, suggesting that the disease is more common in Black patients. Also, although female patients were more likely to be affected overall (63.6%), the degree to which sarcoidosis affects women is greater in Black patients (72% of the Black cohort) than Caucasian patients (56.7%). In this study, women over 40 years were generally affected more than men, but men had a much higher incidence between 35-39 years, over 25% of males were within this age bracket compared with over 15% of women. Rybicki *et al* assessed the highest rate of incidence to be in individuals aged between 20-49 years of age, the highest incidence also being in Black women, followed by Black men (aged 30-39) [95]. The report also highlighted that the highest incidence in Caucasians was later than Black patients, between 40-49 years of age [95]. A report by Judson *et al* also supported these findings, with Black patients being more commonly affected than Caucasians, the average age of onset of the disease being lower in male patients and the age of onset being a decade before in Black patients [48]. The conclusion is that onset usually occurs before a person reaches 50 years; though different regions can show alternate patterns in this, such as Nordic/Scandinavian variation (with bimodal peak incidence in women between 25-29 and 65-69 years [387, 388]) and Japan (bimodal peak incidence in females between 25-39 and 50-70 years, peak incidence for males between 20-34 years [131]). The racial component has been observed in multiple studies and confirms no significant regional or study bias exists within the reports [47, 48, 95].

The clinical pathology of the disease is dependent on factors such as race, geographical location, gender and age; the presentation of specific organs has been discussed previously (Section 1.3 – Clinical Pathology). Overall, the disease tends to be more severe in Black patients, females and older patients, compared with Caucasians, males and younger patients [47, 78, 87, 95, 129, 374, 389]. Chronic and acute cases were also more prevalent in black individuals than white patients and Black patients also tend to have more organs involved [47, 48]. Extrathoracic organ involvement has been described as more common in Black patients [47, 48, 95] and a higher proportion of Black patients have progressive/extensive lung involvement (increased Scadding Score) [48, 95]. Black patients also typically received corticosteroids more often than Caucasian patients, in keeping with the findings that Black patients typically display more severe symptoms [48, 390]. As previously highlighted, women tend to present with more extrapulmonary symptoms, whereas pulmonary symptoms and dysregulated calcium metabolism are more common in men [47, 48]. Older patients will tend to present with more progressive or severe disease with a worse prognosis than younger patients [87, 129]; some symptoms with a poor prognostic outcome, such as cardiac and neurological sarcoidosis, are more commonly observed in older patients (particularly over 40 years of age) [49, 148, 391-393].

Surprisingly, given the high rate of pulmonary involvement in sarcoidosis, the rate of incidence appears to be lower in smokers than non-smokers [91, 394]. Whilst age, gender and race are determinants of disease incidence and severity, it was also found that the disease progression at initial presentation could be dependent on socioeconomic factors [389].

ACCESS suggests a familial link; siblings and family members had relative risk odds ratio of approximately 5 and this relationship was higher in white populations than black [395]. Other research also suggests family members have over 2.5 times the chance of developing the disease than unrelated individuals [396]. First degree relatives display a higher incidence rate relative to the wider population, 1.5% compared with 0.07% respectively, which cannot be explained purely by environmental factors without a genetic component due to spousal incidence being 0 [397]. Another study from Ireland got remarkably similar results, leading the group to estimate the risk to relatives of

sarcoidosis patients is 2.4% [398], though others have reported lower rates amongst relatives [399, 400]. This is further reinforced by genetic studies into twins, showing both monozygotic twins are more likely to develop the disease than dizygotic pairings [401, 402]. Taking all this together, it would seem to suggest that there is a possible genetic component to sarcoidosis and that relatives of sarcoidosis patients have a greater risk of also developing the condition.

1.5.2 The Genetics of Sarcoidosis

Sarcoidosis is suggested to be a generalised term for a number of pathologically related conditions [403], so it would make sense that whilst a number of gene targets have been identified through ACCESS and other studies [395], no simple combination of polymorphisms have been shown to be consistent across all cases. This disease is considered a complex multi-factorial genetic disorder, whose varying clinical manifestations may be representative of different genetic dysfunctions [403]. Twin studies hint that a genetic component may be at work here [401, 402], as does wider reaching familial and genetic studies [395, 396]. Many candidate genes have been suggested to play a role in the disease, coming from genetic linkage studies and genome wide association studies (GWAS). Some of these are summarised in Table 1.6.
Given that sarcoidosis has traditionally been considered an 'auto-immune' disorder, links began to be established between the disease and the Human Leukocyte Antigen (HLA) genes. As the genes responsible for coding the major histocompatibility complex (MHC), this seemed like a reasonable target of study [404, 405]. The HLA region is present on chromosome 6 and despite the MHC genes being reasonably conserved in evolution [406], there are a vast number of HLA polymorphisms and alleles within the human population [407]. Early work had highlighted HLA antigens and genes (particularly B8) were associated with the disease [408, 409]. Grunewald *et al* identified potential HLA-DRB1 haplotypes present in a stratified patient population, with or without Löfgren's syndrome, with HLA-DRB1*01 and HLA-DRB1*03 tending to be more protective, associated with Löfgren's and resolution, whereas HLA-DRB1*11, HLA-DRB1*14 and HLA-DRB1*15 were associated with a chronic, non-remitting course and conferred a risk factor for sarcoidosis [410]. Since then a large number of HLA genes and loci have been

implicated, though there has been movement more towards MHC class II rather than class I, HLA-D rather than HLA-B [404, 411, 412]. Spagnola *et al* concludes that class II are more likely to be of importance in disease pathology [403], though the author concedes that work by Grunwald *et al,* which indicates class I genes independently increase risk, may contradict that assertion [413].

After looking at MHC gene and familial links, Nowack *et al* summarised that "...the hypothesis that the role of an infectious agent triggering sarcoidosis cannot be envisaged without considering genetically linked cofactors" [414]. Despite this, the issue of candidate genes of the HLA linking these to familial patterns has led to conflicting reports without a singular responsible target [404]. Others re-affirm that the region of the MHC is a risk factor, but genes (such as the HLA-DPB1) previously identified are not necessarily responsible in exclusion of others [415]. Studies have shown that certain HLA-DRB1 gene alleles, such as HLA-DRB1*1101, are risk factors across different groups [411, 412]. Overall, this appears a likely region to focus on; differences in end pathology and the heterogeneous nature of sarcoidosis means multiple genes or alleles may contribute to incidence and severity. The HLA genes are thought to be subject to epistatic control (alleles are dependent on other genes) and display a high degree of linkage disequilibrium (whereby alleles are associated with other alleles) [416]. In effect, the extent of the associations of HLA alleles with the disease may be dependent on other genes. An example of this for sarcoidosis is BTNL2.

The BTNL2 gene, encoding the Butyrophilin-like 2 protein, is a member of the Butyrophilin superfamily of proteins structurally related to the B7 proteins [417]. This is of particular interest, as B7-1 and B7-2 (CD80 and CD86 respectively) are costimulatory molecules present on antigen presenting cells during T cell activation, interacting with CD28 or the inhibitory CD152 (CTLA-4) [418]. BTNL2 is structurally distinct, yet still homologous to the B7 proteins [419]. The protein is expressed most heavily on epithelial cells in the intestine (in murine models), though is also expressed in a range of tissue including the lungs and lymph nodes, as well as other cells such as dendritic cells [419]. Whilst the protein has been shown to play a role in T-lymphocyte regulation of TCR signalling through an (as yet) unknown receptor, and also interacts with B-cells [420], the interaction is not through proteins involved in B7 co-stimulation [419]. A glycine to alanine amino acid change in

BTNL2 (variant rs2076530) leads to a truncated protein, conferring a greater risk of developing sarcoidosis that is independent of HLA-DBR1 gene variants [421]. BTNL2 variants have been identified as a risk factor for the disease in other reports [422-426]. Some reports have associated that the rs2076530 variant confers an increased risk with chronic, but not acute, sarcoidosis [423, 427], as well as suggesting a strong linkage association exists between the HLA-DRB1*15 and BTNL2 [422, 427]. Others have noted that it may be a risk factor in Caucasian patients and not Black [422]. It should be noted however that Spagnola *et al* lost this association when Löfgren's patients were excluded and the HLA-DRB1 gene was considered [424], another report also indicated that acute cases were defined more by HLA-DRB1*03 than BTNL2, the former also conferring increased chance of resolution [426].

Other, non HLA/MHC associated genes have also been investigated. These are wide ranging and include genes encoding chemokines, cytokines, co-stimulatory molecules, vitamin D receptors, Angiotensin-converting enzyme and many others (for full reviews, see [403, 428]). There has been a lot of study into these, though no consistent link has been established for these as yet. Interest in innate immune receptor involvement derives from the strong association of NOD2 polymorphisms with Crohn's disease [429, 430], as well as having a role in Blau Syndrome (BS) and Early Onset Sarcoidosis (EOS) [431-434]. In sarcoidosis, there has been no definitive link between this receptor and the disease in aetiological terms, though the severity of pulmonary sarcoidosis may be influenced by loss of function mutations in CARD15/NOD2 [435]. Of the Toll-like Receptor genes, the most likely candidate is TLR4 [436], with some suggestion that chronic manifestations may be associated with polymorphisms in this region [437]. By contrast, the TLR9 gene, which is important for recognising Mycobacterial and *P.acnes* infection (potential aetiological sources of the disease), was not associated with sarcoidosis [436, 438]. The ACE gene insertion/deletion polymorphism in intron 16 has also been characterised extensively, though there is conflicting evidence regarding the contribution it plays in either the elevated serum ACE evident in patients, risk of disease onset or role in disease pathogenesis [230, 247-252]. Another potential gene of importance is annexin A11 (ANXA11), which is involved in apoptosis and where a GWAS identified a polymorphism that was strongly associated with the disease [439]. Annexin A11 is involved in apoptosis and cell proliferation; subsequent studies have also established that

polymorphisms in this gene are associated with the disease and even severity in sarcoidosis [440-442].

Given the importance of TNF-α in the pathogenesis of sarcoidosis, it is unsurprising that the gene for this cytokine has been studied. Polymorphisms of the TNF-α gene (TNFA) and TNF-β gene (TNFB) have both been associated with enhanced release of TNF-α in other conditions, TNFA polymorphisms in the promotor (-307) [443] and TNFB polymorphisms in intron 1 [444]. Whilst the TNFA2 haplotype is more common in Löfgren's syndrome [445, 446], neither TNFA2 or TNFB2 have appeared to correlate with increased TNF-α release from BAL leukocytes or peripheral leukocytes [447]. The -307 polymorphism may not confer an independent risk of Löfgren's however, as there is strong linkage disequilibrium between this and a number of HLA gene alleles [448]. A different polymorphism in the TNFA promoter, -857, has been associated with sarcoidosis patients in one study of British and Dutch patients [446]. It may be that different TNF polymorphisms are risk factors in other populations, such as Japanese and Indian cohorts [449-451]. A large number of other immune factors have been investigated, including IL-1 and TGF-β (for a full review, see [428]).

Table 1.6: Examples of genes investigated in sarcoidosis.
Adapted from [428].

Gene	Function	Risk Allele/Location
HLA-DRB1	Class II antigen presentation	*0301 *1101
HLA-DRB3	Class II antigen presentation	**1501 *0101
Angiotensin Converting Enzyme (ACE)	Vasoconstriction	17q23
HSPA1L	A Heat Shock Protein 70kDa (DAMP)	6q21.3
IFN-γ	Cytokine (class II interferon)	9q22
Toll-like Receptor 4 (TLR4)	PRR, innate immune receptor	9q32
TNF-α	Inflammatory cytokine/adipokine	6q21.3

1.6 Aetiology

Sarcoidosis is best described as a disease of unknown aetiology [1, 452], which has led to slow progress in delineating the pathogenesis, identifying features for diagnostic purposes and adopting appropriate treatments. Whether or not sarcoidosis is actually classified as a single 'disease', or a series of conditions with the same final pathology, is a matter of some discussion. Complicating this is a series of contradictory findings that would, in isolation, lead to radically different conclusions. A great deal of care needs to be taken in determining the aetiology of this complex disease; the mere presence of a factor, such as a bacteria or antigen, or correlation does not necessarily provide a good solid aetiology. There is a temptation to fall victim to 'cum hoc, ergo propter hoc' (correlation equals causation fallacy) in assigning a cause, thus a wealth of evidence and robust associations are necessary. As yet, there is no consensus on the issue, though possible causes are widely discussed [1]. A summary of possible causes is shown in Figure 1.10.
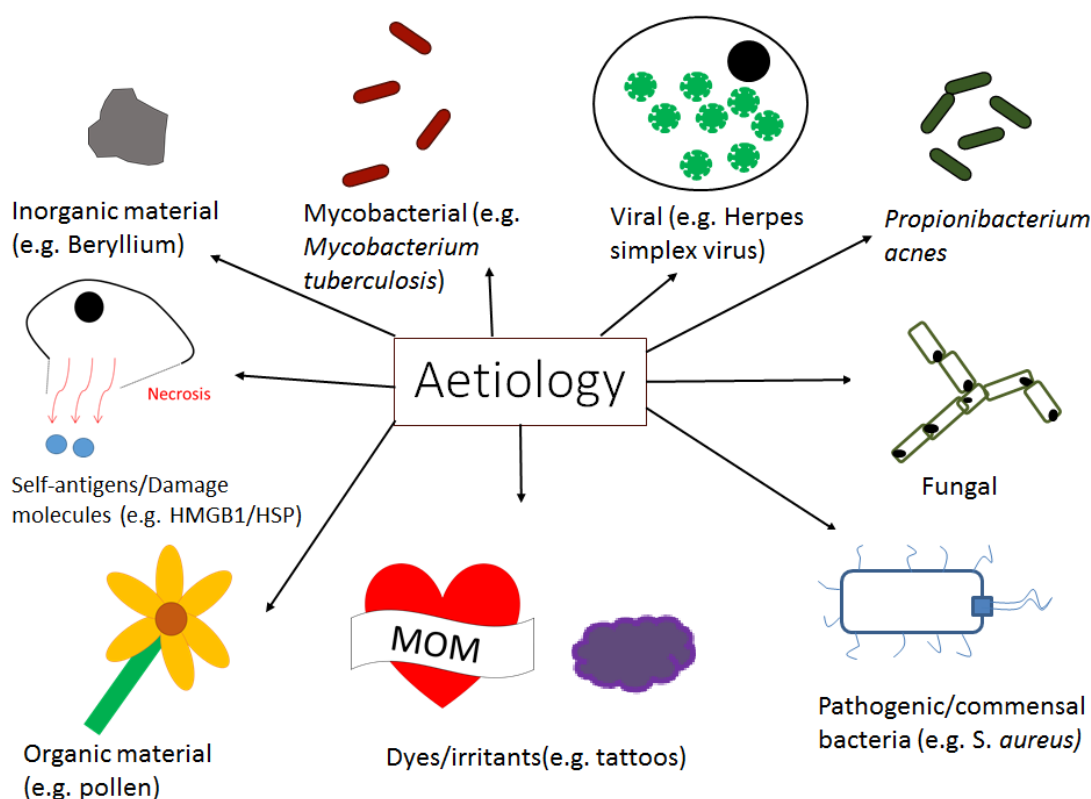
Figure 1.10: The aetiology of sarcoidosis.

Examples of organic and inorganic sources suggested as possible causes of sarcoidosis. *Mycobacterial tuberculosis* and *Propionibacterium acnes* remain the favourite proposed sources of antigen causing sarcoidosis, though other reports suggest non-infectious causes (pollen, inorganics etc.), 'self-antigens', fungi or other possible causes may be responsible for sarcoidosis.

1.6.1 Mycobacterium

This potential cause has been one of the longest and most persistent theories throughout the history of sarcoidosis research, particularly *Mycobacterium tuberculosis* (MTB) which displays some clinical and pathological similarities to sarcoidosis (Section 1.8.2 – Tuberculosis). There are significant differences between the two diseases however, not least in that MTB typically leads to necrotic/caseating granulomata (Figure 1.11) [453], rather than the non-necrotic observed in sarcoidosis.

Initially many thought a direct pathogenic cause might be responsible; the attempts to 'transmit' an infection through sarcoid tissue has been met with conflicting data, both positive [454] and negative [455, 456]. Richter *et al* [455] does concede that a pathogen remnant or super-antigen cannot be ruled out of as a possible cause. This has led researchers to suggest a mycobacterial antigen is present from a now absent or cleared infection that cannot be correctly processed. Research has shown the presence of DNA and RNA from these species can be isolated and amplified from some sarcoid tissue [64, 457, 458]. The species type differs depending on the individual or cohort; studies have shown the absence of *Mycobacterium tuberculosis* but the presence of *Mycobacterium avium* species in tissue [458, 459], whereas other reports did detect MTB in approximately half of tissue samples [460, 461].

Antibody studies in the serum of sarcoidosis patients also reveal immunoglobulins raised against different mycobacterial proteins [462-464]. Studies have identified the *Mycobacterium tuberculosis* catalase-peroxidase (mKatG) as a potential antigen [462, 465]. There are specific T cell mediated immune responses to both mKatG and ESAT-6 in a proportion of patients with sarcoidosis [465-467], though not all display this recognition and some research has pointed to limited or diminished responses to *Mycobacterium tuberculosis* antigens (such as BCG or ESAT-6) [468]. Mycobacterium tuberculosis heat shock proteins have also been shown to induce a greater pro-inflammatory cytokine release in sarcoidosis patients than healthy controls [469]. MTB mKatG antigen has been considered favourable in the search for an aetiology, in part because it has been detected locally in the tissue of over half of sarcoidosis patients, but not in control tissue [462]. Using both ESAT-6 and mKatG in combination, stimulating BAL cells, showed positive

response from over 70% of the patient cohort [470], suggesting that the theory of an MTB antigenic trigger carries some utility. Other mycobacterial species have also been linked to sarcoidosis, including *M. avium* and *M. marinum*, though the majority of research has focussed on MTB associations [471]. All of this evidence has meant that a mycobacterial aetiology for sarcoidosis is still considered a strong possibility.
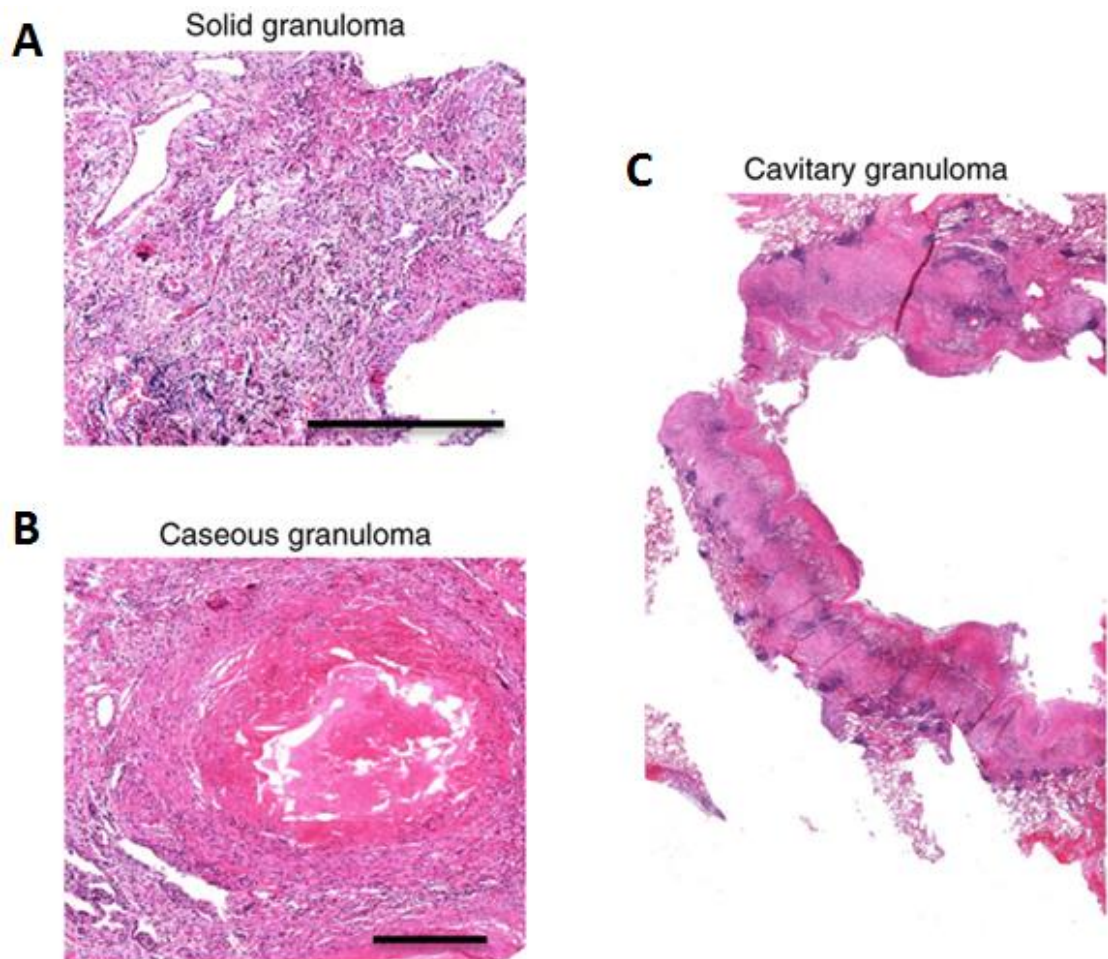
Figure 1.11: Different types of granuloma observed in tuberculosis.

Different manifestations of *Mycobacterium tuberculosis* granulomata. A: Solid granulomata, more typical of earlier stages of infection. B: Caseous (caseating/necrotic) granulomata are the most common presentation, results from active disease and cell death in inflammation. C: Cavity granuloma is a large cavity formed when there is significant caseation, and the clearance of the material leaves gaps within the tissue. Represents severe tissue damage. Figure modified from [472].

1.6.2 Propionibacterium acnes

Another favourite potential aetiological source often suggested is *Propionibacterium acnes* (*P. acnes*), a commensal bacteria that often colonises the body and can lead to acne in some individuals [473]. The precise mechanism by which *P. acnes* induces acne vulgaris is still not fully elucidated, though it is believed to result from an active immune response to bacterial products [473, 474]. There is also a suggestion that different strains of the bacteria exist, pathogenic/opportunistic and commensal, and may define the type of response observed [475, 476]. The controversy surrounding the role of *P. acnes* in disease pathology of acne vulgaris alone is an issue when associating the bacterium with other diseases [473, 476]; similar issues surround links between *P. acnes* and sarcoidosis. There are conflicting reports to support or reject the assertion that *P. acnes* may be the aetiological source of sarcoidosis. It has been suggested that the presence of the bacteria near granulomata in sarcoidosis may indicate that they have a role to play in 'sparking' an initial immune response [477]. The genomic signal and genetic material of *P. acnes* has been detected close to granulomata in tissue [478, 479], a greater percentage of sarcoidosis lymph nodes have been shown to contain the bacteria (77.5% compared with 21.1% of healthy controls) [480] and peripheral blood leukocytes respond to specific *P. acnes* antigens in sarcoidosis more robustly compared with other lung diseases or controls [468, 481, 482]. It is also notable that when comparing the presence of MTB and *P. acnes* genetic material in tissue in TB, sarcoidosis and cancer patients, it was the latter organism that was more commonly detected in sarcoidosis but not that of TB or the cancer control patients [479], supported by others who conclude that *P. acnes* is more common in sarcoidosis [457]. This is contested in another study however, which suggests that *P. acnes* is very common in normal lung tissue as a commensal and that there is no apparent specific strain of the organism linked to sarcoidosis [483]. *P. acnes* antigens or heat-inactivated *P. acnes* have been shown capable of inducing granulomata in murine/rat or rabbit models [484-486], suggesting that the organism may be capable of a similar response in humans.

This has all led Yoshinobu Eishi to suggest that the organism would lead to sarcoidosis as a result of a hypersensitivity response to the bacteria itself or antigens from the bacteria [487]. Care needs to be taken in assigning this aetiology to the disease however, as no

definitive single associative factor could be identified in the ACCESS study (although microbial aerosol exposure did show some correlation with the disease) or in other large patient analyses [1, 93] and the commensal nature of the bacteria means it would likely be present in a number of patients without necessarily being the cause of the disease. The same could be applied to the immune responses to antigens; a recognition of bacterial antigen and activation of immune cells does not guarantee the organism caused the granulomata. Despite this, the data suggests *P. acnes* could be a possible trigger for the disease.

1.6.3 Other Infective Causes

Other groups of organisms and species have been investigated as a possible aetiology for sarcoidosis, including fungal and viral (for reviews, see [68, 488, 489]). Viruses have been looked at, with some suggestions that patients may have elevated antibody titres to specific types, such as Epstein-Barr virus (EBV) [490, 491]. This possible cause is not considered likely however, as viruses have not been associated with other granulomatous diseases, particularly those with non-caseating pathologies (Section 1.8 – Related Granulomatous Diseases). It is therefore more likely that the presence of elevated antibodies to viruses would be more indicative of opportunistic infections during the disease, especially as titres for a number of viruses were also raised in tuberculosis patients [491]. Fungal, protozoal or other causes have been proposed, though the evidence is very limited. Many are proposed based on their ability to induce granulomatous disease (see Section 1.8 – Related Granulomatous Diseases), whilst some indirect evidence may suggest possible groups are worth considering, such as the elevated immune response to fungal cell wall components in sarcoidosis [492]. However, the evidence is more compelling for mycobacterial or bacterial causative agents in sarcoidosis than other infective agents.

Other bacteria have also been investigated and suggested to be possible causes of sarcoidosis, though the evidence of this is limited compared with *P. acnes*. *Rickettsia helvetica* has been linked with the disease, being identified in the granulomata of Swedish patients [493], though serum analysis in another study found no evidence of significant

elevated anti-Rickettsia antibodies in the sarcoidosis cohort [494]. Both serum and BALF samples have shown the presence of antibodies against *Chlamydophila pneumoniae* in sarcoidosis samples [495, 496], though granulomatous tissue in one report did not detect the genetic signature of the bacteria [497]. *Borrelia burgdorferi,* responsible for Lyme disease, has also been associated with sarcoidosis in some studies [498, 499], though there are equally a number of studies in other cohorts of patients where the presence of the bacteria was not found [500, 501]. A study of the microbiome present in BAL from patients with sarcoidosis revealed that the overall diversity of bacterial species was reduced compared with healthy individuals [502]. Interestingly, the same study showed that patients with rheumatoid arthritis also displayed a similar reduction in bacterial flora, suggesting that inflammation itself may contribute to alterations in the microbiome [502].

The ACCESS study found no definitive infective cause by health questionnaire, blood culture or PCR of blood samples that differentiated patients from healthy controls [93, 503], though analysis of blood may not rule out local tissue infection or a resolved infection. The report did conclude that infection was unlikely to be the cause of the disease [503].

Taken together, this has supported the conclusion that either: (a) a pathogenic cause is not the aetiology of sarcoidosis, it may be environmental, (b) a range of pathogenic organisms might be capable of initiating the disease through a common mechanism or (c) different subsets of the disease (geographical or genetic groups for example) respond to different aetiological triggers. Whether or not any of these are suitable to explain the often confused findings is unknown. It is clear from the evidence that a simple, single pathogenic cause is unlikely.

1.6.4 Environmental (non-biological) Agents

It has been argued that many of the variations in epidemiological expression can stem, not from outbreaks of regional infectious diseases, but from localised differences in environmental triggers. Berylliosis gives credence to this idea; the granulomata in the disease is indistinguishable from those present in sarcoidosis and berylliosis is only

differentiated from the latter by identifying the causative agent via a history of exposure [14, 15, 63, 64]. Quite aside from this, the most common organs involved are those exposed to the external environment such as the eyes, skin and lungs [4]. There is also a geographical and temporo-spacial element shown in various studies that may go beyond the genetic predisposition or pathogen explanations [504].

As with berylliosis, occupational exposure to metal has shown increased risks of sarcoidosis [505], as has other inorganic matter like photocopier dust [506]. There have also been other studies identifying at risk occupations such as firefighters and naval personnel and increases in sarcoidosis incidence [507-509]. After the 9/11 attacks, first responders to the twin towers were found with sarcoid like granulomata resulting from their exposure [510], though it should be taken in context that a large range of pulmonary conditions developed in many present there.

It is unlikely that environmental factors alone, without an underlying immune/genetic component, explains sarcoidosis, as family studies identify spouses as having limited risk of developing sarcoidosis compared with relatives [397]. The ACCESS study could not find a strong positive correlation between environmental factors and sarcoidosis incidence rates, only weak associations in some candidates with mould and insecticides [93]. The researchers conclude that different environmental factors are likely to affect different patients, thus there is no single environmental cause. This relies on a more prominent genetic dysfunction being established, leading to a possible hypersensitivity (as in allergic response) or flawed clearance gene(s). As this has yet to be established conclusively, more research is required.

1.6.5 Immune Dysfunction and 'Self-Antigens'

Recent advances have identified immune receptors, such as the Toll-like receptor, and are shedding light on the interactivity of innate and adaptive immune system. As more evidence is compiled, the nature of how the body deals with 'self-antigens' (auto-antigens), circulating proteins or cellular components that are released from metabolism, apoptosis and necrosis, is now better understood [511]. Despite this, the clearance of

material the body would usually consider part of the natural make-up of the organism is complex and has yet to be fully delineated. The possibility exists that, within susceptible individuals, the immune system is responding aberrantly to an inert antigen or antigens, and the outcome is a hypersensitivity to these, in much the same way environmental factors may be causative [511].

Anna Dubaniewicz has proposed that the often contradictory findings observed in sarcoidosis, particularly as regards aetiology, might be explained using the model of interactions between Pattern Recognition Receptors (PRRs) with Pathogen Associated Molecular Patterns (PAMPs) or Damage Associated Molecular Patterns (DAMPs) [512]. In summary, 'danger signals', such as proteins unique to a pathogen or a protein released during necrosis, bind to a PRR like the TLRs (Toll-like Receptors) and activate a downstream immune response as appropriate [511-513]. This would, it is argued, explain why multiple potential antigenic sources (both infectious and non-infectious) are purported to lead to immune responses in sarcoidosis patients, including both mycobacterial and host heat shock proteins [512]. For the disease to progress, there would likely be some form of immune dysfunction in these pathways; the concept of an immune dysfunction is generally considered the underlying issue in sarcoidosis [1]. As serum amyloid A is considered a marker of inflammation present in granulomata in sarcoidosis and can bind to TLR2 [266, 514], the release of which by 'stressed' cells (with HSPs) may actively contribute to the inflammatory process in the disease [512].

Aside from innate immune response, findings by Grunewald *et al* suggests that TCR recognition on clonal T-lymphocytes from patients specifically recognise antigen, a sequence related to the self-antigen vimentin [515]. This had previously been suggested as a possible contributing stimuli, alongside other auto-antigens in antibody screening of patients [516, 517]. Interestingly, there is a suggestion that vimentin might be released by macrophages after TNF-α activation [518] and that vimentin can stimulate PRRs (Dectin-1) to promote inflammation [519]. Grunewald *et al* suggests that the recognition of vimentin by the TCR might be the result of "molecular mimicry", where the antigen epitope is similar to a pathogenic sequence, though the authors have not identified what the source would be [515].

Overall, there may be a range of theories regarding the aetiology should the cause be incidental relative to the immune dysregulation. Some have proposed that sarcoidosis results primarily from this immune dysfunction, and that the aetiological source is variable depending upon the patient [520]. Should this be correct, a single causative agent would likely never be identified across all patients with sarcoidosis.

1.6.6 Deriving an Antigen for Sarcoidosis – The Kveim-Siltzbach Reagent

In the 1940s, Kveim noted that a lymphatic tissue from a sarcoidosis patient could be intradermally injected into the skin of other patients to initiate a condition specific response [37]. It became a diagnostic tool in subsequent years and the source of interest and study by many in the field keen on establishing a cause.

The issue of the Kveim antigen and the importance to aetiology has been widely debated. It is by no means a consistent success; studies on different preparations of spleen or lymph node will reveal anywhere between a 23% positive correlation to over 90% [109, 521-525]. Siltzbach in his validation found 80% positive with less than 1% of controls displaying false positives [20]. Conversely Sones *et al* found only 26.8% of their sarcoidosis patients responded positively to the test, as opposed to 43.8% of TB patients and 14.3% of healthy controls as 'false positives' [521]. The majority of reports suggest a higher percentage of specific response is obtained than lower [109, 523-525]. Attempts to isolate individual components responsible for this response have thrown up different possibilities, but rarely lead to a definitive molecule. Attempts to replicate this effect *in vitro* have also failed [526]. Belief that the active antigen results from mycobacterial presence has been presented [462-464] and refuted [455, 456] by different studies; the active agent remains elusive. This often throws up interesting links, such as the pso p27 psoriasis associated protein as a possibility [527] or even suggestion of nanoparticles [528].

As the understanding of adaptive-innate immune coordination has improved, and antigen presentation elucidated to a much greater degree, the question returns to whether the Kveim Reagent holds antigen(s) that can initiate a condition specific immune response. It

has been shown that CD4 T cells are present consistent with an antigen-specific response [529]. A study looking at the conditions required to 'sterilise' the Kveim antigen is of particular interest here. DNAse, RNAse and other chemical methods failed to inactivate the preparation, and in standard practice the mixture is heated to 56 °C multiple times [20, 530]. This indicates that the active agent would appear relatively stable. In addition, whilst treatment with a chaotrophic agent or a reducing chemical (urea and mercaptoethanol respectively) would not inactivate the solution, a combination of both would [531].

This would suggest a protein antigen origin for an activating factor that relies on secondary or tertiary structure complex for activity. Given what is understood from the mechanism, this may indicate that only the dissolution of disulphide bridges and intermolecular bonding will allow macrophage antigen presentation in a normalised fashion. Further proteomics attempts to isolate antigens have indicated a possible insoluble antigen is responsible [462]. Whether or not this is relevant is dependent upon complementary immune dysfunction; the response is normal in healthy individuals, thus it is likely an aberration must exist in patients susceptible to sarcoidosis.

1.7 Diagnosis, Disease Activity and Treatment

1.7.1 Diagnosis using the Kveim-Siltzbach Antigen

Within a clinical setting, diagnosis of sarcoidosis remains a challenge. In one study, less than 1/5 of patients are diagnosed on the first presentation of symptoms and the diagnosis can take over 6 months [532]. Even when the Kveim test was used, the delayed onset of the papules meant a significant wait was required [533]. An intradermal injection was performed with validated suspension, 4-6 weeks later papules would appear if the test was positive [524, 533]. New preparations could only be validated against existing preparations, which would require a high level of concordance for approval [524, 534]. Previously, Kveim-Siltzbach reagent/antigen was a standard diagnostic practice for sarcoidosis, though it is no longer widely used; fear over cross infection and the perceived limited benefit of the test have both been factors in the diminished use [1, 68]. Another

major factor issue was the lack of a sustainable source of tissue for use within the tests [68]. The long incubation period for development has also lost favour in an age of relatively high throughput, quantitative and quick diagnostics.

A parallel can be seen in tuberculosis, whose initial diagnosis has long been through the Mantoux skin test, heaf test or other similar observed confirmatory tool to establish whether immunity or infection exists [535]. Whilst these have proven useful, the negatives of introducing a foreign agent to invoke an apparent response confirming adaptive immunity are clear.

More recently, the use of blood tests which test for Interferon-γ produced by memory T cells (Interferon Gamma Release Assay – IGRA) and exploiting the antigens derived from tuberculin have become widely used to establish TB infection, both latent and active [536]. This confers a few advantages: latent infection is easily tested, it can be used in immunosupressed patients (though the efficacy suffers in these patients [537]) and can distinguish between infection and vaccination, as the BCG injection contains different antigenic epitopes [536, 538, 539]. There are differing views on the relative merits of each test, based on ELISA (Enxyme-linked Immunosorbant Assay) or ELISPOT (Enzyme-linked ImmunoSpot) for Quantiferon Gold TB and T-Spot TB respectively, though both have some evidence of greater specificity than current tuberculin skin testing [539-544].

The one important factor in both of these tests is antigen selection. Currently, as no aetiology or antigen is known for sarcoidosis, this would present a limiting factor on design of a diagnostic tool similar to that of tuberculosis. In addition, as stated previously, the possibility that there may be multiple different antigens responsible for the end pathology would mean a single, all-encompassing test might be difficult to achieve.

1.7.2 Current Strategy for Diagnosis

Current diagnosis is achieved, in essence, by exclusion (see Figure 1.12). CT-Scans, X-Rays, exploratory surgery and lymph biopsy followed by histological analysis will provide evidence of granulatomous infection, whilst other related conditions are tested and eliminated from consideration in order to derive a diagnosis of sarcoidosis [1, 308]. This therefore requires a multi-disciplinary team assessment, typically radiological, clinical and

histological evidence to confirm the disease presentation is characteristic of sarcoidosis; the histopathology considered essential for accurate assignment of diagnosis [1].
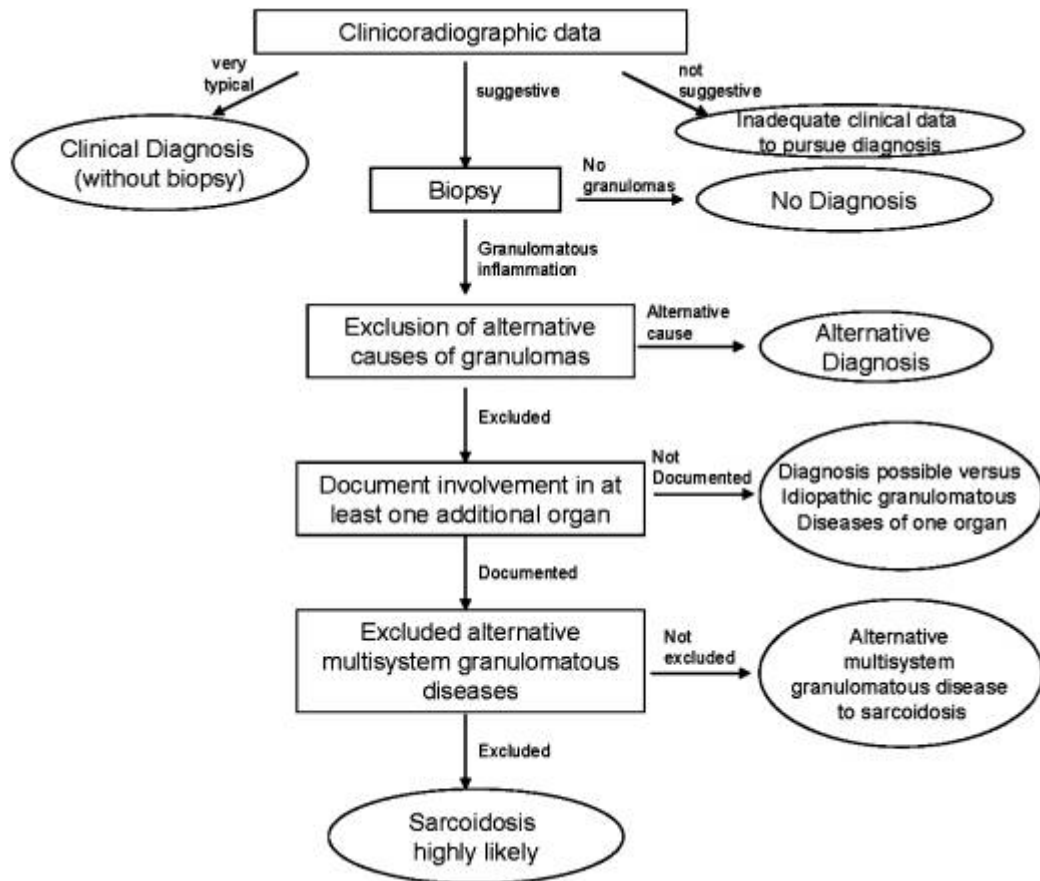
Figure 1.12: A typical diagnostic pathway for sarcoidosis.

A suggested procedure for determining a diagnosis of sarcoidosis. Usually, possible sarcoidosis patients will be identified initially based on radiographic evidence. In some cases, a very typical sarcoidosis chest radiographic pattern and supporting clinical evidence will be used without biopsy, though the advice usually requires a tissue biopsy. Once other causes are excluded and additional organs identified, it is likely that a diagnosis of sarcoidosis can be assigned to the patient. Taken from [307].

However, as an idiopathic multi-system disease, there remains variability in classifying sarcoidosis above other granulomatous inflammatory conditions such as TB [62-64]. Initially, patients present with symptoms which can be indicative of a large number of other conditions, particularly non-specific constitutional symptoms such as fatigue and weight loss (Section 1.3 – Clinical Pathology) [1, 532]. The clinician is unlikely to consider the condition as a primary diagnosis, especially given the low incidence and prevalence of

the disease in the population [1, 532]. An early indicator might be after broad blood tests, which might highlight elevated serum ACE or hypercalcaemia, though many patients may not exhibit either of these and these are not specific enough for sarcoidosis diagnosis in exclusion [47, 201, 230-232]. The majority of patients with sarcoidosis have some form of lung involvement [47], though the presentation of this can vary between patients. Not all patients display reduced lung function (Section 1.3.3 – Pulmonary Sarcoidosis), nor are the patterns observed in the results of lung function tests in patients specific to sarcoidosis [308]. Despite this, the guidance is to perform these tests to establish a baseline at presentation for use in tracking deterioration or improvement in the pulmonary symptoms after diagnosis [1]. A chest X-ray and/or CT scan will identify areas of inflammation in the pulmonary system if the patient has these symptoms [1, 60, 73, 77, 308]. Without biopsy, this could be differentially diagnosed as a cancer, Tuberculosis or other lung pathologies, and would therefore require a biopsy [1]. Ultimately, the result of the biopsy of the organ system with involvement and subsequent histopathological identification of granulomata will typically exclude cancer from the diagnosis; if the granulomata are non-caseating/non-necrotic then the condition of sarcoidosis becomes considered [1].

Generally, to exclude other granulomatous diseases, a display of other organ involvement other than the primary is preferred for diagnosis, though histopathology and biopsy are not an absolute requirement in other organs [1, 137, 308]. Also, patient history has to be considered to exclude pathologically similar conditions such as berylliosis [545-547]. Previously, patients were diagnostically separated into 'Definite/Confirmed', 'Probable' and 'Possible' for sarcoidosis, depending on the criteria used [137, 147]. The system has been refined, with a set of guidelines post-ACCESS that defines organ involvement and the criteria for 4 groups: 'Highly Probable', 'Probable', 'Possible' and 'No Consensus' [548]. In this way, a standardised mechanism for use in diagnostics can be used in subsequent analyses.

1.7.3 Biopsy and Histopathology

Depending on the suspected location of the involvement in sarcoidosis, a biopsy is employed to confirm the presence of non-caseating granulomata [1, 308]. If patients have clinical symptoms and radiographic evidence supporting pulmonary sarcoidosis, the most common presenting symptom, the suggested method of biopsy is the 'transbronchial lung biopsy' (TLB) [1]. Protocols vary, but the probability of securing a positive sample for histopathology is high if 4 biopsy samples are obtained [549]. Compared with open lung biopsy, this method is demonstrably safe and has a low morbidity [550, 551]. Given that higher Scadding Stages are associated with more extensive granulomatous inflammation, it is unsurprising that the probability of obtaining positive sampling increases as the stage increases, though good yields can be obtained in low stages with this method [549, 551-553]. Alternatively, a mediastinoscopy (mediastinal lymph node biopsy) can be used, which tends to provide high sensitivity with low false positive/negative results in diagnosis [554-557], though the method is more invasive than TLB. The use of 'endobronchial ultrasound-guided transbronchial needle aspiration' (EBUS-TBNA), a method employing ultrasound to aid in obtaining a biopsy, has also been used in diagnosis of sarcoidosis [558]. Compared with conventional transbronchial needle aspiration (cTBNA) and TLB, EBUS-TBNA has been reported to benefit from greater sensitivity in the diagnosis of sarcoidosis [558-561], the yield obtained from EBUS-TBNA is similar to that achieved by TLB [562]. This suggests that EBUS-TBNA may become the preferred method to obtain sarcoidosis lung biopsies in the future. Without any chest X-ray indicators or pulmonary symptoms, it is unlikely that a biopsy of that region will be undertaken if, for example, skin manifestations and a biopsy confirms the presence of disease as cutaneous only [1, 137]. However, as the majority of patients will display pulmonary symptoms, a confirmed case of extrapulmonary sarcoidosis may warrant further investigation elsewhere [137].

Classic indicators of sarcoidosis granuloma, aside from the non-necrotic phenotype, include the presence of specific cell types and structures. Whilst these cells and patterns can be present in other diseases and may not appear in every sample observed, they can be supportive of the sarcoidosis assignment in diagnosis [1]. Epithelioid cells [9], macrophages, Langhans' giant multinucleated cells [563], Schaumann bodies [564],

asteroid bodies [565] and peripheral fibrosis around the granuloma are features of the sarcoid granuloma [9, 566]. Conventional work suggests that macrophage-like cells (histiocytes) are predominant inside the central granuloma, though some reports suggest that the most numerous cell type in the sarcoidosis granuloma core are lymphocytes [563]. CD4^{+} T-lymphocytes are principally in the centre of the granuloma and CD8^{+} T-lymphocytes are present in peripheral regions [567, 568].

As mentioned previously, the presence of a non-caseating granuloma alone is not considered sufficient for a positive diagnosis of sarcoidosis. There are also issues regarding the pathology of granulomata that might mean that false positive or false negative diagnostic assignment occurs. In one study, patients with an unassigned necrotising granulomatous pattern based on histopathology alone (23% of the total necrotising granuloma samples taken) could have an aetiology assigned in 60% of unknown cases with additional investigation, leaving 9.2% of the total cases still without a diagnostic cause [569]. What is interesting is that 4 cases were assigned to sarcoidosis once radiographic details were examined and histology reviewed again; these were not identified previously as the granuloma had focal necrotic regions which were therefore labelled as necrotic, but were actually sarcoidosis non-necrotic granuloma [569]. Necrosis within granulomata in sarcoidosis can occur [570-573], conversely non-necrotic granuloma can be present in other granulomatous conditions such as TB [63, 574]. This makes diagnosis based exclusively on histology prone to error, further complicated when rare and unusual conditions such as 'necrotising sarcoid granulomatosis' will present with necrosis yet share features of classical sarcoidosis [575-578].

1.7.4 Lung Imaging – X-rays, CT Scans and PET/CT

Pulmonary involvement remains the predominant clinical finding in those with sarcoidosis [46], therefore identification of the disease in this region remains the most common form of assessment. Traditionally, chest radiographic findings have been used to determine the severity of the disease by use of the Scadding CXR Stage (see Section 1.3.3 – Pulmonary Sarcoidosis) [60]. The value in chest x-rays in defined diagnosis is questionable, the patterns observed in bilateral hilar lymph adenopathy viewed on an X-ray are considered

a classical presentation suggesting sarcoidosis, though can overlap with other inflammatory and cancerous conditions [532]. This is exemplified by a study showing that diagnosis of patients with pulmonary sarcoidosis tends to be longer than those with skin manifestations, ascribed to the difficulty in assigning radiological findings to sarcoidosis rather than other, more common lung pathologies [532]. Additionally, the assignment of stages is subject to interpretation that varies between those assessing it [76] and is not considered useful in defining disease progression [79]. CT scans have proven useful in providing additional details on the state of the disease (Figure 1.13) [73-75], and more recently 18F-FDG PET/CT scans (F-18 fluorodeoxyglucose positron emission tomography) have proven imaging of active inflammation where granulomata are can be achieved [579, 580], though this is not necessarily specific enough for sarcoidosis. For difficult organ systems, such as the heart, PET/CT or MRI (magnetic resonance imaging) might provide details of possible involvement if used on patients with existing confirmation of sarcoidosis in other organs [581]. When available, complimentary use of techniques may be the most appropriate strategy, though cost and time limitations may define the use of methods for individual healthcare providers.
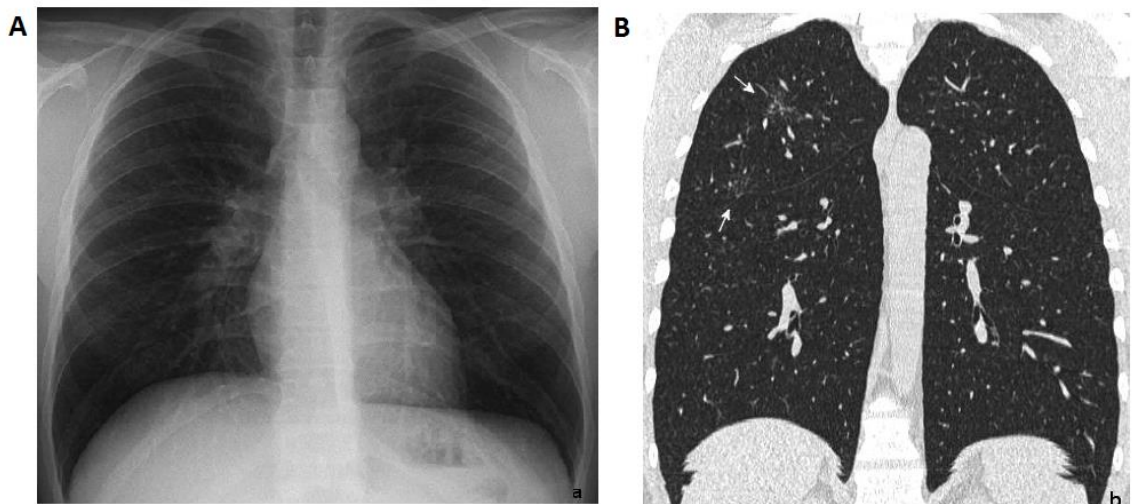
Figure 1.13: Comparison between chest X-ray and CT scan of lungs in a sarcoidosis patient

Lungs in a patient with sarcoidosis but limited lung involvement assessed using radiographic analysis and CT scan. A: Radiograph shows clear lung field, assessed as Scadding Stage 0. B: CT scan of the same lungs showing some inflammation, particularly micronodules (arrows) which are not evident on the X-ray. Taken and modified from [582].

1.7.5 Diagnostic Value of Bronchoalveolar Lavage (BAL)

The general consensus has been that BAL, whilst not specific enough for diagnosis in exclusion, is of use in determining whether a patient potentially has sarcoidosis diagnostically [1, 347]. The use of the technique is common in research, but the value in diagnostics is open to debate. In a comparison with other respiratory diseases, the CD4:CD8 ratio was significantly higher for sarcoidosis, 7.52 mean compared with mean values below 2 for asthma, hypersensitivity pneumonitis and idiopathic pulmonary fibrosis [322]. There are differences in CD4:CD8 lymphocyte ratios in BAL samples between patient sets in one study; it was determined that the prognostic value for defining risk of progression was insufficient [239]. This is supported by another study which goes further, in which the patient cohort were determined to have ratios ranging from 0.5 to 37.3 with a median of 3.35 [348], less than the 3.5-4 suggested previously to be valuable in diagnosis [1, 68]. Ultimately, if only 42% of the patients in this study would display a ratio above 4, the utility of the method in diagnosis needs to be considered carefully [348]. This is reinforced by an assessment in which the use of the CD4:CD8 ratio displayed a very high specificity for diagnosing sarcoidosis (approximately 95%), though the test was not particularly sensitive (approximately 40-50%) [583]. The conclusion would be that the method may have clinical use, though results should not be relied upon exclusively for either diagnosis or to chart the disease progression.

1.76 Evaluating Disease Activity

As mentioned previously, the disease is heterogeneous in presentation and clinical course, with some patients improving without intervention and others deteriorating over time [2, 4]. Determining the disease activity proves challenging in sarcoidosis, as the factors studied are not specific to the disease and often represent a narrow aspect of the clinical pathology. Serum ACE levels are commonly used and cited as a marker of disease activity (Section 1.3.11 - Serum Angiotensin Converting Enzyme (sACE/serum ACE)), though it is concluded that this may really represent the extent of granulomatous burden rather than a true marker of disease activity [232, 243, 246]. Chest X-rays and other imaging may allow the extent of lung involvement to be visualised, though it has been

demonstrated that the Scadding CXR stages are not an effective tool to determine the progression of the disease course [1, 78, 79]. Serum ACE and the Scadding stages do not appear to correlate significantly with each other either [237, 238], suggesting that assessment of disease activity cannot be accounted for by these factors alone. Other blood biomarkers have been researched, though clinically inflammatory markers of disease such as the C-reactive protein (CRP) and plasma viscosity (PV) can be used to determine the extent of inflammatory activity [584-586], though these are again not specific for sarcoidosis.

While factors such as serum ACE, PV, CRP and the Scadding CXR stage have some utility in assessing the status of a patient, alterations in these are unlikely to warrant clinical intervention alone. Use of lung function tests to determine both obstructive and restrictive dysfunctions in a patient are vitally important as a method to track deterioration (Section 1.3.3 – Pulmonary Sarcoidosis) [1]. It is apparent that associations between the lung function and Scadding CXR stage can vary depending on the pulmonary feature under examination [46, 47]. Clinically, deterioration in lung function, such as reduced vital force capacity, acts as an indicator of worsening disease and the requirement for therapeutic intervention in the patient [1, 308]. The current guidance therefore suggests that spirometry be used at initial presentation and in subsequent follow-up to determine whether there is a deterioration in lung function and guide clinical assessment of progress of the condition [1].

1.7.7 Treatments and Therapy

Due to the idiopathic nature of sarcoidosis, treatments have tended towards broader action anti-inflammatory regimes to resolve or abate symptoms. In a large number of cases, as much as 50%, the condition will correct itself without treatment [587]. Assessing who requires corticosteroids can be difficult; whilst patients can be separated into so called 'mild', 'moderate' and 'severe' (Table 1.7), this assigning of a status will often be retrospective rather than predictive. Patients who require intervention are currently treated with corticosteroids as a first line defence [587], choice dependent upon localised inflammatory area and disease severity. As pulmonary sarcoidosis remains the

predominant form of the disease, inhaled corticosteroids have been used in a number of cases [588-590], some research suggesting the activity is achieved through synergistic enhancement of the effect of TNF-α and IFN-γ [591]. Other forms, including systemic and cutaneous forms, require alternate strategies; oral or topical steroid application is common.

Research into the efficacy of corticosteroid compounds in cases of sarcoidosis lacks a true consensus, though most lean towards a necessity for a more specific regimen [591, 592]. Application is limited to those patients displaying a more persistent pathology or a systemic form with multi-organ involvement, as use of glucocorticosteroids has led to potential side-effects in a number of patients [592]. In addition, the re-occurance rate has been shown to be significantly lower in patients who were left untreated, 8% compared to 74% in those given previous corticosteroid treatment; whether this is due to the steroids themselves not allowing natural resolution or the underlying difference in conditions is unclear [390].

A number of anti-inflammatory drugs have been used in sarcoidosis in preference to this steroidal, broad immunosuppressive strategy, particularly when patients display disease steroid resistance. TNF-α is a known pro-inflammatory mediator present in many different conditions and is believed to play an important role in sarcoidosis [593, 594], particularly in the transition of inflammatory to fibrotic disease phenotypes [595]. A range of TNF-α inhibitors/antagonists now exist to attempt a more specific reduction in inflammatory effect; infliximab, ethanercept and adalimumab [596, 597]. One particular drug is Infliximab, a TNF-α antagonist based on blocking antibodies, which has shown varied results [598]. Some studies show improvements in patient conditions, others reveal a negligible effect; there is some evidence these compounds may induce sarcoidosis [599]. Ethanercept has shown a lack of efficacy and was not approved for use [600]. This is particularly relevant, given the inherently broad activity and multiple pathways that can activate TNF-α.

Other drugs used previously in chronic or severe cases include thalidomide [601], hydroxychloroquine [602] and methotrexate [603] amongst others, though consistent results are rarely seen. Whether this is due to the lack of understanding of the

pathogenesis, the presence of various complementary pathways contributing to the disease or an indication of multiple aetiologies contributing to an apparent singular pathology is not clear. It is possible that a greater understanding of the disease will allow a more specific, tailored response.

Table 1.7: *Summary of sarcoidosis presentation separated into mild, moderate and severe.*

Group	Typical Profile	Prognosis
Mild	• Often self-limiting or remitting • Usually short course/acute or limited progression • Likely to involve less organ systems • If pulmonary, mostly Scadding Stage 0 or I • Low reoccurrence rate after resolution	Usually a good prognosis, resolution within 2 years and will not require treatment. Limited organ damage.
Moderate	• Can be acute, but usually chronic or longer course of disease • More likely to involve multiple organ systems • More likely to involve some extrapulmonary symptoms in combination with pulmonary • Usually not progressive, but can persist for extended periods • If pulmonary, mostly Scadding Stage I or II • May require intervention, but associated with low reoccurrence rates after treatment	Reasonable prognosis. Course can be longer, but not likely to lead to organ damage. Responds well to corticosteroid intervention.
Severe	• Mostly chronic and longer course of disease, or acute with rapidly progressive disease • Likely to involve multiple organ systems or be systemic • Usually associated with extensive pulmonary symptoms and some extrapulmonary involvement • Non-remitting or recalcitrant • Usually requires intervention, but is associated with high reoccurrence rates after cessation of treatment • If pulmonary, mostly Scadding Stage II, III or IV. • Progressive	Worse prognosis. Long term and progressive. Causes organ damage. Higher probability of fibrotic phenotype. Some may respond to corticosteroids, others respond poorly. The reoccurrence of disease is high, returning to a progressive disease state. Some will resolve. Higher mortality rate associated with severe disease.

1.8 Related Granulomatous Diseases

1.8.1 Atypical Sarcoidosis Disease Types

Sarcoidosis is a protean disease, particularly in terms of clinical manifestation and pathogenesis. Not only that, but it has variably been described as "a great imitator" or "mimic", as it can display features similar to a wide range of other conditions [99, 604-606]. The lack of known aetiology and a sometimes loose definition of sarcoidosis reliant on diagnosis by exclusion presents other problems, particularly in what constitutes "sarcoidosis" and whether other similar diseases are or are not sarcoidosis [575, 607]. "Classical" sarcoidosis has been described previously, though clearly there is a large degree of variation in this group also. Löfgren's syndrome, an acute form of the disease with better defined clinical features, is also usually acknowledged as being a distinct subset within "Classical" sarcoidosis, and has been discussed in more detail previously (see Section 1.3 – Clinical Pathology). Other forms are less well defined. For the sake of clarity, sarcoid-like reactions will be excluded from consideration, as they are varied responses in malignant disease, infections, drug responses and environmental exposure that lead to clinical sarcoid-like granulomatous manifestations without the other supporting features [607]. Also, as they may have aetiological sources, albeit without a full understanding of the pathogenesis of the granulomatous response, they are unlike sarcoidosis (for a full review, see [607]).

Whilst rare, "nodular sarcoidosis" is defined by the presence of nodules and masses on X-ray or CT scans of lungs which may superficially appear like a neoplasm or cancerous inflammation [575, 608, 609]. This is supported by other clinical findings and granulomatous patterns on histopathological assessment consistent with sarcoidosis; granulomata can be necrotic and non-necrotic [575, 609]. "Necrotising sarcoid granulomatosis" (NSG) is similar to nodular sarcoidosis in presentation on chest imaging, though has been defined by varying amounts of coagulative necrosis in the granulomata (fixed but dead tissue), granulomatous vasculitis/angiitis (small blood vessel inflammation) and other sarcoidosis diagnostic indicators [575-578]. Despite the radiographic differences in presentation between both of these and classical sarcoidosis, it has been argued by Yale Rosen that both nodular sarcoidosis and NSG should be

considered aspects of sarcoidosis with clinical phenotypic differences, rather than separate defined conditions in of themselves [575]. Given the spectrum of other manifestations in sarcoidosis, this would seem to be a reasonable assertion. Sarcoidosis in children is rare and is the subject of discussion in definitions. Traditionally, "Blau Syndrome" (BS), "Early Onset Sarcoidosis (EOS)" and "Juvenile Sarcoidosis" (JS) were terms denoting three particular manifestations, though EOS and BS appear to be similar in presentation but differentiated by the heritability of BS versus the sporadic development of EOS [610]. Juvenile sarcoidosis occurs over the age of 5, usually between 9-15 years of age, and presents clinically in a similar way to adult sarcoidosis, with pulmonary and systemic manifestations [610, 611]. EOS and BS occur under 4/5 years of age and present with skin, eye and joint manifestations, usually progressive, and can lead to long term damage in these patients [612-614]. Blau syndrome has been associated with inherited mutations in the CARD15/NOD2 pattern recognition receptor gene [431]. Sarcoidosis in general does not appear to have a similar genetic component in NOD2 [615], however, sporadic development (not inherited) of CARD15/NOD2 mutations have been shown to be present in EOS patients [434].

1.8.2 Tuberculosis

Tuberculosis accounts for a large number of observed granulomatous disease cases worldwide, with as many as 9 million people and up to 1.3 million deaths estimated for 2012 [616]. *Mycobacterium* tuberculosis (MTB) infection leads to necrotising granulomatous inflammation in lung tissue and nearby lymph nodes (Figure 1.14) and at stages involves a similar profile in immune response to sarcoidosis, weighted towards a Th1 cell-mediated reaction [617-619]. The process underlying the infective process and granuloma formation is not fully understood, with questions regarding whether or not the granuloma has a protective or negative role in the infection [620-622].

MTB infects macrophages in the lung [623, 624], though it is also capable of infecting other cell types, in particular dendritic cells [624]. There is a question over the temporal events of granuloma formation, suggestions have been that early infection results in tissue necrosis before the formation of the granuloma [453]. Work in zebrafish models

suggest early macrophage recruitment and granuloma formation may aid in MTB survival and the spread of the organism [625]. However, this spread/dissemination may be advantageous for the immune system, as it appears to be controlled and allows a robust T cell mediated adaptive response to be initiated [626]. It should be noted that early response may be defined by neutrophil activation as well as macrophage resident response and a subsequent T-lymphocyte activation [627].

It is known that the success of *Mycobacterium* tuberculosis arises from its ability to evade specific immune responses, particularly phagocytosis. MTB disrupts the usual phagosome processes in macrophages/dendritic cells, preventing lysosomal breakdown of the pathogen and allowing intracellular survival [628]. This subversion of the normal phagocytic myeloid cell response and innate immune responses can, in exclusion of adaptive response, initiate the formation of granulomata [620, 629]. These studies do rely on zebrafish models however, which may not necessarily be relevant to organisms with adaptive immune systems; previous work has suggested adaptive T-lymphocyte response is essential in granuloma formation and maintenance (reviewed in [630]). As with sarcoidosis, TNF-α is considered vital in granuloma formation and subsequent integrity [631, 632].

Apoptosis is important within tuberculosis granulomata [303]; the Mycobacterium tuberculosis infection has been shown to induce macrophage apoptosis [633, 634], necessary for inhibiting the growth and proliferation of the pathogen [634, 635]. More virulent strains of MTB are capable of subverting the apoptotic pathways necessary for clearance and inhibiting pro-apoptotic TNF-α activity [634]. Heat shock proteins (HSP) also appear to play a vital role in the apoptotic pathways involved in granulomata [636], with organisms inducing granulomata such as *Toxoplasma gondii* able to utilise these HSPs, such as HSP65, to evade immune responses [637]. In sarcoidosis, more alveolar macrophages express apoptotic receptors in BAL compared with healthy controls [638], conversely the lymphocytes in BAL have been reported to be more resistant to apoptotic stimuli in sarcoidosis [639]. Another study suggested that macrophage-derived cells in the granuloma were pro-apoptotic [640], which would be comparable to that seen in TB. However, there is an element of contradiction between reports, as macrophages have been studied for IFN-γ induced inhibition of cells in tissue, concluding that macrophages

are resistant to apoptosis in granulomata [641]. Monocytes have also been shown to be resistant to apoptosis in sarcoidosis when compared with TB patients [642], suggesting that there may be a core difference between these two diseases in leukocyte responses. It is possible that different methods employed and analyses, as well as areas of focus, may be responsible for these contradictions.
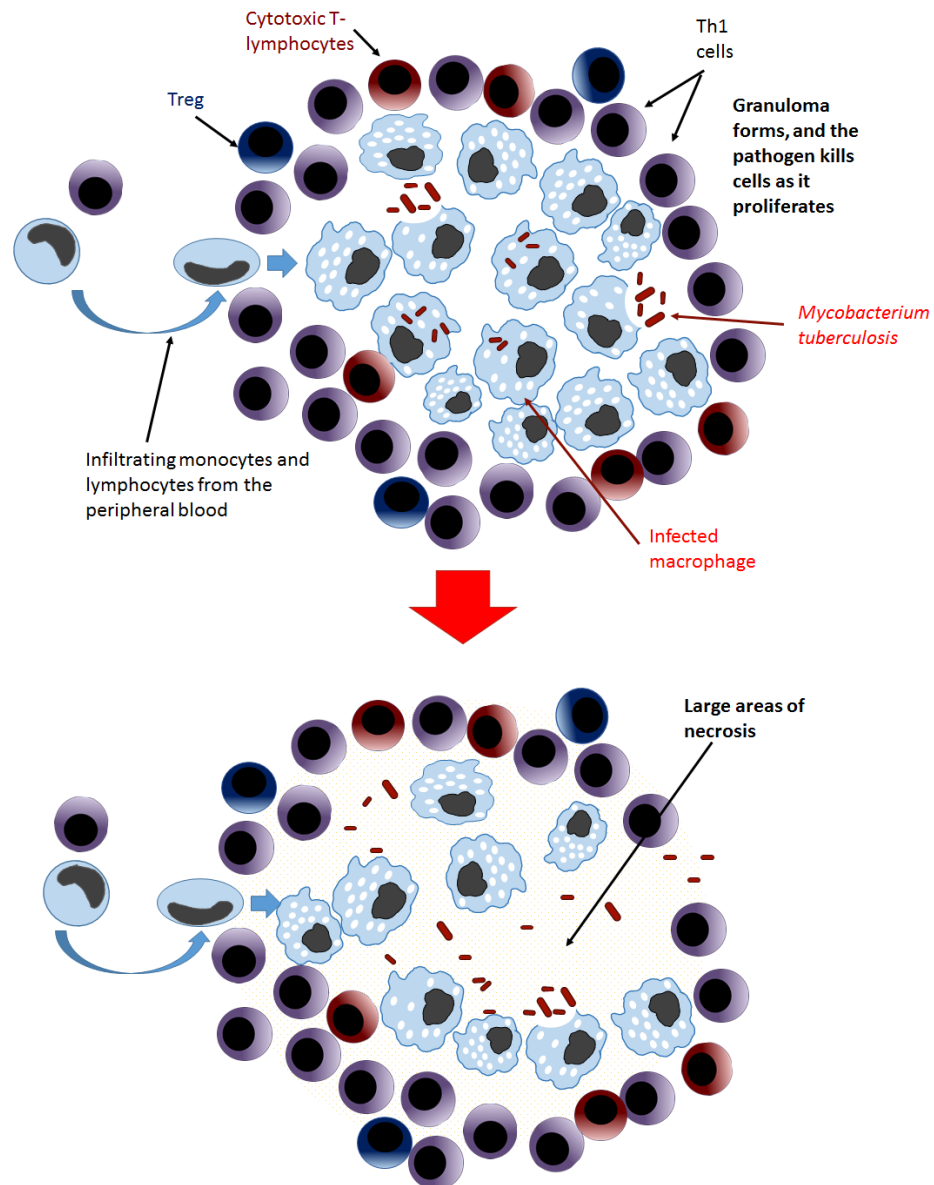
Figure 1.14: Diagram representing a granuloma in tuberculosis.

As *Mycobacterium tuberculosis* is engulfed by macrophages, the pathogen evades phagocytic destruction and proliferates within the cell, leading to the continued collection of macrophages and lymphocytes which form a granuloma. The principle distinguishing feature between TB and sarcoidosis is the central area of necrotic macrophages and mycobacterial infection, neither of which are features of sarcoidosis. Macrophages near the site of infection undergo apoptosis, which may aid in preventing dissemination of the pathogen.

As no infective causative organism has been identified for sarcoidosis (Section 1.6 – Aetiology), the necrotic granulomata seen in tuberculosis suggests a difference exists in the pathogenesis of the two different diseases. A comprehensive study by Kaufmann *et al* compared a range of features of pulmonary tuberculosis and pulmonary sarcoidosis, including gene expression and microRNA (miRNA) expression profiles, as well as serum analysis, in whole blood [643]. The vast majority of both gene and miRNA that were differentially expressed between disease and healthy individuals were the same between TB and sarcoidosis, though some unique gene and miRNA genes were altered between the two, particularly in genes related to protection against MTB. It is also clear from this analysis that, whilst the majority of genes and miRNAs share common alterations in both diseases, there are differences as determined by the differential expression between the diseases (691 genes and 4 miRNAs in this study) [643]. It is also notable that serum analysis between the diseases (and between healthy controls) suggested that TB patients exhibit increased inflammatory cytokines and chemokines necessary for migration of myeloid and granulocyte cells, whereas sarcoidosis exhibited lower neutrophil/eosinophil associated chemokine and cytokine concentrations in serum [643]. Another study by Thillai *et al* sought to characterise BAL and serum cytokine profiles in sarcoidosis and TB patients, compared with healthy controls [644]. Whilst differences in BAL cytokines were minor between TB and sarcoidosis patients, a predominant Th1 profile similar in both disease and higher levels of the Th2 cytokines IL-4 and IL-13 in sarcoidosis, both diseases displayed increased cytokine release from the lung relative to healthy controls [644]. Again, whilst classical Th1 cytokines (TNF-α and IFN-γ) are higher in both TB and sarcoidosis serum, it is the Th2 cytokines (IL-4, IL-5 and IL-13) that are reduced relative to controls, particularly IL-4 in TB [644]. These reports indicate that there is likely a similar underlying immunological mechanism between TB and sarcoidosis, with differences perhaps defined by the absence of a persistent infective agent and defects in sarcoidosis immune response that allow the pathogenesis to mirror some stages observed in mycobacterial infection. This has led some to suggest that sarcoidosis may be the result of TB, though the evidence supporting this assessment remains too limited to conclude this (see Section 1.6 – Aetiology).

1.8.3 Berylliosis

Berylliosis, also known as Chronic Beryllium Disease (CBD), is a granulomatous disease caused by environmental exposure to the metal beryllium [645]. Early identification of the disease was in workers using beryllium in the production of some lights [645], though it has uses in a wide variety of areas, including manufacturing, the aerospace industry and electronics [646]. Exposure to beryllium can be acute, though most will be chronic, either developing pneumonitis or granulomatous inflammation (CBD) [545]. Pathologically, CBD is indistinguishable from sarcoidosis, leading to non-caseating granulomata that can only be separated from classical sarcoidosis by patient history of exposure [546, 547, 647]. A significant proportion of patients are wrongly diagnosed with pulmonary sarcoidosis when they have CBD, though the latter tends to be restricted to the pulmonary system [546, 647]. This similarity goes beyond the clinical presentation; gene expression profiles between berylliosis and sarcoidosis are remarkably similar in both peripheral leukocytes and local lung tissue, though not identical [648]. Interestingly, whilst patients with sarcoidosis can self-resolve [2, 4], patients with beylliosis have progressive, incurable disease [649, 650]. The condition can be managed with treatment, and symptoms can even be reversed using corticosteroids, though the disease is not cured by the drugs [649, 650]. Whilst removal from the exposure may limit the progress of the disease, it will not lead to self-resolution of the condition [649, 650].

Whilst there are differences, berylliosis and sarcoidosis have been inextricably associated, with some questioning whether the former is a subset within "sarcoidosis diseases" or provides aetiological clues for sarcoidosis [651, 652]. Generally CBD is considered separate, though it is the disease most similar pathologically to pulmonary sarcoidosis out of all the granulomatous diseases.

1.8.4 Crohn's Disease

Crohn's disease and ulcerative colitis are the main types of inflammatory bowel disease (IBD), the former a chronic relapsing disease of the gastrointestinal system with an incidence ranging from 0.7 to 9.8 patients per 100,000 in Europe and 3.1 to 14.6 patients

per 100,000 in the U.S. [653, 654], though later studies show an increase to a maximum of 12.7 and 20.2 in Europe and North America respectively [655]. In keeping with the chronic nature of the disease, the prevalence is significantly higher, at approximately 320 per 100,000 people [655]. Granulomata have been considered a differentiating factor between ulcerative colitis and Crohn's disease, though not in of itself a definitive diagnostic indicator in exclusion of other clinical features [656-659]. Not all cases of Crohn's disease will present with granulomata, studies range from 24.3% to 68.9% of patients displaying the granuloma pattern in inflammation [660-662]. Epithelioid granulomata, similar in morphology to those observed in sarcoidosis, are the typical granulomatous presentation [660, 662, 663]. Much like Blau syndrome, NOD2 mutations have been strongly associated with susceptibility to Crohn's disease [429, 430, 664, 665]. As NOD2 recognises muramyl dipeptide (MDP) [666, 667], a bacterial peptide in peptidoglycan, this has supported concepts that Crohn's disease is an overactive response by the immune system to bacteria present in the gastrointestinal system [668, 669]. Although NOD2 gene mutations have not been associated with classical sarcoidosis, the similarity in non-caseating granulomata and cytokine responses suggests that a bacterial aetiology for sarcoidosis may be equally possible.

1.8.5 Other Diseases Displaying Granulomatous Inflammation

A very large array of other diseases can present with granulomata, but chemicals and non-organics can also lead to granulomatous inflammation. Tables 1.8 and 1.9, adapted from a review by D. Geraint James [670], present the most commonly observed infectious and non-infectious causes/diseases with granulomatous components. This is not all-encompassing, as atypical or rare granulomatous presentation can be a feature of other conditions (for reviews, see [69, 670]).

Infectious cases account for the majority of cases of granulomatous presentation. Along with TB, other species of mycobacterium lead to similar granulomatous patterns, suggesting common infective pathways and immune response mechanisms are present [69]. A wide range of other organisms can cause a granulomatous response (Table 1.9), though not always in a similar way. Schistosomiasis (helminth parasites – blood flukes) for

example is characterised by Th2 mediated responses in granulomata, though the parasite encourages the formation of these granulomata around the eggs itself to continue a lifecycle [671]. It should be noted that there is little compelling evidence that viruses of any kind lead to granulomata, with the presence of Herpesvirus/Epstein Barr Virus (EBV) being briefly studied in sarcoidosis [490, 491] and discounted as a cause of HIV associated granuloma annulare [672].

Non-infectious causes are often granulomatosis diseases (vasculitis), hypersensitivity responses in the pulmonary system to foreign agents (for example, Bird Fancier's Lung), an additional response to neoplastic disease or from a disparate collection of immunologic diseases [69, 670]. Whilst most of these disease groups with different aetiologies (or unknown causes) present with granulomata, they clearly have specific clinical pathologies and pathogeneses that differentiate them from each other [69, 670]. Inorganic materials have been associated with granuloma development, as seen in berylliosis, though other materials such as zirconium and silica are capable of inducing granulomatous responses, albeit rarely [673-675]. Specific inks used in tattoos (usually red or purple) have been reported to induce granulomata in a limited number of case reports [676-681], though this is not a common response to tattoos in the vast majority of people.

Table 1.8: Infectious causes of granulomatous disease.

Adapted from [69, 670].

Type	Disease/Cause
Mycobacteria	Mycobacterium tuberculosis
	Mycobacterium leprae
	Mycobacterium kansasii
	Mycobacterium marinum
	Mycobacterium avium
	BCG vaccine
	Buruli Ulcer (Mycobacterium Ulcerans)
Fungi	Cryptococcus
	Histoplasma
	Aspergillus
	Coccidioides
	Blastomyces
	Sporothrix
Bacteria	Brucella
	Yersinia
	Whipple's Disease (Tropheryma whipplei)
	Cat Scratch Disease (Bartonella henselae/Bartonella Quintana)
	Granuloma Inguinale (Klebsiella granulomatis)
	Lymphogranuloma Venereum (Chlamydia trachomatis)
	Listeria monocytogenes
Protozoa	Leishmania
	Toxoplasma
Trematoda	Schistosoma
	Paragonimus
	Chlonorchis
Spirochaetes	Treponema pallidum
	Treponema carateum
	Treponema pertenue
Unknown	Kikuchi Disease (Possibly Viral)

Table 1.9: Non-infectious causes of granulomatous disease.

Adapted from [69, 670].

Type	Disease/Source
Immunological	Sarcoidosis
	Crohn's Disease
	Blau Syndrome
	Langerhans Granulomatosis
	Primary Biliary Cirrhosis
	Hepatic Granulomatous Disease
	Orofacial granulomatosis
	Langerhans Cell Histiocytosis (LCH)
	Lupus Pernio
	Granuloma Annulare
	Chronic Granulomatous Disease (Leukocyte Oxidase Defect)
Vasculitis	Wegener's Granulomatosis (Granulomatosis with Polyangiitis- GPA)
	Lymphomatoid Granulomatosis
	Churg-Strauss Syndrome (CSS) Eosinophilic Granulomatosis with Polyangiitis (EGPA)
	Bronchocentric Granulomatosis
	Horton Disease (Giant-cell Arteritis - GCA)
	Necrotising Sarcoidal Granulomatosis (NSG)
Hypersensitivity Pneumonitis	Bird Fancier's Lung
	Farmer's Lung
	Mushroom Worker's Lung
	Coffee Worker's Lung
	Suberosis (Cork Worker's Lung)
	Bagassosis
Inorganic/Chemical	Beryllium
	Zirconium
	Silica
	Starch
	Talc
	Tattoo Pigments/Inks
Neoplasia	Carcinoma
	Reticulosis
	Pinealoma
	Dysgerminoma
	Reticulum cell sarcoma
	Malignant nasal granuloma

1.9 Summary

Sarcoidosis is best defined as a multi-system granulomatous inflammatory disease of unknown aetiology [1]. Despite being first identified over 100 years ago [27] , there is still a great deal about sarcoidosis that remains either unknown or not fully elucidated, including the immunopathogenesis and the cause of the disease.

Sarcoidosis is predominantly observed in the pulmonary system, but also commonly appears in the eyes, skin, neural and cardiac systems [1-3]. The disease has a heterogeneous clinical presentation, with extrapulmonary organ involvement being associated with worse prognostic outcomes [47, 48, 53, 54]. There are significant differences in organ involvement and severity depending on race, gender and age [93]. Broadly speaking, the disease can be acute or chronic , as well as mild, moderate or severe [3]. The majority of patients will self-resolve without the need for intervention, though others will develop progressive or chronic disease [4].

The disease is characterised by the presence of granulomata [1], inflammatory collections of macrophage-derived cells (histiocytes), including epithelioid cells and Langhans' giant multi-nucleated cells, as well as large numbers of T-lymphocytes [8, 9, 11]. These collectively display a Th1 polarised immune response, releasing large quantities of pro-inflammatory cytokines including TNF-α, IFN-γ, IL-6, IL-12 and IL-2 amongst others [280, 281]. Whilst the exact processes involved in the immunopathogenesis are not fully understood, the current paradigm involves 4 phases: initiation, accumulation, effector and resolution [260]. At present, the working theory is that resident tissue macrophages are incapable of effectively processing a putative antigen necessary for presentation, leading to initiation of the inflammatory activity [1, 260]. Macrophages activate T-lymphocytes, which in turn recruit other leukocytes to the site of inflammation [1, 260, 282], though the persistence and inability to process the antigen leads to differentiation of macrophages and formation of the granulomata [9, 11]. This granuloma formation is driven by TNF-α [297-301], though other cytokines such as IFN-γ are also important mediators for this [298, 301]. It is possible that resolution occurs once Th2 cytokine profiles exert themselves in unison with IL-10 or other immunosuppressive factors [315], though progressive fibrosis is associated with TGF-β expression [316]. Both macrophages

and T-lymphocytes are typically identified as being vital in the immunopathogenesis of the disease. Despite the elevated pro-inflammatory action of tissue resident cells in the disease, peripheral blood leukocytes are variably described as being either anergic [21-25], no different from healthy controls [26] or display heightened immune responses depending on the report [16-20], with the reduced responses being predominantly reported [1, 68]. This 'paradox' of increased local response versus reduced peripheral response has yet to be fully explained [68].

Genetic polymorphisms have been associated with the disease, mostly MHC genes (HLA) and other loci related to immunological factors, such as TNF-α [403-405, 446]. Some familial studies have indicated that there may be an inherited component to the disease [395, 396, 401, 402], though it would likely be as a complex multi-factorial profile [403]. The incidence can vary widely between countries, with European and American populations displaying much larger prevalence of the disease compared with East Asian groups [3]. The adjusted incidence ranges from 10.9-35.5 per 100,000 people, Black populations will tend to have higher incidence rates than Caucasians [48].

The aetiology remains elusive, *Mycobacterium tuberculosis* or other mycobacterial species are considered to be strong possibilities for an infective cause, as is *Propionibacterium acnes* [461, 462, 477]. Non-infective environmental causes are also touted as possible sources for initiating the inflammatory events [1, 503]. A large number of other diseases exist with granulomatous inflammation, including those with both infective causes (TB, schistosomiasis etc.) and those environmental causes (beryliosis etc.), as well as those with undetermined causes (Crohn's disease and others) [670]. Diagnosis is difficult in sarcoidosis and is made by exclusion of other causes of the identified clinical presentation [1, 308]. A combination of chest X-ray, clinical indicators (serum ACE levels and others) and the presence of non-caseating granulomata observed in biopsied tissue are required for a positive diagnosis, after excluding other possibilities [308]. Chest radiographic inflammatory involvement has traditionally been stratified using the Scadding CXR Stages, ranging from 0-IV, with 0 being limited inflammation to IV being advanced fibrotic disease [60]. Serum ACE in the blood has long being used as guide for aiding in sarcoidosis diagnosis, though not all patients will display elevated ACE levels and it is generally considered more a guide of granulomatous burden than a prognostic tool

[232, 237-239, 243]. Most patients do not require treatment; those that do are often prescribed immunosuppressive corticosteroids [587-590], though there is a need for more effective therapeutic options for sarcoidosis patients.

Overall, the disease is considered to be one with either an overactive immune response in activation or a dysregulated immune disorder, though the conflicting evidence for peripheral immune responses in sarcoidosis remains an issue in the disease. As sarcoidosis could be considered systemic disease, especially given that multiple organs can be present, it would suggest that specific features of leukocytes may be altered in peripheral blood in sarcoidosis to explain the paradox.

1.10 Hypothesis and Aims

The principle hypothesis under examination is that sarcoidosis is a disease that can display either elevated or diminished peripheral blood immune responses depending on the context. Specific stimuli may be capable of initiating increased pro-inflammatory responses in peripheral blood leukocytes, possibly as a result of hyperactive inflammatory activation or dysregulated immune responses.

The project has the following aims:

1. Identify differences in the proportion of leukocyte populations between sarcoidosis patients and healthy controls using peripheral blood mononuclear cells, with a particular focus on T-lymphocyte and monocyte subsets.
2. Characterise the inflammatory cytokine responses of whole blood leukocytes to non-specific immune activators in patients with sarcoidosis.
3. Investigate the role of innate immune receptor activation in peripheral blood leukocytes in sarcoidosis.
4. Characterise regulatory receptor expression and activity in sarcoidosis leukocytes.

- Aims 1 and 2 will feature in Chapter 3 – Peripheral Blood Leukocyte Immune Responses to Lectins and Mitogens in Sarcoidosis.
- Aim 3 will be the focus of Chapter 4 - Innate Immune Inflammatory Receptor Activity in Peripheral Blood.
- Aim 4 will be presented in Chapter 5 - Regulatory Immune Mechanisms in Sarcoidosis Peripheral Blood Leukocytes.

The outcomes will aim to answer the following research questions:

1. Are the proportion of specific T-lymphocyte and monocyte subsets different in sarcoidosis?
2. Is pro-inflammatory cytokine release from whole blood leukocytes reduced or enhanced in sarcoidosis in response to different immune activators?
3. Is innate immune receptor expression and activation altered in sarcoidosis?
4. Is there regulatory receptor dysfunction/alteration in expression on leukocytes in sarcoidosis?

The project aims to better understand peripheral blood leukocyte immunology to potentially identify factors that may aid in prognostic evaluation. As a pilot study, this would provide avenues for future research and identify areas that could possibly be targeted for therapeutic intervention or explain why some subsets of patients display more severe phenotypes than other patients.

Chapter 2 General Methods

2.1 Ethical Approval

Separate ethical approval was obtained for healthy controls and sarcoidosis/latent tuberculosis patients for blood samples and tissue. For healthy controls, local ethical approval was obtained from the Hull and York Medical School (HYMS), reference: 'HYMS 12 01 – The Immunology of Sarcoidosis'. Healthy volunteers were taken through a 'Patient Information Sheet' (version 1.1, 20/05/2013) outlining the background to the study, details of the study, contacts, risks, consent and other pertinent information detailed on the sheet (Appendix A2.1). For healthy controls, up to 30ml of blood was taken, as detailed in the information sheet and consent form. The 'Research Participant Consent Form' (version 1.1, 20/05/2013) required initials by each separate point highlighted and a signature by both the volunteer and researcher. The first sheet required volunteer details, including age, gender, medications being taken and smoking status. The second sheet ensured donors understood the details of participation in the study and agreement to take part. Finally, a 'Research Participant Debriefing Sheet' (version 1, 8.2.13) was provided after donation with details for contact if there were questions or issues.

Sarcoidosis and latent TB patients were consented through the NHS research ethics committee, using the 'Immunology of Lung Fibrosis' LREC approval (08/H1304/54). The ethics were amended through the official 'Notice of Substantial Amendment' to include lymph node tissue approval and an increase in blood samples to be obtained to 30ml (2013 LREC IPF Application v.3, 27/03/2013). Patients were given the 'ILD study Patient Information Sheet' (version 1.2, 29.03.13) and the aspects of the information were verbally explained, including details of the study, aims of research, what donation means and contact details (Appendix A2.2). The 'Consent Form' (version 1.2, 29.03.13) required initials from the patient by each point, as well as signatures from the patient, researcher and person taking the blood sample. For latent TB patients, patients were instructed to initial all boxes, with the exception of requests for bronchoalveolar lavage (BAL), lung/lymph node biopsy, pleural fluid, induced sputum and exhaled breath condensate, as these were not required for this study. Sarcoidosis patients were asked to initial all boxes with the exception of bronchoalveolar lavage (BAL), pleural fluid, induced sputum

and exhaled breath condensate, but were consented for lung/lymph tissue for existing samples from routine diagnosis.

All forms were stored, details were kept confidential and samples obtained were given anonymised identifiers for use in this study. Healthy controls were given 'D' prefixes followed by a number (D1, D2 etc.), latent TB were identified as LTB (LTB1, LTB2 etc.) and sarcoidosis patients were provided the SA identifier (SA1, SA2 etc.) to ensure anonymity.

2.2 Cohorts and Donor Selection

Individuals were recruited for the research in three independent groups: 'Heathy Controls', 'Latent TB Patients' and 'Sarcoidosis Patients'. All subjects were 18 years of age and above. Exclusion criteria for all groups included pregnancy, those with an immunodeficiency or chronic condition (aside from TB or sarcoidosis for those respective groups) such as HIV, diabetes, cancer etc. and those currently taking immunomodulatory medications, particularly corticosteroids.

2.2.1 Healthy Controls

All healthy volunteers were between the ages of 18-75 years old. Donors were defined as healthy by the absence of any diagnosed chronic conditions or acute medical conditions at the time of donation. All attempts were made to ensure donors were non-smokers and Caucasian, in order to match the typical sarcoidosis patient demographic. Where donors were smokers, this was recorded in patient details. Attempts to age match and gender match were attempted. Healthy controls had the number of peripheral blood mononuclear cells (PBMCs) counted as standard, but serum angiotensin converting enzyme (sACE) levels were not assessed. Data on age, medications, gender and smoking status were collected for all donors, linked to their anonymous identifier.

2.2.2 Sarcoidosis Subjects

Sarcoidosis subjects were all between the ages of 18-75 years of age. The diagnosis was by a multidisciplinary team assessment, summarised in Figure 2.1, principally confirmed by the presence of non-caseating granulomata within inflamed tissue. Typically, this was through a mediastinal lymph node biopsy or lung biopsy, though skin and other organ restricted sarcoidosis pathologies were diagnosed through biopsy of the appropriate area. Details of age and gender were recorded, as well as details of clinical findings and sACE levels. All patients were Caucasian and recruited locally in the Hull and East Riding region. PBMC number was also recorded for all patients.
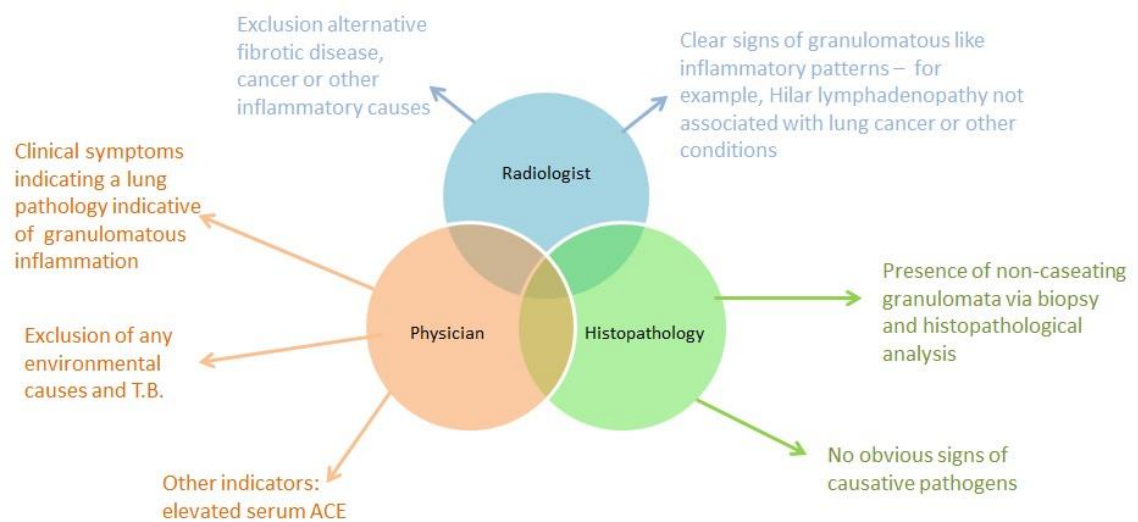
Figure 2.1: Multi-disciplinary team assessment criteria required for a positive determination of sarcoidosis.

Principle confirmation relies on histopathological analysis of tissue biopsies for confirmation of non-caseating granulomata and exclusion of other explanatory causes. Radiologist and physician will contribute with supporting evidence confirming a positive diagnosis of sarcoidosis, based on features agreed in the 'Statement on Sarcoidosis' in 1999 [1].

2.2.3 Latent Tuberculosis Subjects

Latent tuberculosis (Latent TB) subjects were defined as those individuals with a positive test result in either the QuantiFERON TB Gold In-Tube Test (Cellestis) or the T.Spot TB test

(Oxford Immunotec). Latent TB patients were not, at the time of donation, undertaking a course of anti-tuberculosis medication but may have been in receipt of other medication. Patients were of various ethnicities, due to the difficulties in obtaining donors diagnosed as having latent TB who have not started medication. These donors were used for limited numbers of experiments, and were not the primary patient group under study.

2.3 Antibodies

Antibodies were stored as per the manufacturer's instructions and those which required reconstitution were reconstituted as suggested in the associated material data sheets. Stock concentrations varied, but the same clones and antibody type were used to ensure consistency. For all antibodies, matched isotypes were obtained and used at the same concentrations as the primary target antibody.

2.3.1 Antibody Selection for Flow Cytometry

All flow cytometry experiments were completed using a Becton Dickinson Biosciences (BD Biosciences) FACSCalibur^{TM}, which uses two lasers for excitation: at 488 nm and 635 nm. Using this, the flow cytometer could detect 6 parameters; the forward scatter (FSC), side scatter (SSC) and 4 fluorescence channels labelled FL1, FL2, FL3 and FL4. FL1, 2 and 4 were used in experiments, with filters 530 nm, 585 nm and 661 nm respectively. Each potential fluorochrome has excitation and emission spectra, with wavelengths specific to each type (see Figure 2.2, taken from [682]). Three fluorochromes were selected; each were detectable in a different fluorescence channel. Fluorescein isothiocyanate, FITC, was detectable in FL-1. Phycoerythrin, PE, was detectable in FL-2. Allophycocyanin, APC, was detectable in FL-4. Where possible, combinations of PE and APC or FITC and APC were used in pairings to prevent spectral overlapping from FL-1 into FL-2 and FL-2 into FL-1, which would require compensation. Each antibody fluorochrome has properties, including the brightness of the fluorescence produced. The stain index is a measure of the relative fluorecence difference between positive and negative populations in staining with a particular fluorochrome over the breadth of the fluorecent spread in a negative

population [683, 684], D/W (Figure 2.3). Examples of stain index values are displayed in Table 2.1 (adapted from [685]). Using this index as a guide, the brightest fluorochrome with the highest stain index (PE) was typically selected for low abundance proteins or those of particular interest, for example, CD200R. APC and FITC were selected for higher abundance proteins or those used in basic phenotyping, for example, CD3. Combinations were selected based on this principle, usually comprising PE and APC combinations only.
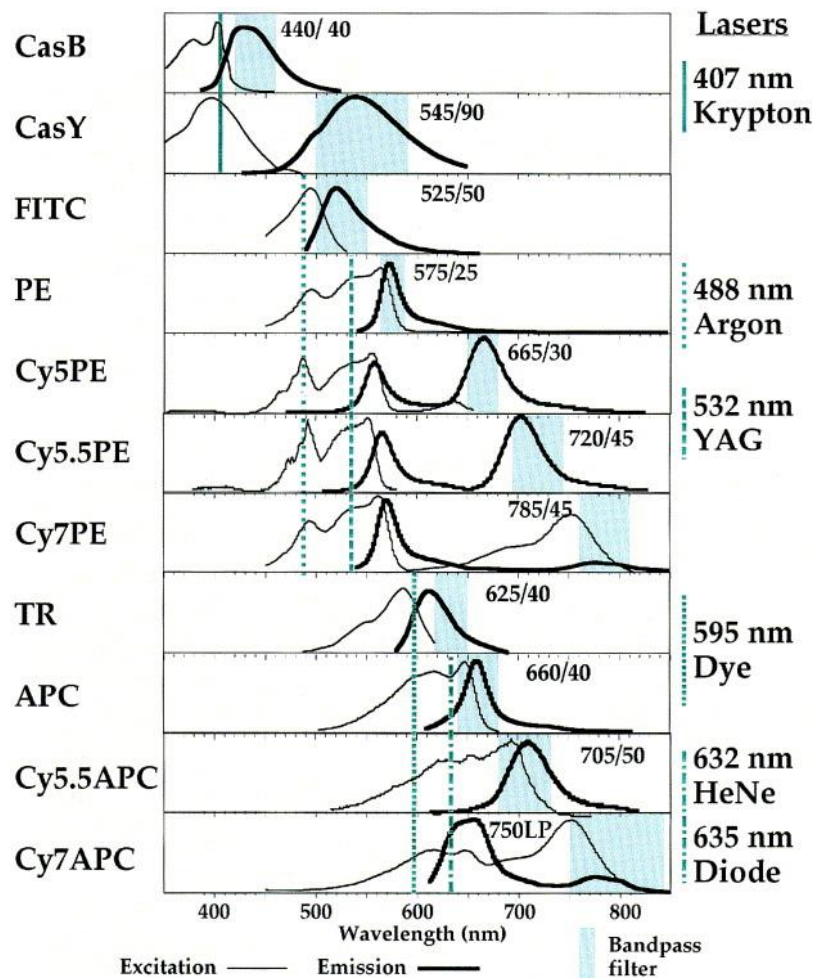
Figure 2.2: Commonly used fluorochromes with their respective excitation and emission spectra.

Excitation (thin black lines) and emission (thick black lines) spectra. Lasers emit different wavelengths, the FACSCalibur utilises the 488 nm Argon and 635 nm Diode lasers. The Argon laser excites PE and FITC fluorochromes and the Diode laser excites APC. Where emission spectra overlap between FITC and PE, neither displays any significant spectral overlap with APC. Taken from [682].
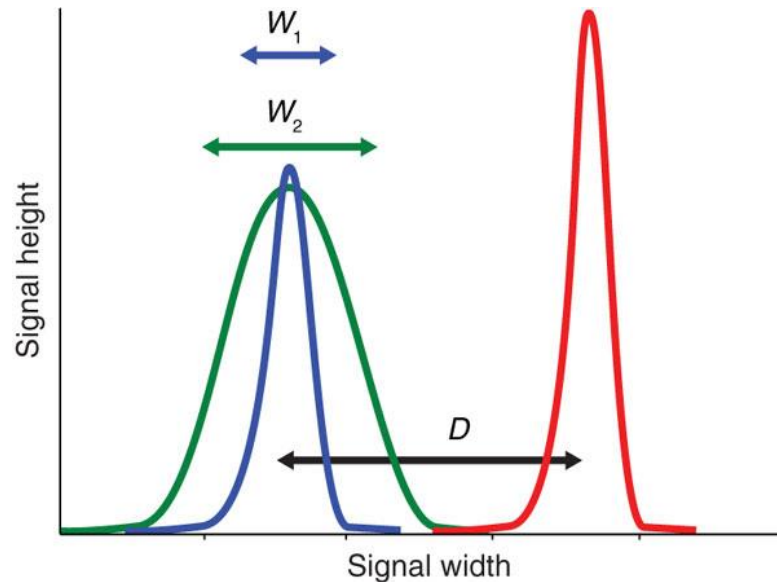
Figure 2.3: Stain index for fluorescent antibodies.

The distance between the mean signal fluorescence of the positive population (red line) and the negative population (blue line), D, broadly determines the signal brightness. The stain index is this brightness divided by 2 standard deviations of the width of the negative population, W_2. Thus Stain Index = D/W_2. Taken from [685].

Table 2.1: Example fluorochromes present on conjugated antibodies and the corresponding stain index

Fluorochrome	Stain Index
PE	302
APC	278
PerCP-CY5.5	107
Pacific Blue	80
FITC	56
PerCP	37

2.3.2 Antibodies for Immunophenotyping

All antibodies for immunophenotyping were obtained from Biolegend. All were monoclonal antibodies and single conjugated constructs. Unless otherwise stated, the antibodies were anti-human mouse IgG1 (Immunoglobulin G). Antibody pairings were selected that typically featured fluorescent conjugated tags that required little

compensation in order to increase the reliability of the quantitative data acquired. For example, a FITC (FL1) or PE (FL2) antibody would be paired with an APC (FL4) antibody. When 3 were required, antibodies would be selected with FITC, PE and APC fluorescent tags. Table 2.2 summarises the antibodies used for immunophenotyping, alongside a 'typical' stock concentration, the isoform the antibody and the fluorophore.

Table 2.2: Antibodies used for immunophenotyping of PBMCs and the isotype controls

Target Protein	Host Species	Isotype	Fluorophore	Clone	Reference Number	Additional Notes
CD3	Mouse	IgG1, κ	APC	UCHT1	300412	
CD4	Mouse	IgG1, κ	APC	RPA-T4	300514	
CD8a	Mouse	IgG1, κ	PE	HIT8a	300908	
CD14	Mouse	IgG1, κ	APC	HCD14	325608	
CD14	Mouse	IgG1, κ	FITC	HCD14	325604	
CD14	Mouse	IgG1, κ	PE	HCD14	325606	
CD16	Mouse	IgG1, κ	APC	B73.1	360706	
CD25	Mouse	IgG1, κ	FITC	BC96	302604	
CD45RA	Mouse	IgG2b, κ	FITC	HI100	304148	
CD45RO	Mouse	IgG2a, κ	PE	UCHL1	304206	
Isotype Control	Mouse	IgG1, κ	APC	MOPC-21	400122	Control for CD3, CD4, CD14 and CD16 APC
Isotype Control	Mouse	IgG1, κ	FITC	MOPC-21	400110	Control for CD14 and CD25 FITC
Isotype Control	Mouse	IgG1, κ	PE	MOPC-21	400114	Control for CD8a and CD14 PE
Isotype Control	Mouse	IgG2b, κ	FITC	MPC-11	400307	Control for CD45RA
Isotype Control	Mouse	IgG2a, κ	PE	MOPC-173	400212	Control for CD45RO

2.3.3 Antibodies for Intracellular Flow Cytometry Analysis of Cytokine Release

All antibodies for cytokine analysis were purchased from Biolegend. All were single conjugate antibodies with matching isotypes. Fluorophores were either PE or FITC for the target cytokine and paired with an APC expressing antibody for immunophenotyping, for

example, TNF-α PE antibody with a CD3 APC antibody. Where possible, these were monoclonal IgG1 isoforms. The antibodies selected are displayed in Table 2.3.

Table 2.3: Antibodies used for intracellular staining of cytokines in flow cytometry

Target Protein	Host Species	Isotype	Fluorophore	Clone	Reference Number	Additional Notes
IFN-γ	Mouse	IgG1, κ	FITC	4S.B3	502506	
IFN-γ	Mouse	IgG1, κ	PE	B27	506507	
IL-6	Rat	IgG1, κ	PE	MQ2-13A5	501107	
IL-10	Rat	IgG1, κ	PE	JES3-9D7	501404	
TNF-α	Mouse	IgG1, κ	PE	Mab11	502909	
Isotype Control	Mouse	IgG1, κ	FITC	MOPC-21	400138	Control for IFN-γ FITC
Isotype Control	Mouse	IgG1, κ	PE	MOPC-21	400140	Control for IFN-γ and TNF-α PE
Isotype Control	Rat	IgG1, κ	PE	RTK2071	400408	Control for IL-6 and IL-10 PE

2.3.4 Antibodies for Innate Immune Receptor Analysis

For RAGE and NOD2 staining, primary antibodies were unconjugated and required secondary antibodies in the protocol (see Section 4.2). The RAGE antibody was obtained from Santa Cruz (RAGE (A-9):sc-365154) and targeted a region common to RAGE, ES-RAGE (endogenously secreted RAGE) and sRAGE (soluble RAGE) isoforms on the N-teminal end of the receptor. NOD2 antibody was purchased from Biolegend as a mouse IgG2a (NOD-15, 625802). A purified mouse IgG1 isotype control was used for RAGE (MOPC-21, 400102) and a purified mouse IgG2a used for NOD2 (MOPC-173, 400202). TLR4 was targeted using a single conjugated PE mouse IgG2a antibody (HTA 125, 312806) with a matched isotype control antibody (MOPC-173, 400212), both obtained from Biolegend. The Secondary antibody was a polyclonal FITC anti-mouse raised in goat from Sigma Aldrich (F0257).

2.3.5 Antibodies for Regulatory Immune Receptor and Ligand Analysis

For the surface regulatory receptors and their cognate ligands, single conjugate antibodies were obtained from Biolegend. All were monoclonal, and with the exception of the FITC CD47, all were PE conjugates. The details of these antibodies are displayed in Table 2.4.

Table 2.4: Regulatory receptor and cognate ligand antibodies used for cell surface staining by flow cytometry

Target Protein	Host Species	Isotype	Fluorophore	Clone	Reference Number	Additional Notes
CD47	Mouse	IgG1, κ	FITC	CC2C6	323106	
CD172a/b (SIRPα/β)	Mouse	IgG1, κ	PE	SE5A5	323806	
CD200 (OX2)	Mouse	IgG1, κ	PE	OX-104	329206	
CD200 Receptor	Mouse	IgG1, κ	PE	OX-108	329306	
CD210 (IL-10R)	Rat	IgG2a, κ	PE	3F9	308804	
Isotype Control	Mouse	IgG1, κ	FITC	MOPC-21	400110	Control for CD47 FITC
Isotype Control	Mouse	IgG1, κ	PE	MOPC-21	400114	Control for SIRPα/β, CD200 and CD200 receptor PE
Isotype Control	Rat	IgG2a, κ	PE	RTK2758	400508	Control for IL-10R PE

2.3.6 Antibodies for CD200L/CD200R Functional Studies

For functional studies, all antibodies were unconjugated. CD200R and CD200L blocking antibodies were obtained from R&D Systems and reconstituted as per the manufacturer's instructions. The CD200R was a goat polyclonal (AF3414) with a matching goat polyclonal isotype from Santa Cruz (normal goat IgG: sc-2028). The CD200L antibody was a mouse

anti-human IgG1 monoclonal antibody (R&D #325531) matched with a LEAF purified mouse IgG1 κ isotype control antibody from Biolegend (BALB/c, 400124).

2.3.7 Antibodies for Immunohistochemistry

Staining of CD200R and CD200L was undertaken using antibodies suitable for immunohistochemistry (IHC) of Formalin Fixed Paraffin Embedded tissue (FFPE), aside from an initial screening of CD200L with a monoclonal IgG1 CD200L from Biolegend (OX-104, 329202) and matched control, stated to be suitable for IHC with fresh-frozen samples. CD200R IHC suitable antibody was obtained from Abcam as a rabbit polyclonal (ab198010) with control rabbit polyclonal IgG from either Santa Cruz (sc-2027) or Dako (X 0903), though these were not successful. Final antibodies used in the IHC for CD200L were a polyclonal goat antibody (AF2724) and the normal goat IgG control (AB-108-C), both obtained from R&D. CD200R staining was achieved using mouse monoclonal anti-human CD200R (IgG1, OX108, MCA 2282T) from AbD Serotec/Biorad. A matched mouse monoclonal IgG1 antibody obtained from Biolegend (MOPC-21, 400101) was used as a control.

2.4 Immune Stimulants and Cytokines

A range of immune stimulants were obtained which included proteins from the lectin family and mitogens, proteins defined as either Pathogen Associated Molecular Patterns (PAMPs) or Damage Associated Molecular Patterns (DAMPs) and the cytokines TNF-α and IFN-γ. All of these were reconstituted and stored as per the manufacturer's instructions. Stock concentrations were made, and aliquots frozen to prevent freeze/thawing derived degradation of the stimulants.

2.4.1 Mitogens and Lectins

Phytohaemagglutinin (PHA) was obtained from Sigma-Aldrich as PHA-P, a raw mixture of PHA isoforms including PHA-L and PHA-E (Lectin from *Phaseolus vulgaris* – red kidney bean). This was reconstituted in sterile autoclaved 1X PBS at stock concentrations of 10mg/ml and 1mg/ml. Stock aliquots and subsequently diluted working concentrations were stored at -20°C until use.

Wheat Germ Agglutinin (WGA) was obtained from Cambio (*Triticum vulgaris- WGA*) and reconstituted in deionised water at 10mg/ml stock concentrations and stored at -20°C until use.

The mitogen Staphylococcal Enterotoxin A (SEA) was obtained from Sigma-Aldrich (Staphylococcal enterotoxin A, from *Staphylococcus aureus* – S9399), and carefully reconstituted in a class II safety cabinet with appropriate personal protective equipment (P.P.E.) with deionised water. Aliquots were stored at 10mg/ml and 1mg/ml concentrations at -20°C until use.

2.4.2 Cytokines

Both inflammatory cytokines were obtained from Promokine, recombinant TNF-α (C-63722) and recombinant IFN-γ (C-60724). Both were reconstituted with PBS at a stock concentration of 100μg/ml and stored at -20°C until required.

2.4.3 Pathogen Associated Molecular Patterns (PAMPs)

Lipopolysaccharide (LPS) from Escherichia .*coli* was obtained from Sigma Aldrich, two different forms labelled 'LPS1' (L2630) and 'LPS2' (L3129). Unless otherwise stated, 'LPS' applies to LPS2, the alternative LPS1 was used as a comparison between the different forms. There was no significant difference between these two forms of LPS in initial testing, thus LPS2 was used for subsequent assays. These were reconstituted with PBS at 1mg/ml stock concentration. The NOD1 ligand γ-D-Glu-mDAP (IE-DAP), a mixture of γ-D-Glu-D-mDAP and γ-D-Glu-L-mDAP isoforms, was obtained from InvivoGen and reconstituted with PBS at 10mg/ml. Muramyl dipeptide/ N-Acetylmuramyl-L-alanyl-D-

isoglutamine hydrate (MDP), the NOD2 ligand, was reconstituted in PBS at 1mg/ml. The fungal antigen, Beta-glucan/ β-1,3-Glucan from Euglena gracilis (Sigma, 89862-1G-F) was reconstituted at 20mg/ml in PBS, though this was not a complete dissolution. The resulting mixture contained particulate matter, which would act more akin to zymosan particles. The TLR3 agonist Polyinosinic–polycytidylic acid, Poly I:C, was reconstituted in dH$_2$O at 10mg/ml (Sigma P1530-25MG). All PAMPs were stored at -20°C in aliquots until required.

2.4.4 Damage Associated Molecular Patterns (DAMPs)

A range of DAMPs were selected that agonise the receptor for advanced glycation end-products (RAGE). HMGB1/ HMG-1 human recombinant expressed in E.*coli* was obtained from Sigma (H4652) and reconstituted in PBS at 1mg/ml. Three members of the S100 protein family were used, S100A12, S100B and S100A9. S100A12 (Human S100A12 / CAGC / Calgranulin-C Protein) was obtained from Sino Biological (11143-HNAE-50), S100B/ S100 beta peptide was obtained from Abcam (ab30380), S100A9 (Human S100A9 / CAGB / P14 Protein) was obtained from Sino Biological (11145-HNAE-50). All S100 proteins were reconstituted in sterile PBS at 100µg/ml. All DAMPS were stored at -20°C until required.

2.5 Additional materials

RPMI 1640 media was obtained from Lonza and supplemented with 10% heat inactivated foetal bovine serum (FBS, Gibco) and an end concentration of 100 units/ml penicillin and 100µg/ml streptomycin (Lonza). Predominantly, 96 well round bottom culture plates were used to culture PBMCs. Whole blood was incubated in 2ml skirted round bottom centrifuge tubes. Histopaque 1077 (Sigma) was used for separation of PBMCs from whole blood. PBS tablets (Oxoid) were used to make the 1x PBS solution, which was autoclaved prior to use. FACs Blocking Solution was made using 1x PBS and 0.5% protease free high grade bovine serum albumin (BSA, Fisher Scientific). FACs fixing solution was made using 4% paraformaldehyde in 1xPBS solution, dissolved at 60°C until the solution is clear,

followed by sterile filtration at 0.2µm. The solution was stored at 4°C until required and replaced after 1 month to prevent excess aldehyde formation. FACs permeabilisation solution was made fresh for each test with 90% methanol solution (diluted with deionised water) and cooled in a spark-proof freezer at -20°C prior to use.

ELISA materials not supplied with kits included the ELISA wash buffer (0.05% Tween 20 in 1xPBS (Fisher Scientific), ELISA coating buffer (0.84g of $NaHCO_3$ and 0.356g of Na_2CO_3 in 100ml of deionised H_2O), assay diluent (1% BSA in 1xPBS), 96 well Nunc Maxisorb plates (Fisher Scientific), 1M Sulphuric Acid (VWR) and TMB substrate solution (Vector Laboratories).

Studies in CD200 ligand stimulation used recombinant human CD200 Fc Chimera (2724-CD-050, R&D Systems) and a control recombinant human IgG1 Fc (110-HG-100 R&D systems), reconstituted as per the manufacturer's instructions.

2.6 Culture Conditions

Whole blood undergoing incubation and isolated peripheral blood mononuclear cells (PBMCs) were placed in a 37°C and 5% CO_2 incubator for the required time. Samples were contained to prevent contamination of other material in the incubator.

2.7 Peripheral Blood Mononuclear Cell Isolation

Blood was taken using a vacutainer system with eclipse needles or butterfly needles (BD Biosciences) in 6.5ml sodium heparin vacutainer tubes and processed within 1 hour of collection. Heparinised whole blood was transferred to a 50ml falcon tube and diluted 1:1 with sterile 1xPBS, typically this was 10ml of blood mixed with 10ml of PBS, but could range between 5-15ml (10-30ml total). The mixture was carefully layered over Histopaque 1077 in a 1:1 ratio (20ml of blood over 20ml of Histopaque) using a sterile Pasteur pipette, ensuring that two distinct layers were observed, as shown in Figure 2.4. The 'gradient' (blood and Histopaque) was centrifuged at 600g for 30 minutes without

braking, which resulted in the layers shown in Figure 2.4. The PBMC layer was removed with a sterile Pasteur pipette, re-suspended with RPMI media up to 10ml total and centrifuged at 800g for 8 minutes. This wash was repeated and the end PBMCs re-suspended in 2-5ml of RPMI media as appropriate. The total PBMC concentration was adjusted to $2x10^6$ cells/ml after haemocytometer counts for use in other experiments.
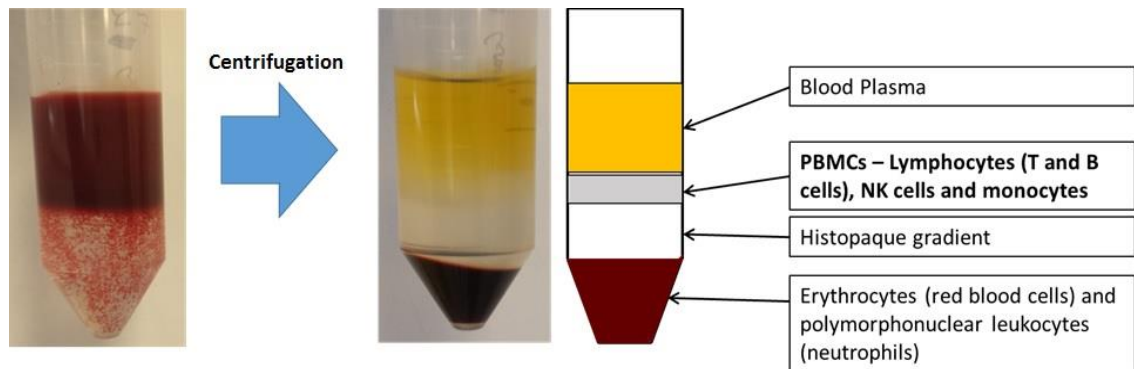
Figure 2.4: Isolation of peripheral blood mononuclear cells from whole blood.

Whole blood is layered over Histopaque 1077 gradient media and centrifuged in a method derived from the first Ficoll-Paque method developed by Böyum *et al* [686]. Layers form after centrifugation, with erythrocytes and granulocytes at the bottom, as these are dense, followed by the Histopaque gradient. The less dense PBMCs form a layer, also known as a 'Buffy Coat', with plasma on top. The PBMCs can be carefully extracted from this Buffy Coat using a pipette with minimal perturbation.

2.8 Antibody Titration

Prior to use, antibodies were titrated to establish the optimum concentration for use with the PBMC cell preparations. This was optimised using healthy PBMCs at $2x10^6$ cells/ml using the extracellular staining method (see Section 2.9). Two parameters were used to calculate the optimum antibody concentration, the geometric mean fluorescence intensity (Geomean) and the percentage of positive cells. The minimum concentration required 100% of cells positive for the marker to be shown as positive in the staining. The geometric mean, a measure of the expression of a protein on each cell, was calculated and plotted in the form of a line graph. The optimum concentration was the greatest concentration of the linear portion of this graph, an example is displayed in Figure 2.5.

Based on these results, 10µg/ml was selected as the optimum concentration for the PBMC preparations to allow saturation of the antibody without significant non-specific binding as a background signal.
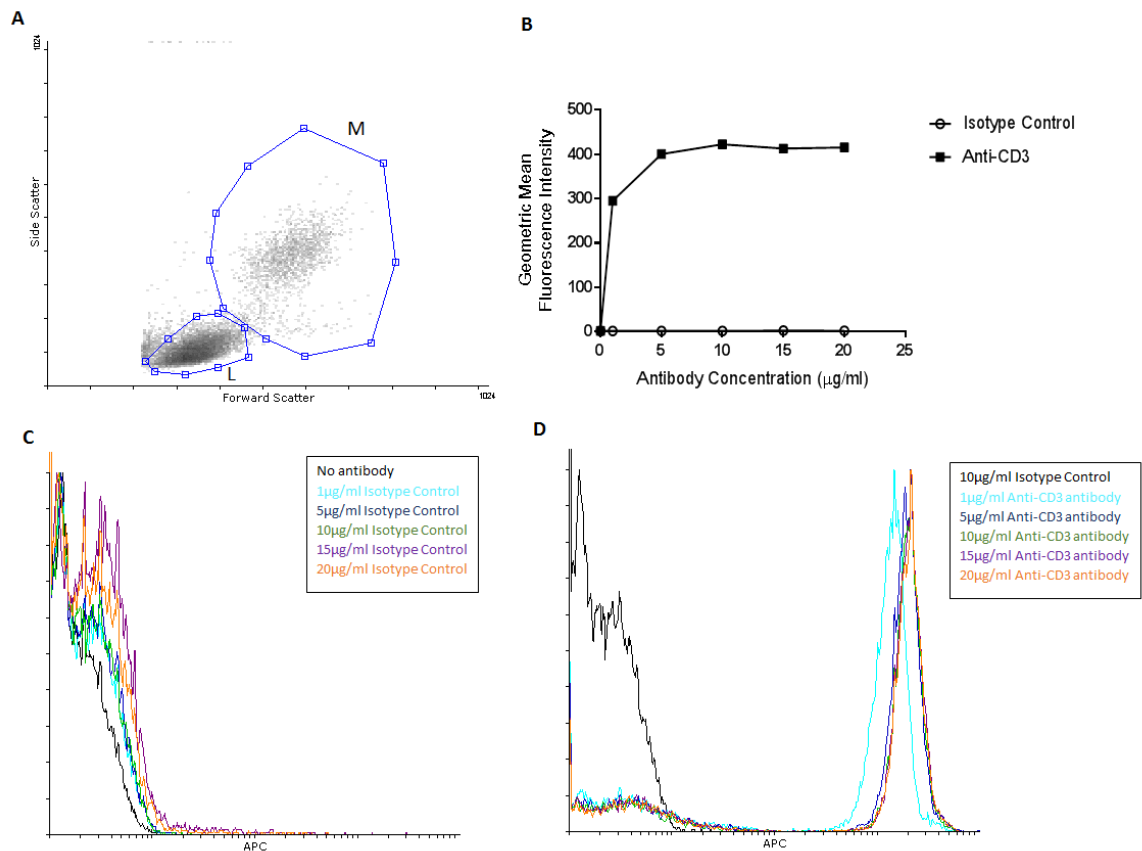
Figure 2.5: An example of an antibody titration using an APC conjugated anti-CD3 antibody on healthy control PBMCs.

A: A density gradient of PBMCs visualised using forward scatter (FSC) and side scatter (SSC), with the lymphocytes labelled 'L' gated based on size and low granularity and the monocytes labelled 'M' gated with greater size and granularity. **B**: Titration curve displaying antibody concentration of the anti-CD3 APC conjugated antibody (X axis) and the geometric mean fluorescence intensity (Geomean) ,used as a relative measure of expression, shown for positive lymphocytes (T-lymphocytes). The antibody is saturated at 5-10 µg/ml of antibody. **C:** Histogram of isotype control antibody APC fluorescence. Increased concentrations above the 10 µg/ml can increase non-specific binding (purple and orange lines). Y axis is cell count. **D:** Histogram displaying an isotype control antibody with positive anti-CD3 staining of lymphocytes. Saturation occurs at 5-10 µg/ml of antibody (cyan and blue lines respectively). Y axis is cell count.

2.9 Extracellular Staining of PBMCs for Flow Cytometry

PBMCs were prepared as appropriate for individual experiments; all were seeded at $2x10^6$ cells/ml in the wells of a 96 well round bottom plate ($2x10^5$ cells per cell). The process outlining the principles of flow cytometry is summarised in Figures 2.6 and 2.7. After the culturing/incubation, the plate was centrifuged at 800 x g for 5 minutes to pellet the cells from the solution, followed by two washes by re-suspending cells in 100μl of FACs blocking solution and centrifugation at 800 x g for 5 minutes. Cells were re-suspended in FACs blocking solution and left to block for 1 hour at 4°C. After the plate was centrifuged again, antibody solution was added at 10μg/ml (50μl per well) for 1 hour at 4°C (on ice). Each target antibody was duplicated with an isotype control at the same concentration, and a well of unstained cells. For unconjugated primary antibodies, a secondary antibody step was added with a FITC secondary incubated, after the primary, for 1 hour at 4°C at 5μg/ml. After being washed twice with FACs blocking buffer, cells were re-suspended in 100μl of FACs blocking buffer, transferred to FACs tubes and made up to 200-300μl of solution with FACs blocking solution. Samples were analysed using a FACSCalibur flow cytometer and Cellquest Pro software (BD Biosciences).

2.10 Intracellular Staining of PBMCs for Flow Cytometry

Figures 2.8 and 2.9 display the steps involved in a typical intracellular staining procedure, which differs from extracellular methods. Cells were prepared as necessary for different experiments and extracellular staining of cell surface markers was completed as described previously (Section 2.9) prior to intracellular staining of the target for standard protocols. After extracellular staining, cells were treated with 100μl of fixation solution (4% paraformaldehyde solution) for 20 minutes at room temperature in the dark. After washing 2 times with FACs blocking solution, cells were permeabilised with ice cold 90% methanol for 30 minutes at 4°C. After washing 3 times with FACs blocking solution, cells were stained with 10μg/ml of the primary conjugated antibody for 1 hour at 4°C. Cells were washed, re-suspended in FACs blocking solution and prepared for analysis as described previously (Section 2.9).
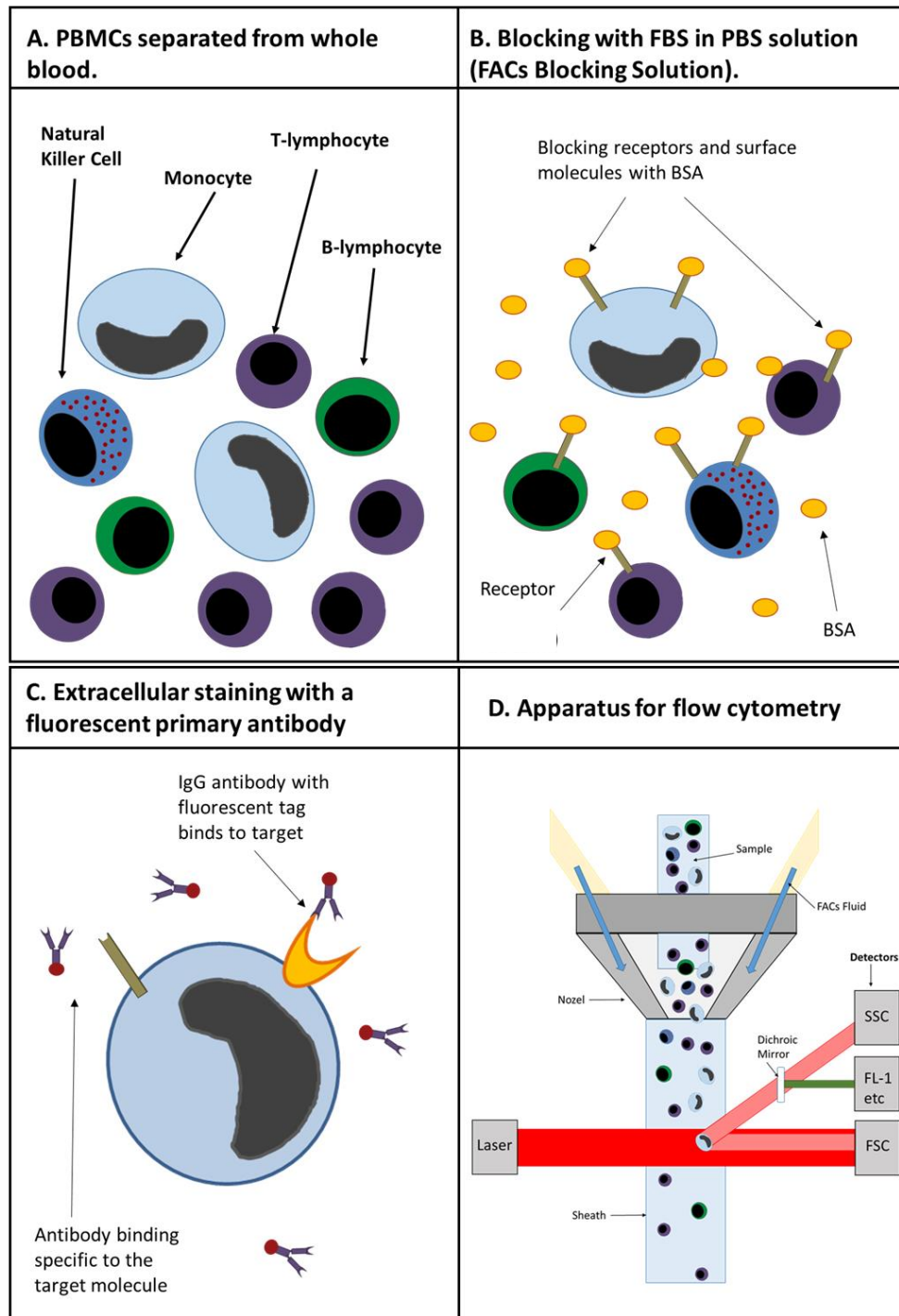
Figure 2.6: Stages in the extracellular staining of PBMCs for flow cytometry

A: PBMCs contain a range of leukocytes, including T and B lymphocytes, monocytes and natural killer cells. **B:** Bovine serum albumin (BSA) solution is used to block potential non-specific binding of the target antibody to receptors and cell surface targets. **C:** Single conjugated IgG antibodies are used to bind specifically with high affinity to the target protein. **D:** Cells are passed at high pressure through the flow cytometer with 'FACs Fluid', through the laser(s) which detect the cells and refracts and diffracts lights to the detectors.

A. Forward and side scatter

Forward Scatter (FSc) Detector = Size

Diffracted Light

Refracted and Reflected Light

Side Scatter (SSc) Detector = Granularity

Laser

B. Excitation and emission of the fluorescent conjugate on the antibody

Fluorescent signal emitted: e.g. APC

Laser source

C. Detection of a fluorescent signal

Background fluorescence, autofluorescence or spectral overlapping signal

FL-1

Strong fluorescent APC emission signal

FL-2

FL-4

Fluorescent signal emitted: e.g. APC.

D. Interpreting data

Isotype 1% Boundary

No Expression

Count

FL-4 Fluorescence Intensity

Low Expression

Count

FL-4 Fluorescence Intensity

High Expression

Count

FL-4 Fluorescence Intensity

Figure 2.7: Detecting the parameters of cells in flow cytometry.

A: PBMCs pass through the flow cytometry laser, diffracting light for FSC to provide size, and refracted/reflected light is detected for SSC as a measure of granularity. **B:** The fluorochromes on antibodies bound to the cell are excited by the laser, emit light of the specific wavelength corresponding to the fluorochrome. **C:** Emitted light is filtered through dichroic mirrors, allowing fluorescent signals to be detected by the appropriate detector, for example, FL-4 for APC. **D:** Fluorescence signals indicate the level of expression of the protein. Low fluorescence indicates low expression, increased fluorescence indicates a higher level of expression.
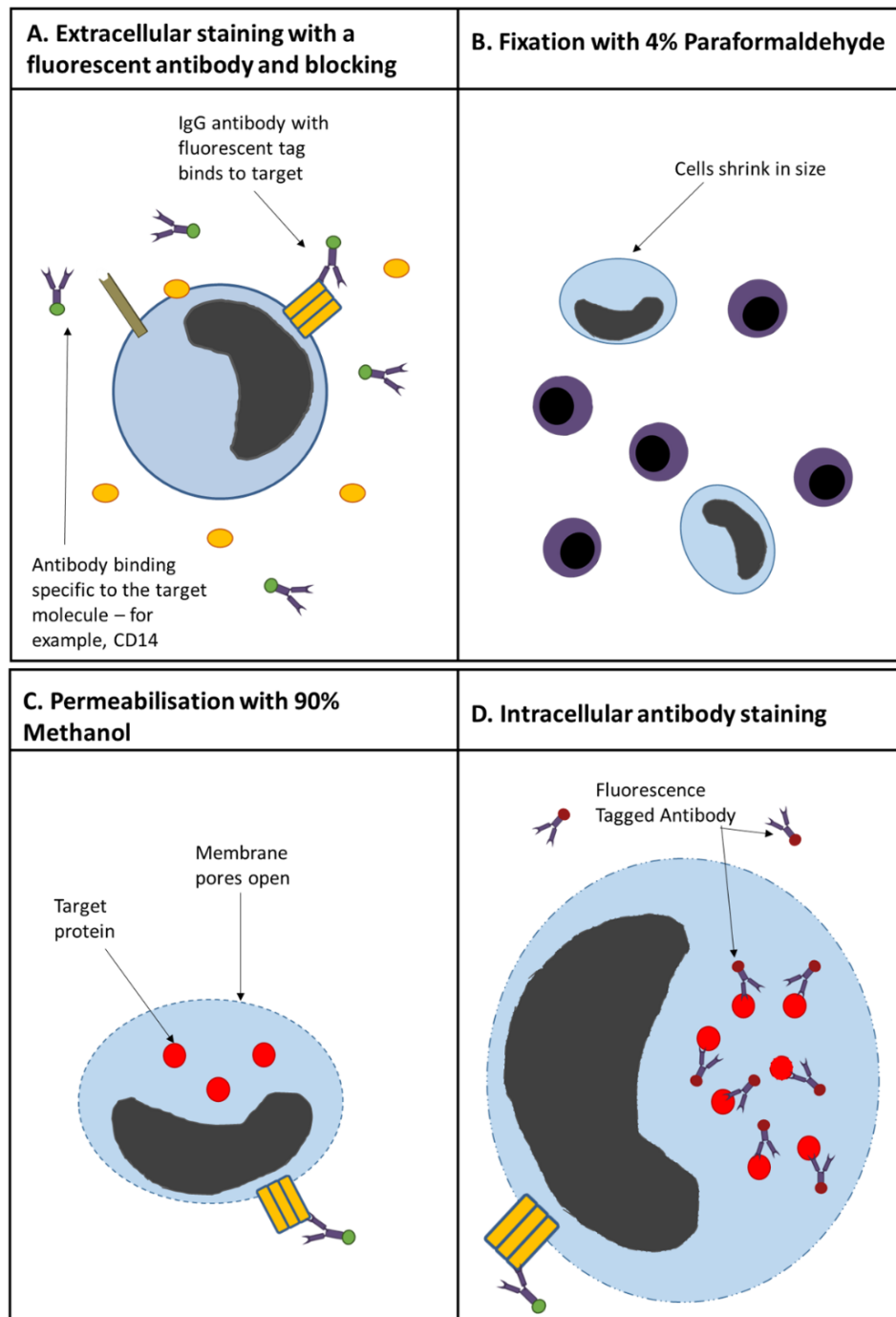
Figure 2.8: Stages in the intracellular staining of PBMCs for flow cytometry

A: Cells are prepared in a similar manner initially, with extracellular markers stained using a standard protocol. **B:** Cells are fixed in paraformaldehyde, which preserves the cell and prevents additional cellular activity. **C:** The cell membrane is made permeable with ice cold methanol, which will allow the passage of antibodies into the intracellular environment. **D:** Antibody staining occurs, after intracellular blocking with BSA. The antibodies bind specifically to the intracellular target protein, for example, a cytokine or intracellular receptor.

A. Fluorescent Excitation and Emission	B. Histogram analysis
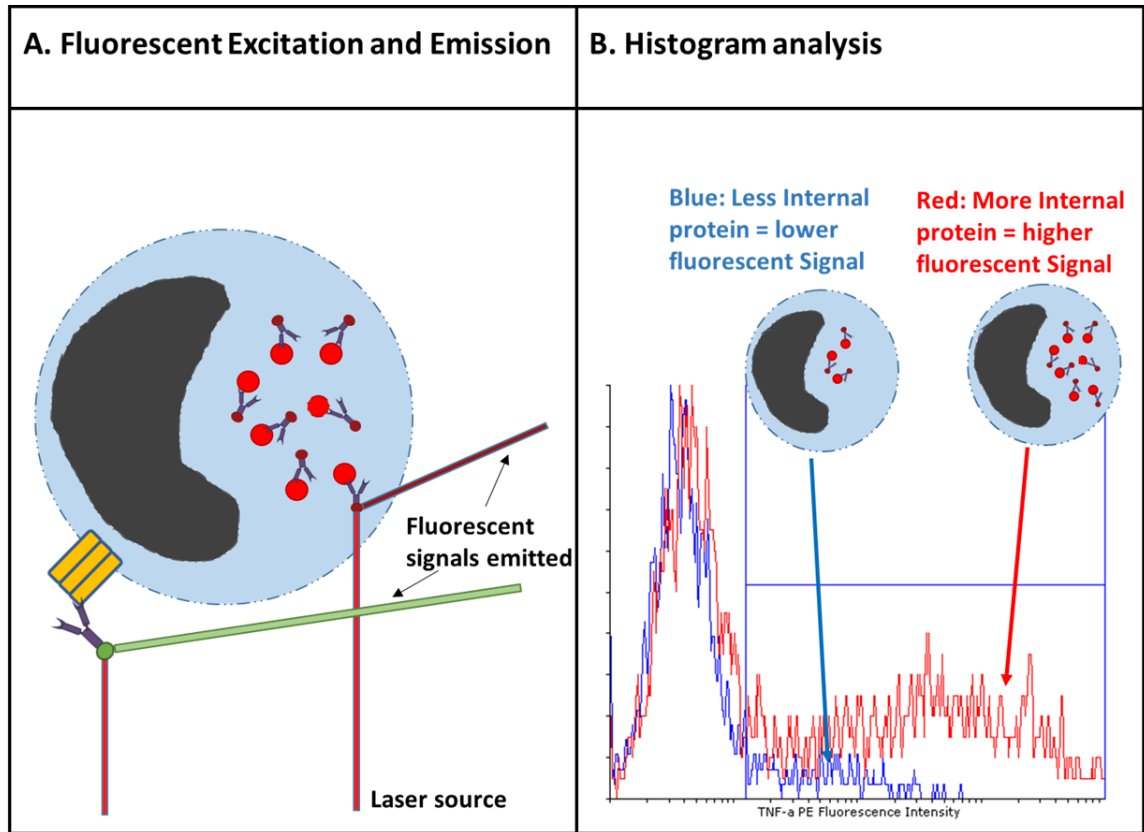
Figure 2.9: Fluorescence detection in intracellular staining for flow cytometry.

A: Cells bound with multiple antibodies are excited by the laser source, but emit fluorescence at different wavelengths corresponding to the fluorochrome. Therefore, positive identification of the proteins and the levels of expression can be determined using a flow cytometer. **B:** An example histogram displaying signals from internal proteins in two different samples (in this case, the cytokine TNF-α). The increased fluorescent signal observed in the red line suggests the cells have a greater protein concentration internally than those from the blue line. Additionally, the area under the graph suggests a greater percentage of the red sample are positive for the protein. Y axis is cell count.

2.11 Flow Cytometry Data Analysis and Interpretation

Initial parameters, regions and settings were optimised using Cellquest Pro software (BD Biosciences). For robust analysis, 10,000-50,000 total events were captured, which excluded events below thresholds established and assessed to be cell fragments and waste material. On rare occasions where the cell number within the field of analysis was low, a reduced number of events were recorded and analysed. A dot plot was used for cell size and granularity, determined by forward scatter (FSC) and side scatter (SSC)

respectively. The field of vision and settings were adjusted to incorporate the total cell populations, excluding outlying duplicate or clumped cell masses with very high granular or size properties. An example of this is shown in Figure 2.10.

Voltage settings for the channels were set using isotype control antibodies, where the histogram displaying fluorescence for the control was visible and peaked below 10^1 on the logarithmic scale. FL-1 to FL-4 were the channels available for detection: FITC conjugated antibodies were detected using FL-1, PE using FL-2 and APC using FL-4. Compensation was required when FITC and PE antibodies were used in unison, FL-1 minus FL2 and FL2 minus FL1 percentages determined based on initial compensation experiments with isotype controls and target antibodies in different combinations. Depending on the fluorescent profiles, this compensation was adjusted for different antibody compensations to prevent fluorescence 'bleeding through' from particular antibody conjugates into nearby detection channels.

Files were saved and transferred to 'Flowing Software 2.5.0' (Perttu Terho) for further analysis. Samples could be represented in different formats; in dot-plots with quadrants (Figure 2.10), in density plots (Figure 2.11) and histograms (Figure 2.12).

The data was analysed appropriately for the specific parameter under investigation, selecting the most appropriate format for representation as required. Interpretation is mentioned in the specific method sections (see Sections 3.2, 4.2 and 5.2), in general the lymphocyte and monocyte gates were determined using FSC and SSC dot plots, shown in Figure 2.10, and then further analysis by either histograms or quadrant analysis using dot plots or density/intensity plots.

Quadrant or histogram gates were set at 1% of isotype positive fluorescence for IgG1 antibodies, or 2% for IgG2 antibodies (to account for greater Fc receptor binding of the latter antibody). Values for percentage positive cells or the geometric mean fluorescence intensity (Geomean) were transferred to excel spreadsheets after initial analysis and organised for subsequent graphical and statistical analysis.
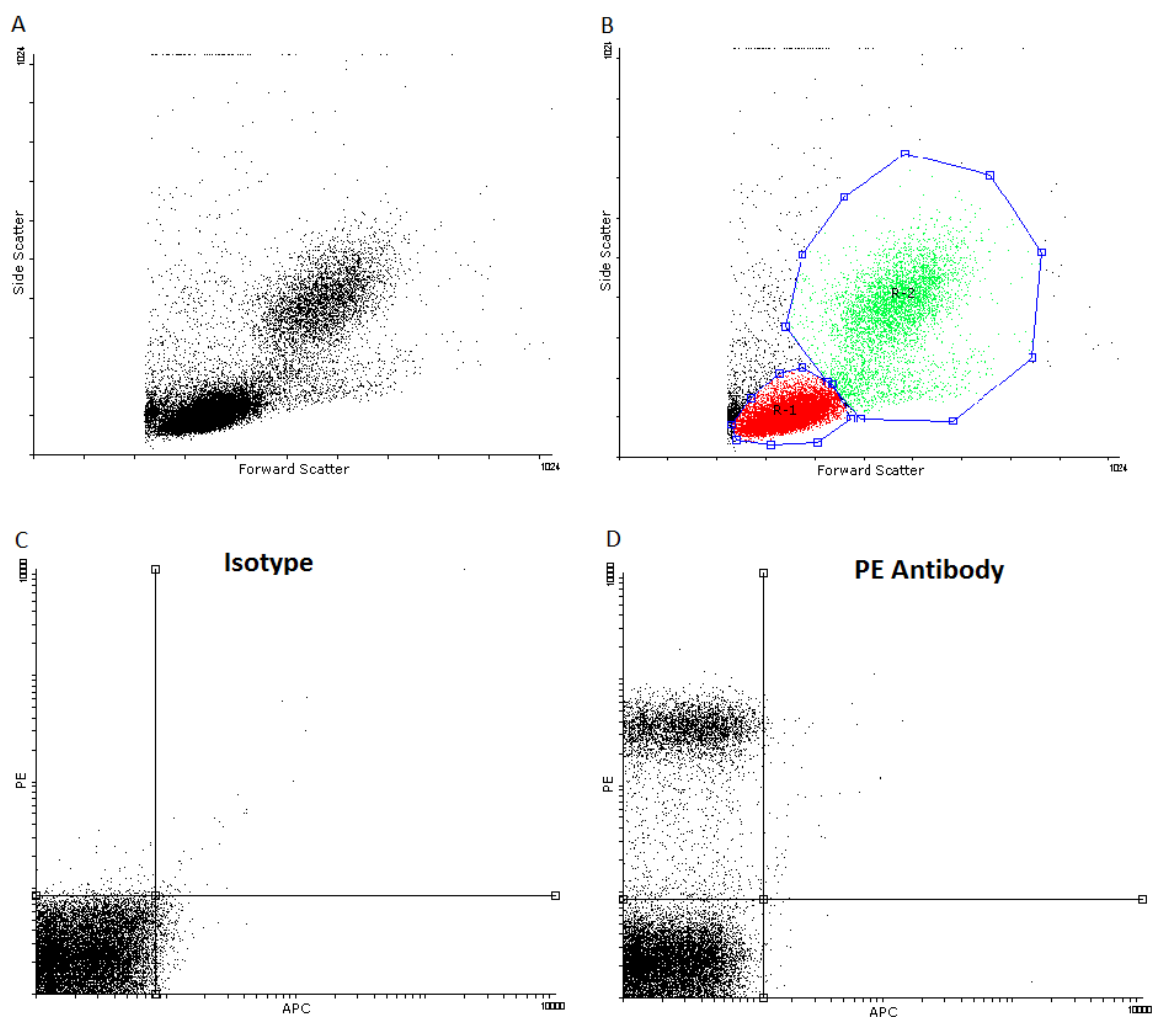
Figure 2.10: Examples of the dot plot format for representing flow cytometry data.

A: PBMCs from a healthy control**.** **B**: PBMCs from a healthy control gated for the lymphocytes (R-1, red cells) and monocytes (R-2, green cells). Gating based on size and granularity; previously confirmed using CD3, CD19, CD56 and CD14 differential staining. **C:** PBMCs stained with a PE isotype control antibody, displaying a quadrant. The right-bottom and left-top quadrants were set to include 1% of the cell populations each. **D:** PBMCs stained with a PE conjugated antibody, a proportion of the cells are represented in the left-top quadrant, described as positive for the particular protein. Cells in the left-bottom quadrant are negative for the protein.
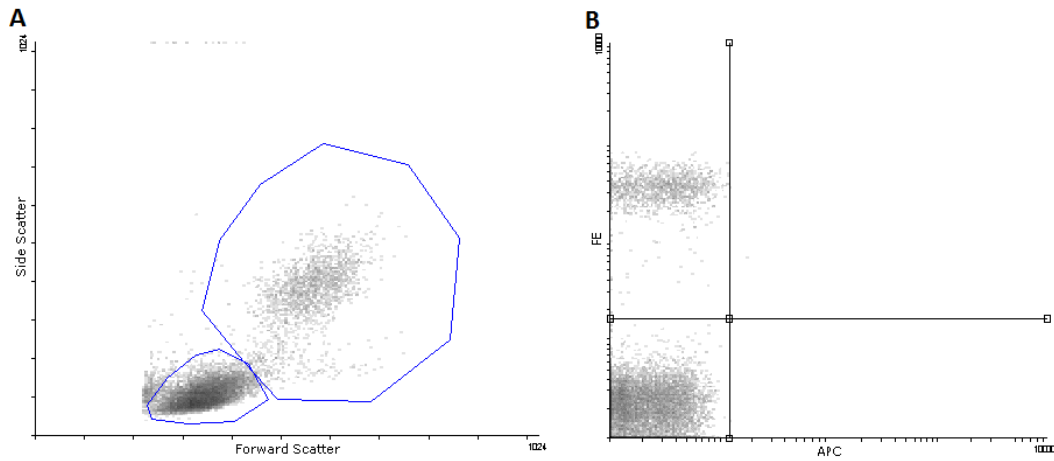
Figure 2.11: Examples of the density plot format for representing flow cytometry data.

A: PBMCs from a healthy control gated for the lymphocytes and monocytes, for the data represented in Figure 2.10B. **B:** PBMCs stained with a PE conjugated antibody, a proportion of the cells are represented in the left-top quadrant, described as positive for the particular protein. For the data represented in Figure 2.10.
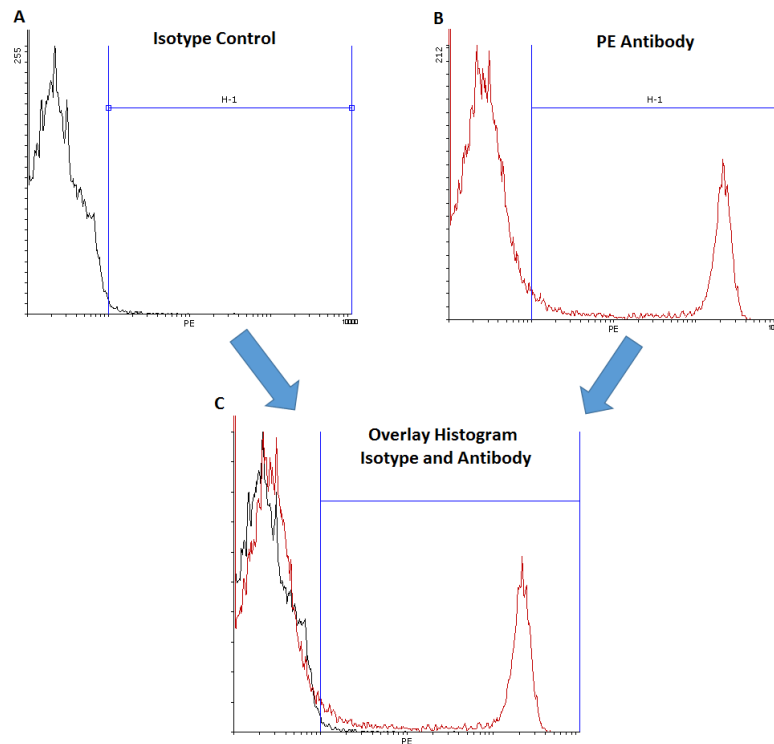
Figure 2.12: Examples of the histogram format for representing flow cytometry data.

A: isotype control antibody for PE staining healthy control PBMCs. H-1 region was set at 1% of the population within this gate. **B:** PE antibody staining of PBMCs, the peak represented with H-1 is the primary positive population, with lower expression observed within the H-1 region to the left of this population. **C:** An overlay histogram comprising both the isotype control (black line) and PE antibody (red line) from **A** and **B**. Maximum peaks have been adjusted to be equal in height for both samples, thus these do not represent comparative counts, but allows for the comparison of relative fluorescence. Y axis represents cell count.
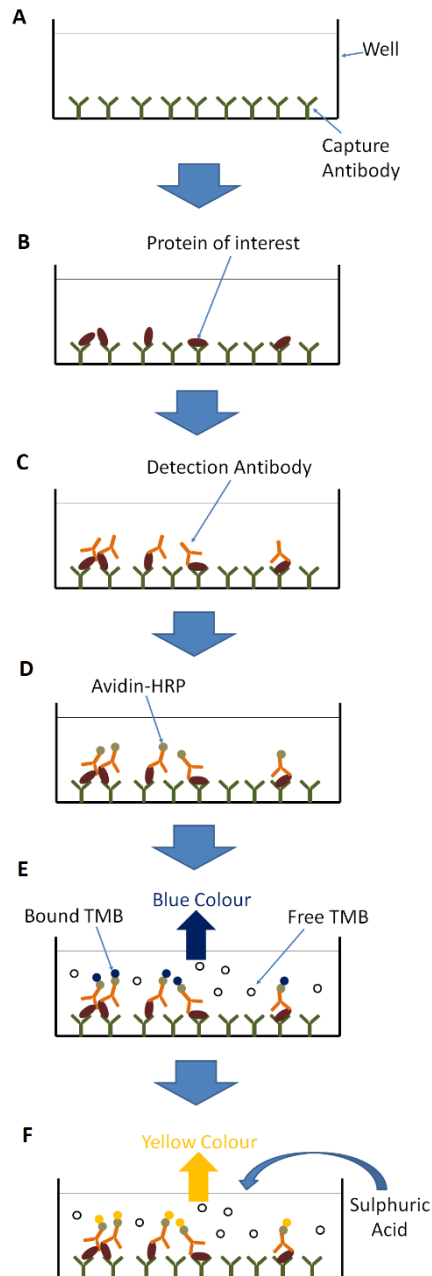
Figure 2.13: The stages of a standard sandwich Enzyme-linked Immunosorbant Assay (ELISA).

A: The wells of a 96 well plate are coated overnight at 4°C with a capture antibody with an affinity for the protein of interest, for example, TNF-α. BSA is then used to block the surface of the plate to prevent additional non-specific binding of proteins to the well surface. **B:** The sample plasma (or cell supernatant) is added to wells, allowing the antibody to bind to the target protein. **C:** The biotinylated detection antibody, with a different epitope to the capture antibody, then binds to the target protein. **D:** The Avidin-HRP binds to the biotinylated detection antibody. **E:** TMB is added, the blue colour indicates the presence of the HRP, therefore also indicates the presence of protein. The amount of blue colour is proportional to the protein concentration. **F:** An acid 'stop' solution is used to halt the progress of the reaction. The plate can then be read using a spectrophotometer.
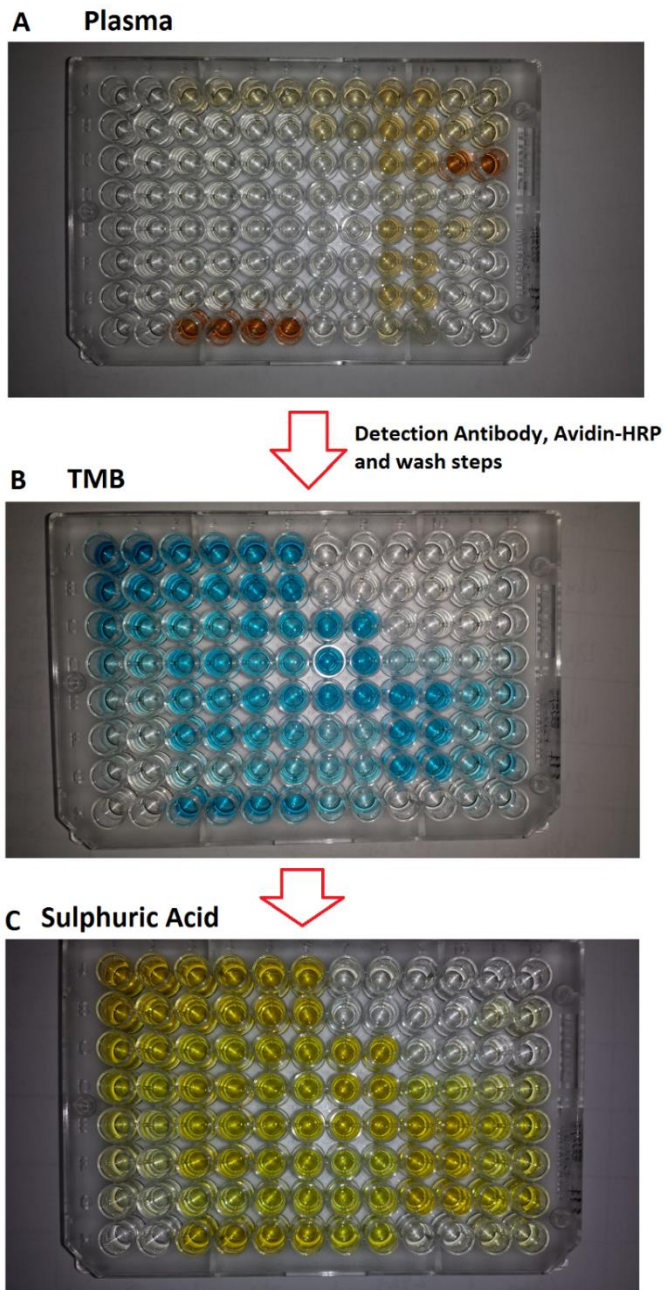
Figure 2.14: A sample plate in different stages of a standard TNF sandwich ELISA protocol.

A: Plasma is added to the plate in either a concentrated (yellow and orange wells) or diluted form (clear wells). Columns 1 and 2 (furthest left columns) are used with serially diluted protein standards, in this case, recombinant TNF-α protein. **B:** After sample incubation, detection antibody and avidin-HRP steps (with washes in between), TMB solution is added. Wells with the darkest blue have the greatest concentrations of the protein. **C:** After the stop solution, the yellow colour corresponds to the blue colour seen previously and therefore the greater the yellow colour, the more protein was present in the original sample.

2.12 Whole Blood Assay Procedure

Heparinised blood samples were transferred from vacutainer tubes to a 50ml falcon tube prior to any assays. Stimulants were added to 2ml round bottom skirted microfuge tubes before any blood was added. Each stimulant volume was maintained at 10.1μl and 1 ml of blood was added to each tube, 1% of the volume. Tubes were incubated at 37°C for the required length of time, either 16 hours or times between 1-72 hours for time course experiments. After incubation, tubes were centrifuged for 6 minutes at 3000 x g and the plasma extracted using a sterile pipette and transferred to labelled 1.5ml microfuge tube. Depending on the sample, there would be approximately 400-600μl of plasma, which was stored at -20°C for future analysis.

2.13 Sandwich Enzyme-Linked Immunosorbant Assay (ELISA) of Plasma and Cell Cultured Supernatants

Sandwich ELISA systems were used to quantify cytokine concentrations present in plasma and cell supernatant, both of which were thawed at 4°C prior to use. The principle behind the sandwich ELISA method is outlined in Figure 2.13. A Maxisorb Nunc 96 well plate was incubated overnight at 4°C with a 'Capture Antibody' in coating buffer with an epitope suitable for the target cytokine. After each step, wells were washed with 0.5% Tween 20 in 1x PBS solution, 4 washes with 300μl per well. 'Assay Diluent', 1% purified BSA in sterile autoclaved 1x PBS, was used to block the remaining surface of the wells for 1 hour at room temperature. Plasma samples or cell supernatant samples were used neat, or diluted to the requisite degree, for example, 1:100 dilution. 100μl of the sample was added to the wells in duplicate, along with duplicate samples of a known protein standard concentration in 2 fold dilutions to create a standard curve. The samples were incubated at room temperature for 2 hours to allow binding of the cytokine to the capture antibody. After washing, a 'Detection Antibody' diluted in assay diluent was used to bind to the protein captured on the plate. This antibody recognised a different epitope from the capture antibody, though it still binds specifically to the target protein. The antibody was also biotinylated and was incubated in the wells at room temperature for 1 hour. After washing, Avidin-HRP (Horseradish peroxidase) was added to the wells for 30 minutes,

which bound to the biotinylated secondary antibody. After washing, including an additional 1 minute of incubation in the washing buffer (soaking time), the plate was ready for the addition of TMB substrate solution (Tetramethylbenzidine). The solution contains a buffer solution, the TMB for colour changing and hydrogen peroxide solution. When the hydrogen peroxide is added, the HRP enzyme breaks this down to H_2O utilising TMB as a hydrogen donor, producing the distinctive blue colour. The more enzyme present, the more TMB is oxidised, thus acting as a guide for relative concentrations. The addition of 1M sulphuric acid halts the reaction, producing a yellow colour that can be detected at 450nm (see Figure 2.14). Using the relative absorbance of the standard protein concentrations, a standard curve can be used to calculate the concentration of cytokine present in the unknown samples (see Figure 2.15). This is adjusted for dilution factor and as the mean averages of the duplicated samples. Plates were analysed using a Multiskan FC Microplate Photometer (Thermo Scientific). Concentrations were recorded using Microsoft Excel and analysed using Graphpad Prism software.
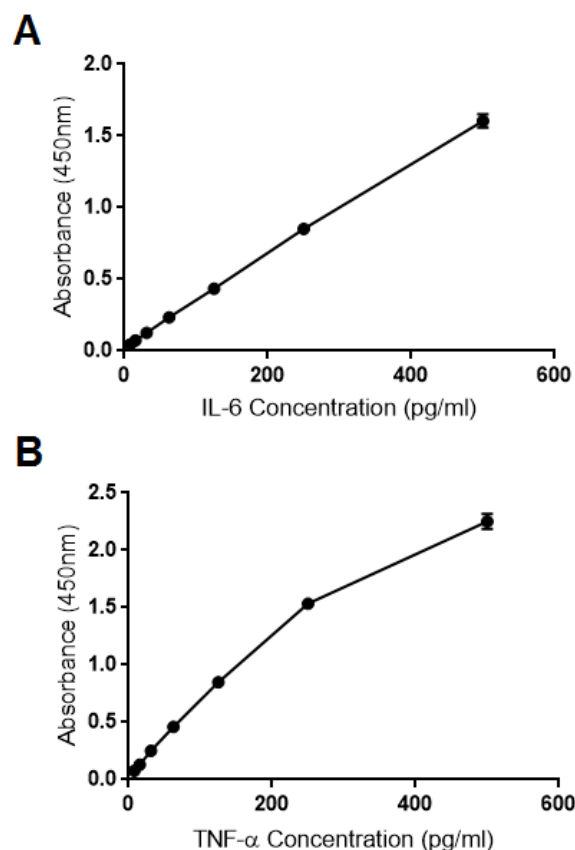
Figure 2.15: Representative examples of ELISA protein standard curves

A: IL-6 protein standard curve for an ELISA. The range is 7.8 pg/ml to 500 pg/ml. **B:** TNF-α protein standard curve for an ELISA. The range is 7.8 pg/ml to 500 pg/ml. Data represented with 2 values for each point and presented as mean ± standard deviation.

2.14 Haematoxylin and Eosin Staining

Lymph node biopsies used for clinical diagnosis were previously stored as formalin fixed paraffin embedded (FFPE) samples. These were prepared at 5µm on slides by Amy Glover (Histotech, York University) for 5 individual sarcoidosis subjects.

Slides were labelled with an appropriate identifier and placed in a metal rack ready for Haematoxylin and Eosin staining. Slides were dewaxed in Histo-Clear II (Fisher Scientific) at 37°C for 10 minutes, followed by two 10 second washes in fresh Histo-Clear II pots. Slides were subsequently rehydrated by three 10 second washes in 100%, 90% then 70% alcohol (industrial methylated spirits, IMS). Samples were washed with gently running tap water for 1 minute, to remove excess alcohol.

Nuclear staining was achieved by a 5 minute incubation with Harris Haematoxylin (Sigma #HHS32-1L). The excess stain was removed with a 30 second wash with running tap water followed by a 10 second wash with acid alcohol solution (70% IMS + 1% concentrated hydrochloric acid) and a 1 minute wash with tap water. Cytoplasmic staining was achieved by either 2 minutes or 8 minutes staining with 1% Eosin (Sigma #E4382). Eosin was washed away with 1 minute in running tap water. Slides were dehydrated with three 10 second incubations with 70%, 90% then 100% alcohol (IMS), followed by three 10 second washes in Histo-Clear II. Slides were coverslip mounted using Histomount and visualised using a microscope and image capture.

2.15 Immunohistochemistry Protocol

A standard procedure was used to stain slides for CD200L and CD200R, adapted from a method outlined in [687]. Slides were labelled, each had a primary and isotype control, and one additional negative control slide (without antibody incubation) was used in each run to ensure there was no non-specific colour staining.

Slides were dewaxed and rehydrated using Histo-Clear and 100%, 90% and 70% alcohol (IMS) as described previously in H&E staining (Section 2.14). Endogenous peroxidase was blocked with methanol/hydrogen peroxide solution (0.6% hydrogen peroxide, methanol solution) for 20 minutes, followed by a 2 minute wash under running tap water. Heat

induced antigen retrieval using 1% low pH Antigen Unmasking Solution (Vector Labs #H-3300) in distilled water was used to prepare samples in a pressure cooker. Slides were subsequently washed using tap water and transferred to 1x TBS solution (Tris-buffered Saline solution).

Slides were transferred to Sequenza coverplates (Shandon) and assembled to allow solutions to flow over the sample. These assembled Sequenza coverplates were transferred to Sequenza holders and 1x TBS solution used to prevent slides from drying whilst other slides were assembled. Slides were incubated with 1x TBS for 5 minutes once all Sequenzas were assembled and non-specific binding was blocked using prediluted blocking serum (Vector Labs Horse serum, #PK-7800) for 10 minutes, followed by washing with 1x TBS for 5 minutes. Endogenous avidin and biotin were blocked using the appropriate solutions for 15 minutes each (Vector labs #SP-2001), with 5 minute washes with 1x TBS after each incubation. The target antibody and the isotype control antibody were diluted at an appropriate concentration in 1.5% Blocking Serum (Horse serum, Vector labs) and 100µl of each added to slides as appropriate with one antibody omitted slide for 90 minutes at room temperature. 100µl of prediluted biotinylated pan-specific universal secondary antibody (Vector labs #PK-7800) was added to slides for 20 minutes and subsequently incubated with streptavidin/peroxidase preformed complex solution (Vector labs #PK-7800), washed with 1x TBS solution after each incubation. Slides were removed and treated with DAB/peroxidase solution for 20 minutes to develop colour, 5 minutes incubation with copper sulphate, 20 seconds of counterstain with Harris Haematoxylin and washed with acid alcohol solution. Slides were dehydrated using 70%, 90% and 100% alcohol and Histo-Clear solution. Slides were coverslip mounted using Histomount and visualised using a microscope and image capture.

2.16 Data Analysis and Statistical Interpretation

Depending upon the experiment type, data was recorded using Microsoft Excel spreadsheets where preliminary analysis took place. As previously mentioned, flow cytometry data was analysed using Flowing Software 2.5.1 (Peter Terho) and data recorded on Excel subsequently. Graphical representation using dot plots, histograms and density plots from flow cytometry were exported from the Flowing Software. All other

graphical representations were completed using Graphpad Prism 6 or 7 software. Data was presented with a mean and either standard deviation (SD) or the standard error of the mean (SEM) values. For data with a significant left or right skew, data was presented with a median and values could be used for the interquartile range (IQR).

Statistical analysis of data was completed using Prism 6 or 7 software, the type of tool chosen suitable for the data. All data underwent normality testing using D'Agostino & Pearson Omnibus Normality test or Shapiro-Wilk normality test as appropriate. The data was deemed to be of a normal distribution if the healthy cohort was not statistically skewed utilising this testing, whereas disease cohorts could be abnormally distributed. This allowed for the possibility of different disease subsets not aberrantly influencing statistical power of the tests being undertaken. For two independent data sets, if the data is normally distributed, a Student's T-test was used (parametric). For data not passing normality tests, a Mann-Whitney U test was used (non-parametric). Although rarely applied, data with highly abnormal distributions that were not appropriate for testing with the Student's T-test or the Mann-Whitney U test, the Kolmogorov-Smirnov test was used which more effectively assessed significance for changes in distribution. One-way ANOVA (one-way analysis of variance) was used for 3 or more independent data sets, or Two-way ANOVA (two-way analysis of variance) used for two or more independent data sets with multiple data points, as in the case of PHA dose curves with 8 concentrations in healthy and sarcoidosis groups. Both these are parametric, so in non-normal distributions Kruskal-Wallace tests were used. To allow for multiple comparisons to be assessed in these more complex data sets, post-hoc analysis was performed using either Sidak, Tukey's or Bonferroni multiple comparison tests as appropriate. Any other multiple comparison test would be stated within the figure if it were not belonging to these three tests. Statistical significance is assessed using p value determination as calculated using the appropriate statistical test previously mentioned, ranging from $p=<0.05$ to $p=<0.0001$ for significant data.

Chapter 3
Peripheral Blood Leukocyte Immune Responses to Lectins and Mitogens in Sarcoidosis

3.1 Introduction

Human blood contains a mixture of different cell types and proteins which can vary in composition between individuals and dynamically alter in response to environmental and physiological changes. The blood consists of plasma, erythrocytes (red blood cells), platelets and leukocytes (white blood cells). The majority of the volume is taken up by the plasma, platelets and erythrocytes; leukocytes make up approximately 1% of the volume (**Figure 3.1**). This will consist of a range of different leukocytes, broadly split between polymorphonuclear cell types, neutrophils and eosinophils, and mononuclear cells such as monocytes and lymphocytes (**Figure 3.2**) [688]. Neutrophils generally make up the vast majority of the total circulating leukocytes, both neutrophil and eosinophil numbers can vary significantly between individuals [688]. Peripheral blood mononuclear cells (PBMCs) account for the rest of the leukocyte populations, approximately 30-40% (**Figure 3.2**). Immune response and peripheral blood constituents can be significantly different between the genders and racial groups, so such factors need to be taken into account when considering peripheral leukocyte populations [689]. For a disease profile, alterations in peripheral immune leukocyte responses and circulating proteins can act as an indicator of systemic involvement or define the progress of a particular condition.

~ 55% Plasma

~ 44% Erythrocytes and Platelets

~ 1% Leukocytes
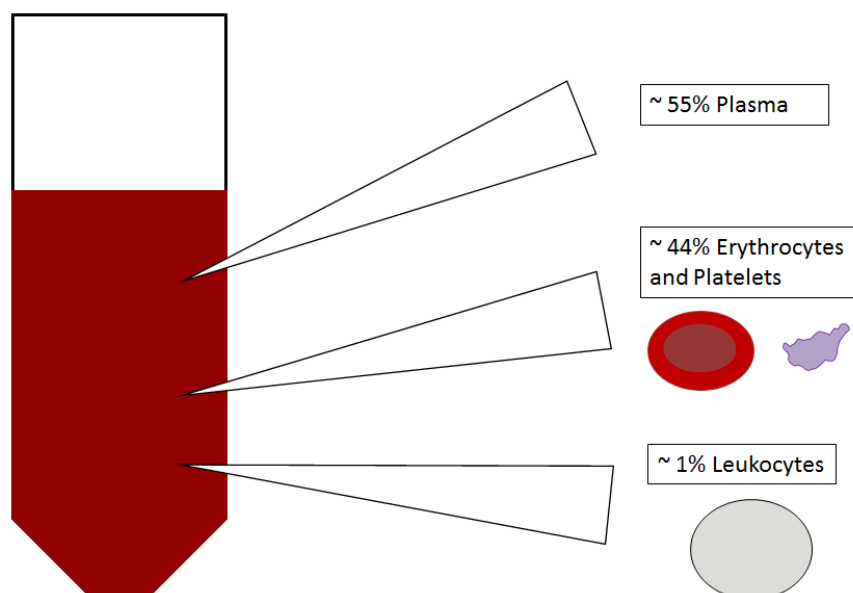
Figure 3.1: Constituents of human blood

The majority of the volume in blood consists of plasma, including proteins like albumin and immunoglobulins. Erythrocytes (red blood cells) and platelets are the predominant cellular constituents present. Approximately 1% of the volume consists of leukocytes (white blood cells/immune cells), though the number can vary depending on the individual.
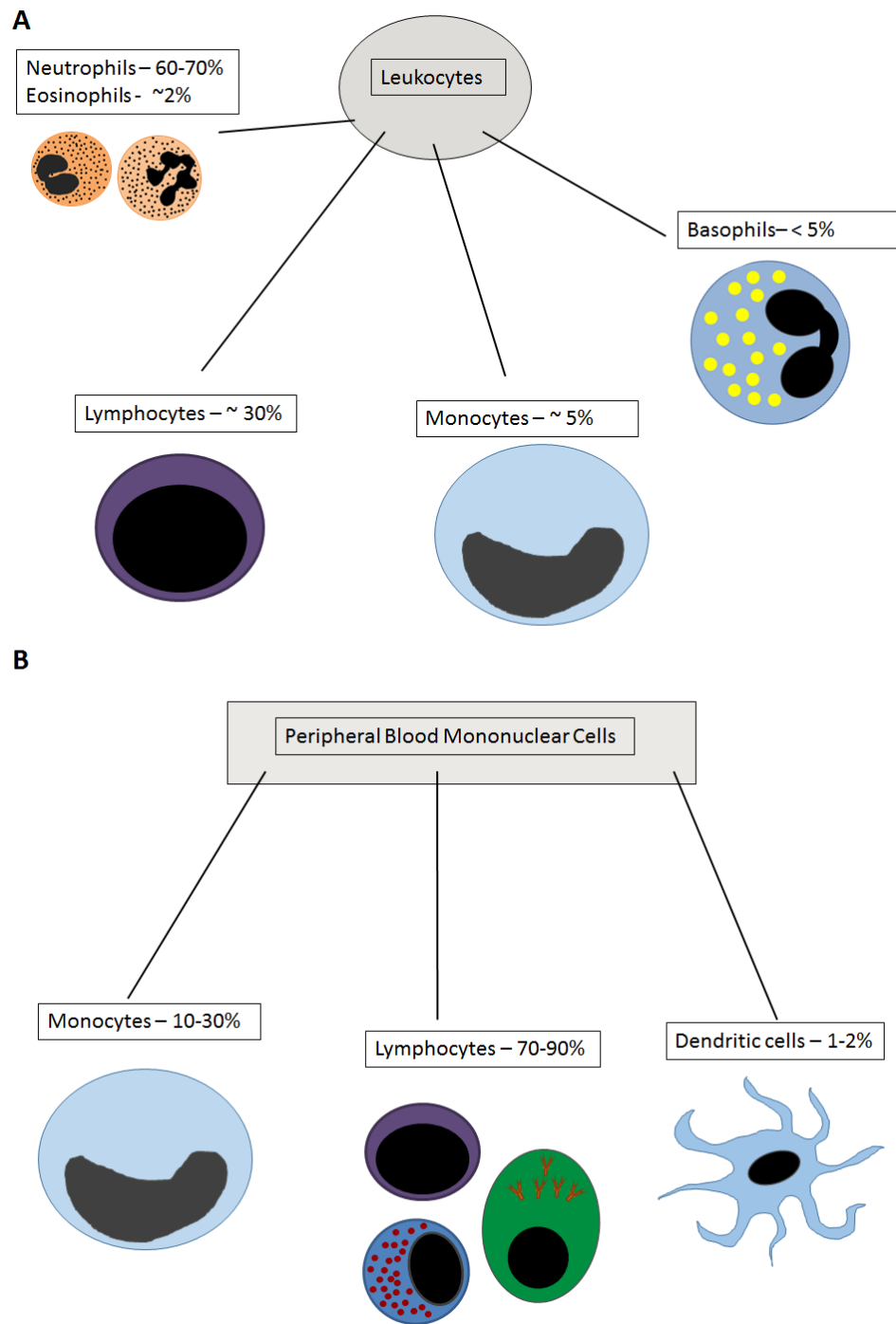
Figure 3.2: Leukocyte populations in human blood

A: Neutrophils are the most numerous cell type present in human leukocyte populations, though this can vary significantly between individuals. Lymphocytes, including T-cells, B-cells and Natural Killer Cells (NK cells), are also commonly observed in peripheral blood. Monocytes are also reasonably well represented in healthy blood. Basophils are less common, at most 5% of leukocytes belong to this group. **B:** Peripheral Blood Mononuclear Cells (PBMCs) consist mainly of T-lymphocytes and monocytes, but B-lymphocytes, plasma cells, NK cells and low numbers of dendritic cells are also typically represented to varying degrees.

The peripheral blood immune responses in sarcoidosis have usually been described as being diminished or anergic when compared with controls [21-25]. This contrasts greatly with the elevated immune responses in affected tissue in the disease, typically from bronchoalveolar lavage cells in pulmonary sarcoidosis [16, 18, 280, 317, 322]. The peripheral blood system is not diminished in a universal way however, with specific stimuli/antigens reported to be capable of stimulating greater responses in patients than healthy controls [19]. The contradictory reports can make the study of the disease difficult, as base principles cannot be established for peripheral immune responses where an aetiological trigger is not defined and where different cohorts of patients also display pathologies based on racial and gender composition [1, 48].

Immune cell activators can include cytokines, proteins, organic and inorganic ligands that can work in a variety of ways, both specifically or a more general manner. In the peripheral blood, broad acting immune activators can be used to assess the immune competency of leukocytes in either *in vivo*, *ex vivo* or *in vitro* settings. In a multi-cell system, "lectins" and "superantigens" are useful for defining whether a generalised anergy exists or not.

Lectins are a group of carbohydrate binding proteins usually present in specific plant species [690]. These proteins can have useful properties; some will be able to agglutinate specific or general cell types, others can activate immune response [691]. One such protein, phytohaemagglutinin (PHA), is capable of both [691, 692]. Found in red kidney beans, the molecule has long served researchers exploiting the erythrocyte agglutinating and immune activating properties [691]. Along with concanavalin A, PHA is the most commonly used and heavily researched lectin; yet the full range of interactions PHA has with different receptors is still not fully elucidated. PHA-P, the raw protein from *Phaseolus vulgaris*, actually consists of two predominant isoforms, PHA-E and PHA-L [693-695]. PHA-E selectively agglutinates erythrocytes, whereas PHA-L is responsible for the leukoagglutination and mitogenic activity (Figure 3.3) [692-695]. Different isolectins with combinations of PHA-L and PHA-E subunits within the tetrameric structure are also present, as well as mucoprotein forms (PHA-M). Traditionally considered a T cell activator, binding through the T cell receptor (TCR) [696, 697], it is also capable of binding to other immune receptors and may interact with a variety of different molecules and

cells for activity [698, 699]. Others have different modes of activity; wheat germ agglutinin (WGA), for example, preferentially binds to myeloid cells and appears to lack the mitogenic activity of PHA, but will induce cytokine release without the proliferation [698]. Staphylococcal enterotoxin A (SEA) is a superantigen, a potent polyclonal activator of immune response through the MHC-TCR complex (major histocompatibility complex – T cell receptor) that bypasses classical antigen specific requirements [700, 701]. Accessory cells displaying MHC complexes, usually MHC class II molecules such as those found on macrophages, monocytes and dendritic cells, are an absolute requirement for T-lymphocyte activation [700]. Use of lectins and superantigens can provide a basic way of assessing overall immune competency within a system, and therefore become a useful tool in general immunological study.
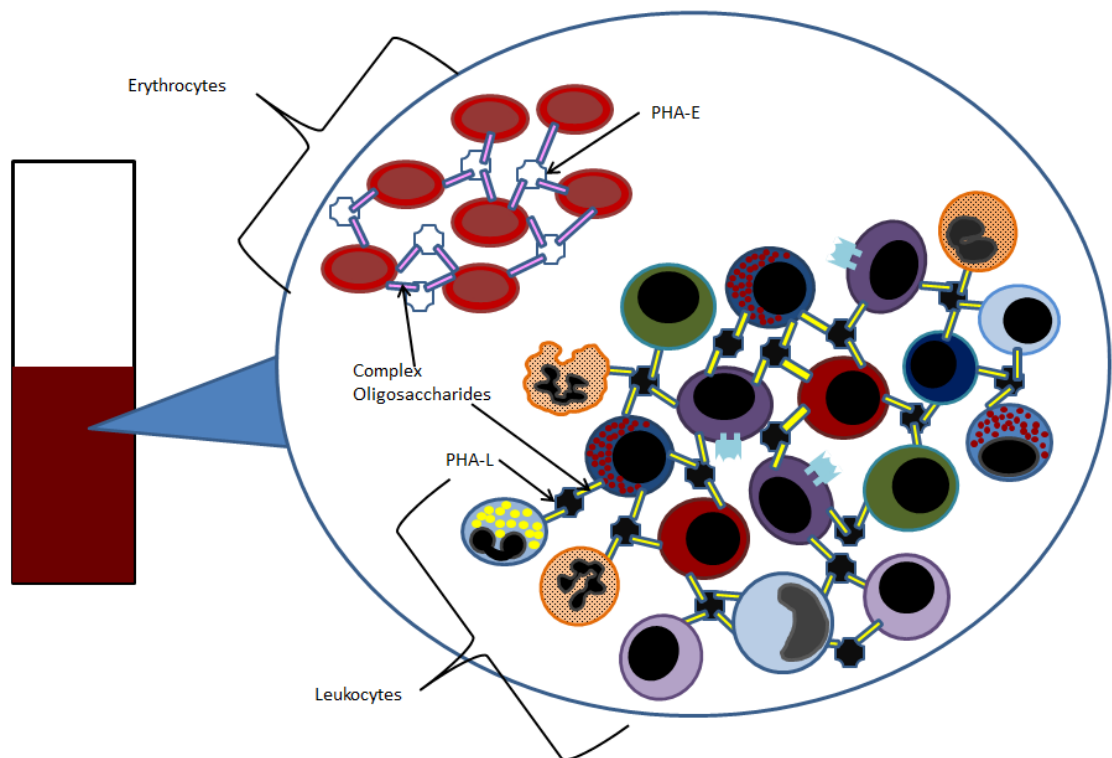
Figure 3.3: Agglutination of whole blood by phytohaemagglutinin: PHA.

PHA-P is a raw mixture of different isoforms of PHA, which both agglutinate cells. PHA-E, an erythroagglutinating form (shown in white), binds to cell surface carbohydrates present on erythrocytes. PHA-M is a mucoprotein form (not shown). PHA-L, the leukoagglutiniating form of PHA (shown in black) binds components on the leukocytes and activates inflammatory and mitogenic pathways associated with immune response. PHA-L is non-specific, binding to a variety of different leukocytes for the agglutination of cells.

In this chapter, the leukocyte populations in the blood of both sarcoidosis patients and healthy controls were characterised to identify any significant differences in the proportion of specific subsets of cells between the two groups. This was a well characterised cohort of patients, all diagnosed by consensus in multidisciplinary meetings comprising a respiratory clinician with an interest in interstitial lung disease, a thoracic radiologist, and a thoracic pathologist. The patients with sarcoidosis were therefore not subject to the high degree of variability that might be a feature of other previous reports that are more inclusive in recruitment [47]. Rather than relying on preconceived assumptions based on these previous conflicting reports, the release of pro-inflammatory and anti-inflammatory cytokines in response to broad acting immune activators (PHA, WGA and SEA) were quantified in order to confirm whether sarcoidosis patients displayed diminished or enhanced peripheral leukocyte responses. The principle aim was to provide a basis to inform further investigations into the inflammatory and regulatory mechanisms in the disease.

3.2 Methods

3.2.1 Immunophenotyping of Peripheral Blood Mononuclear Cell Leukocyte Populations

PBMCs were isolated from whole blood as previously described (Section 2.7) and the protocol for extracellular staining for flow cytometry was followed (Section 2.9). PBMCs were freshly prepared after isolation and a 1 hour incubation at 37°C. Antibody combinations were used to ensure effective fluorescent detection. Appropriate isotype controls were used for all the combinations. Live cell staining was completed by flow cytometry and the results interpreted as previously described.

CD3^{+} cells were identified as T-lymphocytes, producing a clear single modal peak. CD4 staining of mixed PBMCs produced a bimodal distribution, CD4^{high} and CD4^{low} (Figure 3.4), representing CD4^{+} T-lymphocytes and monocytes respectively. For accurate determination of CD4^{+} T cells, the lymphocytes were gated based on forward and side scatter (size and granularity) in order to filter only T cells with the marker. Dual staining

with CD8a and CD3 allowed CD8a$^+$ T-lymphocytes to be separated from other CD8a$^+$ lymphocytes.
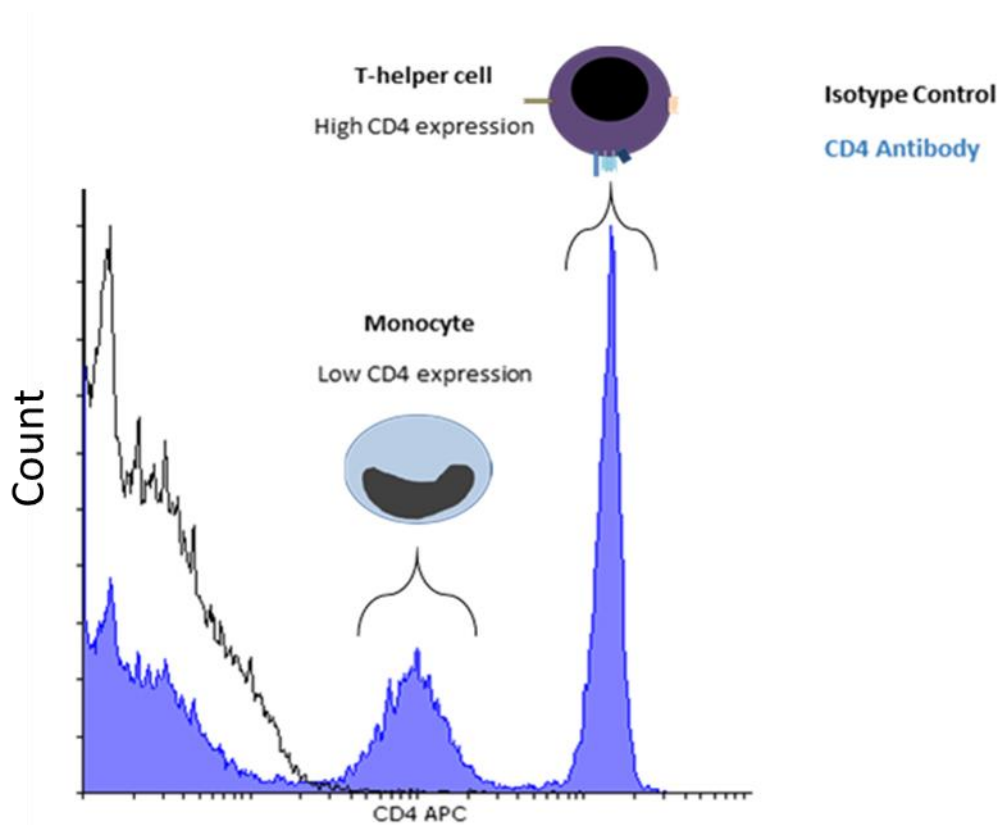
Figure 3.4: Representative histogram of CD4 expression on human peripheral blood mononuclear cells

PBMCs were stained with an isotype control antibody (black line) and an anti-CD4 antibody (blue filled). The fluorescence, measured in FL-4 (CD4 APC, x axis), and the cell count (y axis) displays a bimodal positive distribution. The peak highlighted as T-helper cells express high levels of CD4, whereas a distinct peak with lower fluorescence (closer to the isotype control) represents lower monocyte expression of CD4. The total cell count for each population is represented by the area under the peaks.

CD45RA and CD45RO were used to differentiate naïve T-lymphocytes (CD3$^+$ CD45RA$^+$ CD45RO$^-$) and memory T-lymphocytes (CD3$^+$ CD45RA$^-$ CD45RO$^+$). CD45RA and CD45RO staining required compensation for FITC and PE overlapping, typically FL1-FL2 = -2.5% and FL2-FL1 = 17.5%. **Figure 3.5** shows the gating required for the individual populations, CD45RA$^+$ CD45RO$^-$, CD45RA$^-$ CD45RO$^+$ and CD45RA$^+$ CD45RO$^+$ (double positive). This was validated using isotype controls compared with cells stained with only one of the targets at a time, either RA or RO, and the results of positive populations calculated as follows:

Total double positive cells = Total CD45RA$^+$ cells + Total CD45RA$^+$ cells - Total CD3$^+$ cells

Actual CD45RA$^+$ cells = Total CD45RA$^+$ cells – Total double positive cells

Actual CD45RA$^-$ cells = Total CD45RA$^-$ cells – Total double positive cells
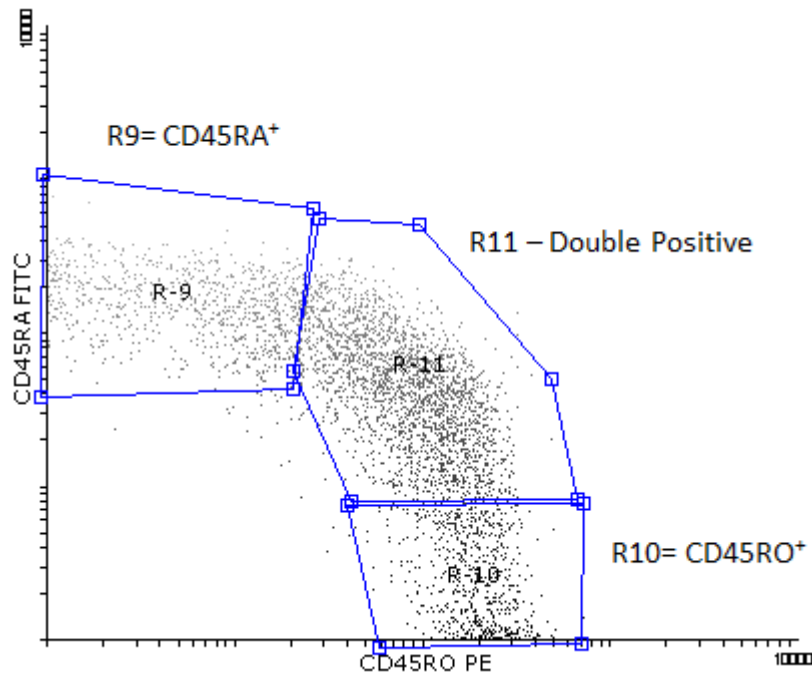
Figure 3.5: Representative gating for CD45RA and CD45RO staining of lymphocytes.

PBMCs were stained with anti-CD45RA, anti-CD45RO and anti-CD3 antibodies, then the lymphocytes gated to separate these cells from monocytes. CD3$^+$ cells were gated using quadrant analysis, and the population of T-lymphocytes then displayed as shown above. Isotype controls and density plots were used to establish the border of R10 to R11 and R9 to R11 populations. R9 is CD45RA$^+$ CD45RO$^-$, R10 is CD45RA$^-$ CD45RO$^+$ and R11 is double positive CD45RA$^+$ CD45RO$^+$ T-lymphocytes.

This produced values for the 3 populations. As these were IgG2 antibodies (IgG2b for CD45RA and IgG2a for CD45RO), 2% was the isotype threshold used to account for increased background binding by monocytes, positive cells displayed fluorescence beyond this gating. CD25$^+$ T-lymphocytes were determined with dual staining with either CD3 or CD4 in the mixture. Total geometric mean fluorescence intensity ('geomean') was used as a measure of relative protein expression on cell surfaces; the calculated geomean was the geomean of the antibody – geomean of the isotype control on a logarithmic scale. Percentage of CD25 positive cells were calculated using gating set at 1% of the isotype.

Monocytes were initially differentiated using the CD14 marker, positive cells in PBMCs were identified as being specifically this cell type. Cells predominantly displayed high CD14 expression, but some produced lower (Figure 3.6). To further differentiate cells, CD16 was used in unison with the CD14 marker. CD16 is positive on a number of lymphocytes, so these were excluded from analysis prior to interpretation. An example of the gating used to establish the populations is shown in Figure 3.7. Monocyte CD25 expression was calculated in the same manner as T-lymphocyte CD25, except monocytes were selected by gating of the population by size and granularity, as described previously. B-lymphocytes were determined using CD19 on lymphocyte gated cells, though these were also typically CD3$^-$. Natural Killer cells were identified using CD56 and CD3, they were determined as those cells which were CD56$^+$ CD3$^-$. NKT cells were defined as CD56$^+$ CD3$^+$. Further staining with CD16 also highlighted a sub-population of CD16 expressing NK cells, and staining with CD8a, CD56 and CD16 was used to validate the populations seen in the staining with CD56 and CD3. A summary of the main leukocyte markers is presented in the appendix (Table A3.1).

3.2.2 Calculation of Peripheral Blood Mononuclear Cell Concentration from the Blood

PBMCs isolated from blood, as described previously, were re-suspended in RPMI of a fixed volume. In a validating step, as freshly isolated PBMCs were used, 10µl of cell suspension was mixed with 10µl of Trypan Blue solution to establish cell viability. In initial counts, there was no significant cell death. Thus, it was concluded that cells would be subsequently counted excluding Trypan Blue solution. Therefore, 20µl of cell suspension was placed on a haemocytometer with a coverslip. The larger square was used on the haemocytometer, the number of cells counted within each and this was repeated for a total of 3 counts. The average of the 3 counts was taken which was the value of x10^4 cells/ml in the cell suspension. This was adjusted with appropriate dilution as required.

The following calculation was used to determine the total PBMC number per ml of blood:

$$\text{Total PBMCs/ml Blood} = \frac{\text{Concentration of Cell Suspension x Volume of re-suspension (ml)}}{\text{Starting Volume of Blood (ml)}}$$
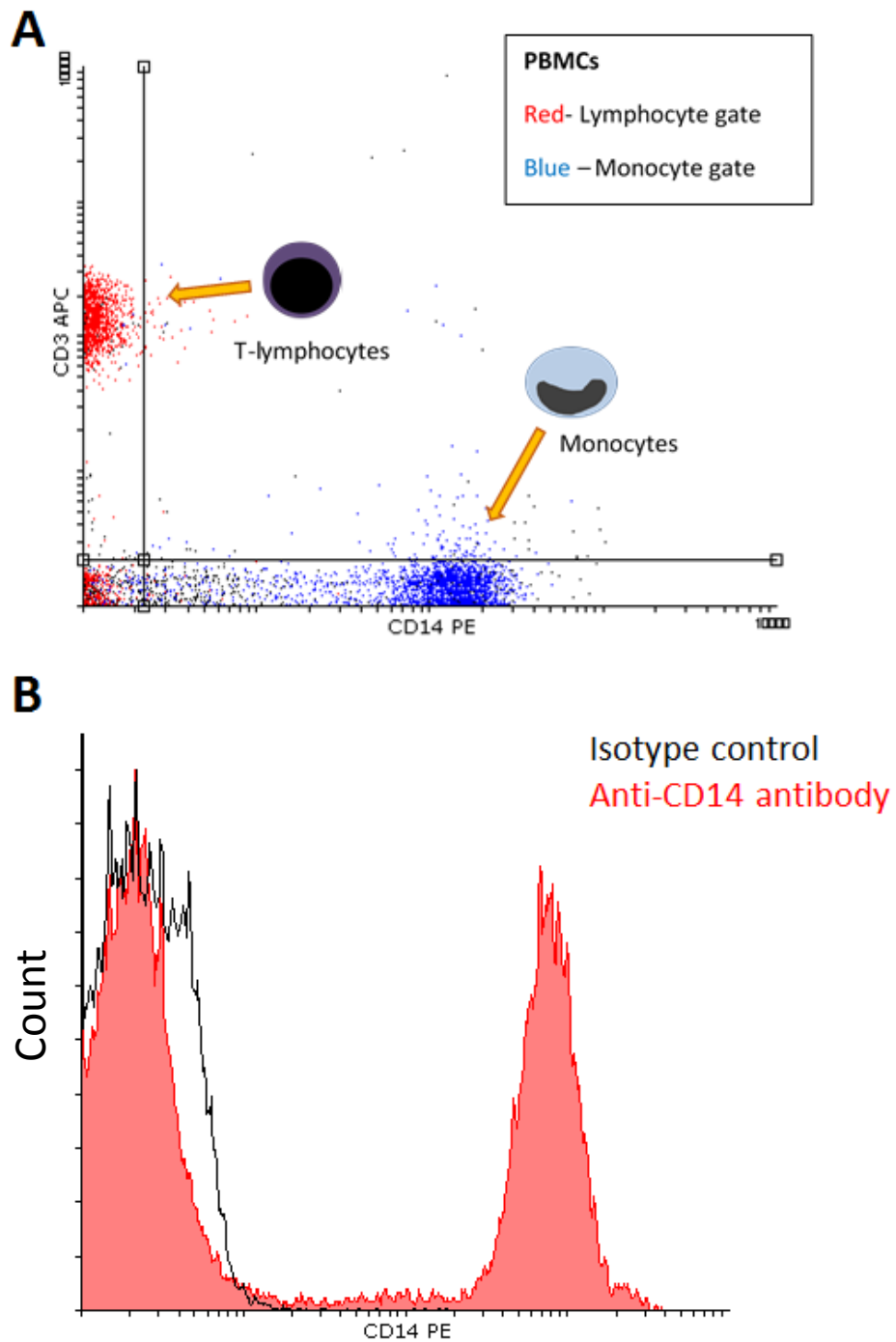
Figure 3.6: Representative flow cytometry analysis of monocytes with CD14.

A: Dot plot of PBMCs stained with anti-CD3 and anti-CD14 antibodies. Quadrant set at 1% positive of isotype control. Cells are identified as T-lymphocytes in the upper left quadrant (CD3$^+$) and monocytes in the lower right quadrant (CD14$^+$). Red and blue cells are those within the lymphocyte and monocyte gates respectively, determined using the FSC and SSC plot. **B:** Representative histogram displaying CD14 expression, CD14 histogram normalised against the control antibody histogram to match peak height. Lower CD14 expression exists on monocytes below the primary peak.
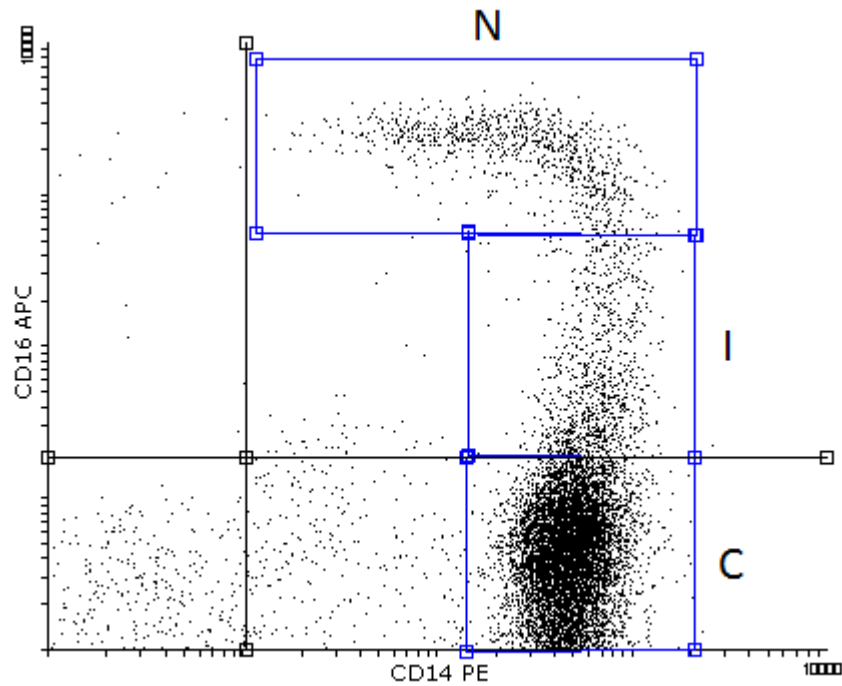
Figure 3.7: CD14/CD16 flow cytometry dot plot of monocyte gated PBMCs to represent gating strategy for monocyte subsets.

Cells were stained with anti-CD14 and anti-CD16 antibodies and quadrants established using isotype controls and density plots. The populations were gated as shown, with 'C' representing 'Classical Monocytes' (CD14^{++} CD16^{-}), 'I' representing 'Intermediate Monocytes' (CD14^{++} CD16^{+}) and 'N' representing 'Non-classical Monocytes' (CD14^{+} CD16^{++}).

3.2.3 Calculation of PBMC Leukocyte Population Numbers

The percentage of lymphocytes and monocytes present in PBMCs were calculated using lymphocyte gated populations (forward and side scatter gating) and CD14^{+} stained cells in flow cytometry. The lymphocyte population and monocyte populations were added together for a total number of events, each population as a percentage was calculated by dividing the population number by the total number of events x 100. Using this, total number of cells/ml of blood was calculated using the PBMC concentration:

Population concentration (cells/ml of blood) = PBMC concentration x (population % /100)

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All other cell populations were calculated using these figures for total lymphocyte or total monocyte concentration. For example, total CD3$^+$ T-lymphocytes were calculated as a percentage of the total lymphocyte population and converted to a concentration of T-lymphocytes/ml of blood for the sample. Each donor sample was calculated individually for each parameter and subsequently used to calculate mean or median values.

3.2.4 Whole Blood Assay – Stimulation with PHA, WGA and SEA

Whole blood had been taken from subjects as previously described (Section 2.1) in sodium heparin blood collection tubes (BD Bioscience). Stimulants (PHA/WGA/SEA) were pre-diluted with sterile 1x PBS in the concentrations required for each experiment. Typically for PHA this was 0.1, 0.3, 1, 3, 10, 30 and 100 µg/ml, for WGA this was 1, 3, 10, 30 and 100 µg/ml and 0.1, 0.3, 1, 3 and 10 µg/ml was used for SEA in dose response experiments. 10.1 µl of the appropriate concentration of stimulant was added to the bottom surface of a 2ml skirted round-bottom microcentrifuge tube. 1000 µl of whole blood was then added to each tube, sealed and the samples placed upright in an incubator (within a container) at 37°C for 16 hours. After the incubation, plasma was extracted as described previously (Section 2.12) and the labelled samples stored for future use.

3.2.5 PHA stimulation of PBMCs for Soluble Cytokine Release

Whole blood had been taken from subjects and then the PBMCs extracted and counted as previously described (Section 2.7). For experiments comparing whole blood stimulation to PBMC stimulation, the PBMCs were re-suspended in RPMI media equivalent to the volume of whole blood the cells were initially separated from (for example, 10ml of blood = 10ml of RPMI). For fixed PBMC number extraction, PBMCs were counted using the haemocytometer and the concentration adjusted to 1-2 x10^6 cells/ml in RPMI media. PHA stimulation occurred as described previously (Section 3.2.4) and incubated for 16 hrs in the incubator. After this incubation, the supernatant was extracted from the tube (Section 2.12) and stored at -20°C until required.

3.2.6 Whole Blood Assay – Timecourse Stimulation with PHA

Whole blood was prepared as described previously and PHA was pre-diluted to stimulate samples with either 10 µg/ml or 30 µg/ml. 10.1 µl of the appropriate concentration of PHA was added to tubes, 8 tubes per concentration, and 3 tubes had 10.1 µl of PBS control added. 1000 µl of whole blood was added to all the tubes, and with the exception of 1 tube labelled 0 hrs (with PBS), the samples were incubated at 37°C. The 0 hr tube was centrifuged and the plasma extracted immediately as described previously (Section 2.12). The remaining 2 PBS control tubes were incubated for 24 hrs and 72 hrs; at each time point the plasma was extracted and stored immediately at -20°C. PHA sample tubes were incubated for the following times at 37°C before plasma was extracted: 1, 3, 6, 12, 16, 24, 48 and 72 hrs. All samples were stored in 1.5ml microcentrifuge tubes at -20°C until required.

3.2.7 ELISA Analysis of Plasma Samples from WBA Stimulations and PBMC Supernatant

ELISA was performed as previously described (Section 2.13). Samples were run in duplicate on the plates and were diluted appropriately with assay diluent after optimising the system for each cytokine. If a value was outside the range of detection, the sample would be run later using a different dilution factor to compensate. Dilution factors ranged from 1:1 to 1:5000 depending on the cytokines tested and the stimulant concentration used. This could vary depending upon the individual tested and whether healthy or sarcoidosis samples were analysed. IL-6 and IL-8 in response to 100 µg/ml would typically require the greatest diluting, whereas TNF-α and IL-10 required the least. The most concentrated samples used in the assay were 1:1 or 1:2 dilutions. The 'detectable limit' for an ELISA is the range of the protein standard concentrations included as part of the ELISA. For example, if an ELISA as a minimum concentration standard of 7.8 pg/ml and a maximum of 250 pg/ml, then the detectable limits are 7.8 pg/ml and 250 pg/ml. Concentrations determined using the absorbance below or above these values were not used and required diluting (if too high) or listed as not readable (if below). If the absorbance was below the detectable limit for these samples, the value would be recorded as 0 pg/ml. Taking the dilution of samples into consideration, the minimum

detectable concentrations for each ELISA were as follows: IFN-γ = 15.6pg/ml, IL-10 = 7.8pg/ml, TNF-α = 15.6pg/ml, IL-6 = 15.6pg/ml, IL-8 = 31.2pg/ml, IL-4 = 7.8pg/ml and IL-12(p70) = 31.2pg/ml.

3.2.8 Intracellular Staining of PBMCs for Cytokines after Stimulation

PBMCs were isolated and adjusted to a concentration of 2 x10^6 cells/ml of RPMI media. These were then seeded to a 96 well round bottom plate with 2 x 10^5 cells per well and left at 37°C for 1 hour prior to stimulation. PHA was pre-diluted as required so that final concentrations would be 1 μg/ml or 10 μg/ml within each well. Brefeldin A was also prepared so that 5 μg/ml would be obtained in the final concentration. Wells were labelled in triplicate for each concentration, each lane representing a different antibody combination. PBS control wells (in triplicate), no PHA control wells (in triplicate) and no Brefeldin A control wells (in triplicate) were also used. For the stimulated wells, PHA and Brefeldin A (10 μl each) were added to the 80 μl cell suspension at the following time points (from first to last): 16 hrs, 12 hrs, 6 hrs, 3 hrs, 1 hrs and 0 hrs. Between each time point, cells were incubated at 37°C.

After the incubation, cells were prepared using the method outlined previously (see Section 2.10). For cell surface staining, 10 μg/ml of antibody was used, the 3 lanes for each sample set corresponding to: lane 1 = APC isotype control, lane 2 = APC anti-CD3 antibody, lane 3 = APC anti-CD14 antibody. Cells were subsequently fixed and permeabilised, and each lane incubated with intracellular antibodies at 10 μg/ml: lane 1 = PE isotype control, lanes 2 and 3 = PE anti-TNF-α/IL-6/IL-10/IFN-γ antibody (as required). Samples were then subsequently prepared as previously stated (Section 2.9 and 2.10) and analysed using the appropriate methodology (Section 2.11).

3.2.9 Correlations between Factors

Data obtained in experiments could be correlated with data from other experiments belonging to the same individual donor. Individual samples were used for different experiments, so correlations would typically have lower numbers than the individual

experimental results. For two sets of continuous data, correlation plots as X-Y scatter were used to determine the correlation, be it positive or negative. Data was typically assessed using a 'Spearman's Rank Correlation test' for significance (represented with a p value), as at least 1 data set was usually not Gaussian ('normal') in distribution. The R^2 value, determined using the 'Pearson Product-moment Correlation Test' was included for clarity and to highlight the degree of linearity of data. For categorical/limited discrete data correlated with continuous (or discrete) data, such as Scadding CXR stages with serum ACE, groups were compared using standard Mann-Whitney U or Student's T-tests (or ANOVAs/Kruskal-Wallis tests for more groups) between categories rather than scatter plots.

3.3 Results

3.3.1 Cohort Details

Three cohorts, 'Sarcoidosis Patients' (n=30), 'Healthy Controls' (n=31) and 'Latent TB Patients' (a sample size n=5) were defined as previously described (Section 2.2). Previous comprehensive epidemiological studies had suggested women were more likely to suffer from the disease than men, even in Caucasian populations, and that the peak incidence is typically between 20-49 years of age [95]. Initial recruitment sought to obtain healthy donors predominantly between these ages and more skewed towards women than men to a similar degree.

In contrast to previous reports, the recruited sarcoidosis patients were skewed towards males, 53% male and 47% female in sarcoidosis compared with 42% male and 58% female in healthy controls, though the difference between the cohorts were not statistically significant (p=0.45, Fisher's Exact Test). The median age of the sarcoidosis cohort was 10 years higher than healthy controls (Table 3.1). Despite attempts to age match later in the study to accommodate the higher observed age in patients, there was a statistically significant difference between the median age of healthy controls and patients with sarcoidosis (Appendix A3.1, p=0.019, Student's T test). The range of ages included in the study were similar, 20-72 in healthy controls compared with 28-73 in patients with

sarcoidosis. The latent TB patient cohort had a much lower median age of 31 years and a range between 18-68 years old. Both gender and age factors were impacted by the recruitment strategy and exclusion criteria (such as significant health conditions and corticosteroid use versus age and gender). Subsequent data collected would be correlated with age and gender to identify whether differences were statistically significant or a result of gender and age differences between the cohorts.

Table 3.1: Demographics and clinical data for healthy subjects and patients with sarcoidosis. Data are presented as median (range).

Category	Healthy	Sarcoidosis
Number	31	30
Age (years)	41 (20-72)	51 (28-73)
Male/female	13/18	16/14
CXR Stage (%) 0 I II III	-	7 33 43 17
Serum ACE activity (reference range 8-65 U/L)	-	84.5 (25-356)
C-reactive protein (reference range 0-8 mg/L)	-	3.8 (0.5-64)
Plasma viscosity (reference range 1.5-1.72 m.Pas.s)	-	1.68 (1.49-1.93)

3.3.2 Clinical Features of Patients with Sarcoidosis

Clinical information for patients with sarcoidosis were collected and are shown in Table 3.1. The Scadding CXR Stage was included to determine the relative stage of the disease; a measure of the degree of granulomatous inflammatory mediastinal pulmonary involvement based on radiographic assessment. This scored 0-IV, where 0 was normal chest presentation and IV included extensive fibrotic inflammatory disease, based on guidelines previously established [60]. Organ involvement considered mediastinal lymph nodes as a separate organ system from parenchymal lung involvement, as patients may display involvement in both or just one of these systems. Serum Angiotensin Converting Enzyme (ACE) was included as an indicator of disease activity. 1 patient was on ACE inhibitors for a concomitant condition. Plasma Viscosity (PV) and C-Reactive Protein (CRP) concentrations in blood were also used as indicators as serum inflammatory markers. All features were included with reference ranges for 'normal', though these were not taken for healthy controls.

The organ involvement and associated symptoms (hypercalcaemia/altered calcium) of the whole cohort was collected and collated. All 30 patients had either lung or thoracic lymph node involvement and 21 patients (70%) had both lung and lymph node involvement. The majority had 2 or 3+ organs involved, 25 patients (83.3%), whereas 5 patients (16.7%) had only 1 organ involved. Only 2 patients had 4 organs involved, these would be included within descriptions of '3 organs' to denote 3 or more organ systems, rather than separated into their own group. The most common organs/symptoms included lungs (25 patients), lymph nodes (27 patients), skin (3 patients) and altered calcium metabolism (3 patients). Eyes (2 patients) and bones (2 patients) were seen and the following were identified in only 1 patient: liver, spleen, lacrimal glands and parotid glands. One patient had been described as having Lofgren's syndrome. Extrapulmonary symptoms were recorded in 43% of the patient cohort. The median age of patients with 2 organs involved was lower than those with 1 or 3 organs involved, 48.5 years (IQR 16) compared with 51 (IQR 24.5) and 54 (IQR 9) respectively (Appendix A3.2**),** though this was not statistically significant. Patients with extrapulmonary symptoms tended to be older than those with pulmonary restricted organ involvement, a median age of 54 years (IQR 12.5) compared with 50 years (IQR 32) respectively, though the difference was not statistically significant

between the cohorts (Appendix A3.3, p=0.71, T test). Gender seems to play an important role in the number of organs involved, as shown in Figure 3.8. The vast majority of female patients (93%) had a minimum of 2 organs involved compared with males (75%). Observation of 3 organs effected were also more common in the former, 50% of female patients compared with only 25% of male patients. Additionally, extrapulmonary symptoms were observed more in female patients (57.1%) than male (31%) (Figure 3.9, p= 0.0003, Fisher's Exact Test).
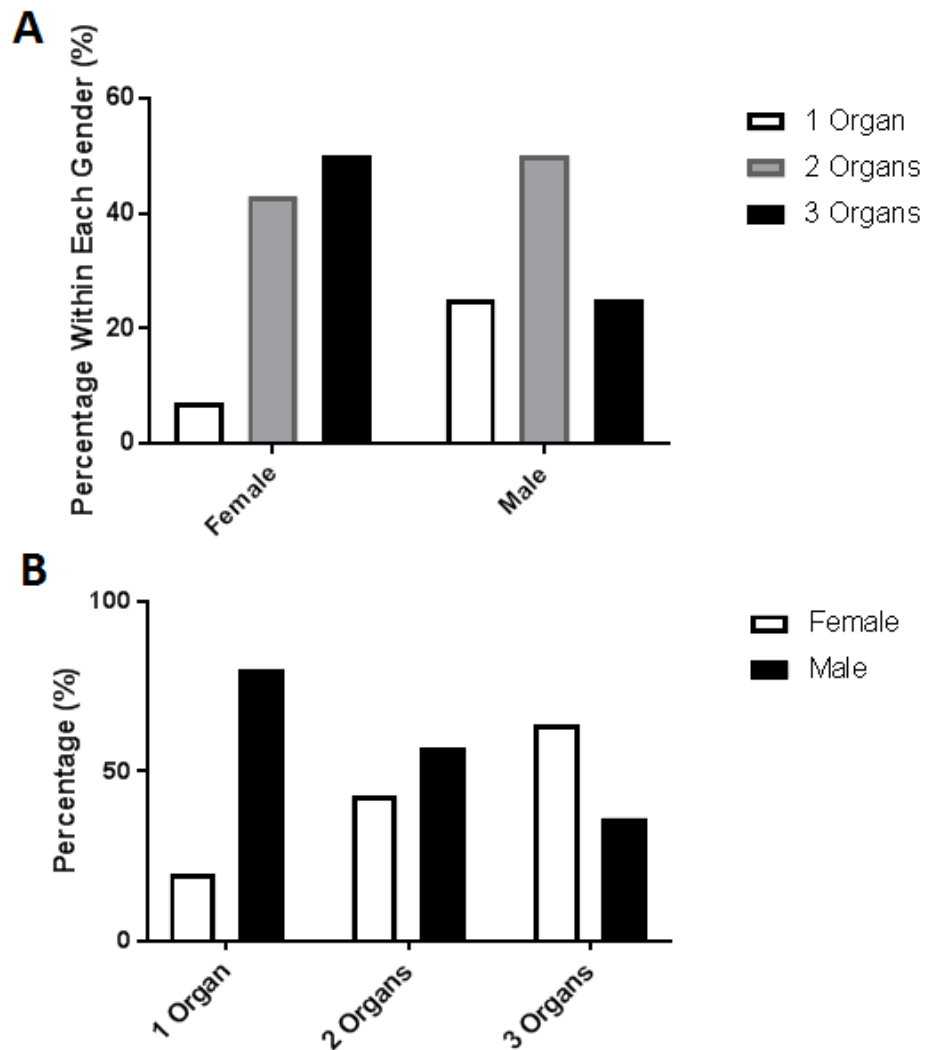
Figure 3.8: Gender differences in the number of organs involved in patients with sarcoidosis.

A: The percentage of male and female patients exhibiting 1, 2 or 3 organs within each gender, female patients tend to exhibit more than 1 organ involved, whereas 2 organs are most common in males. **B:** The percentage distribution of male and female patients within each group, showing that the majority of patients with only 1 or 2 organs involved are male, whereas female patients make up a greater proportion of those with 3 organs involved. Total cohort n = 30: n = 16 male, n = 14 female.
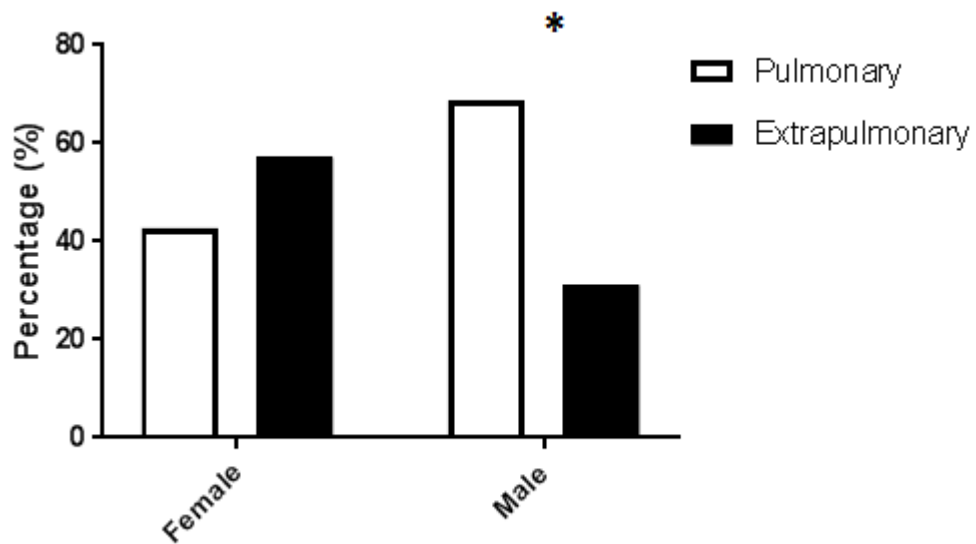
Figure 3.9: Percentage of sarcoidosis patients within each gender displaying extrapulmonary organ involvement.

Sarcoidosis patients described as having pulmonary symptoms only (white bars), including lung and mediastinal lymph nodes, or extrapulmonary symptoms (black bars), including skin, eyes and other manifestations. Total cohort n = 30. Gender: n = 16 male, n = 14 female. Pulmonary restricted total n = 17, extrapulmonary total n = 13. * P = 0.0003, Fisher's Exact Test.

All 30 patients with sarcoidosis had their Scadding CXR Stage defined. Only 2 patients (6.7%) of the cohort had no observed radiographic lung inflammation (Score 0). The majority of patients were Stage I and II, a total of 23 patients (76.6%), suggesting that the cohort comprised mainly of those with mild/moderate disease profiles. 5 patients had stage III (16.7%), parenchymal lung presentation restricted, and there were no stage IV patients recruited. The decision to exclude patients undergoing corticosteroid therapy could account for the lower numbers of stage III and absence of stage IV patients included in this study. The median age was not significantly different between stages (Appendix A3.4), though the range tended to decrease with increasing radiographic staging. The overwhelming majority of those with stage III scores were male, 80% (Figure 3.10). Within the male patient group this accounted for 25% of patients compared with only 7% of female patients. Male patients had equal numbers staged at I and II (37.5%), compared with female patients who were more heavily weighted towards stage II (50%) rather than stage I (29%), but also had 2 patients with stage 0 (14%) (Figure 3.10).
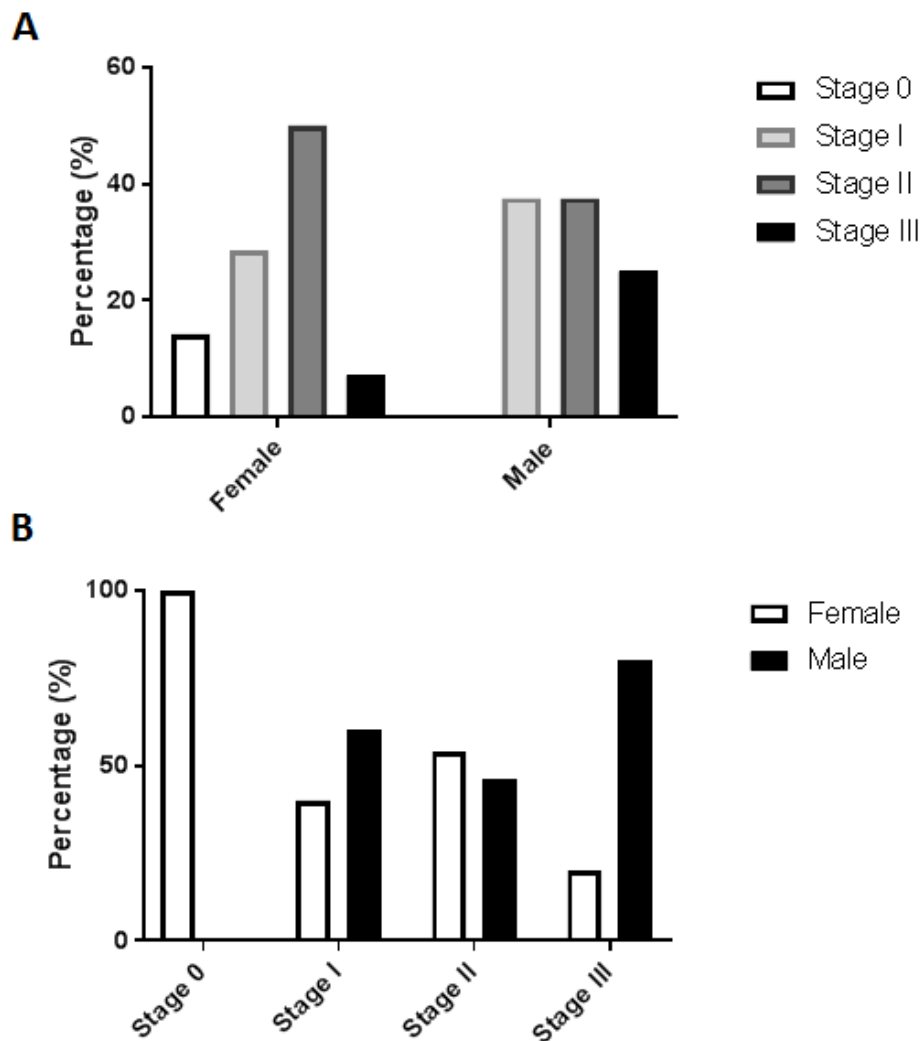
Figure 3.10: Gender differences and radiographic Scadding stages in patients with sarcoidosis.

A: Stages 0-III displayed as a percentage within each gender. Males tended to have more advanced staging, though 50% of female patients were stage II. **B:** Percentage of each stage comprising male or female patients. Male patients made up the majority of both stage I and stage III patients, whereas female patients were the majority of stage 0 and II. Total cohort n = 30. Gender: n = 16 male, n = 14 female.

Patients with a higher Scadding CXR score would tend to have a larger number of organs involved (Figure 3.11). A Scadding score of III indicates parenchymal lung restricted, and thus those with stage III had less organs involved than stage II. Specifically, stage II had a minimum of 2 organs, whereas stage I had only 2 individuals with 3 organs involved. Extrapulmonary symptoms correlated with a higher Scadding CXR stage, 77% of those with extrapulmonary symptoms were stages II or III, as opposed to pulmonary restricted patients who only had 47% with these radiographic scores (Figure 3.11).
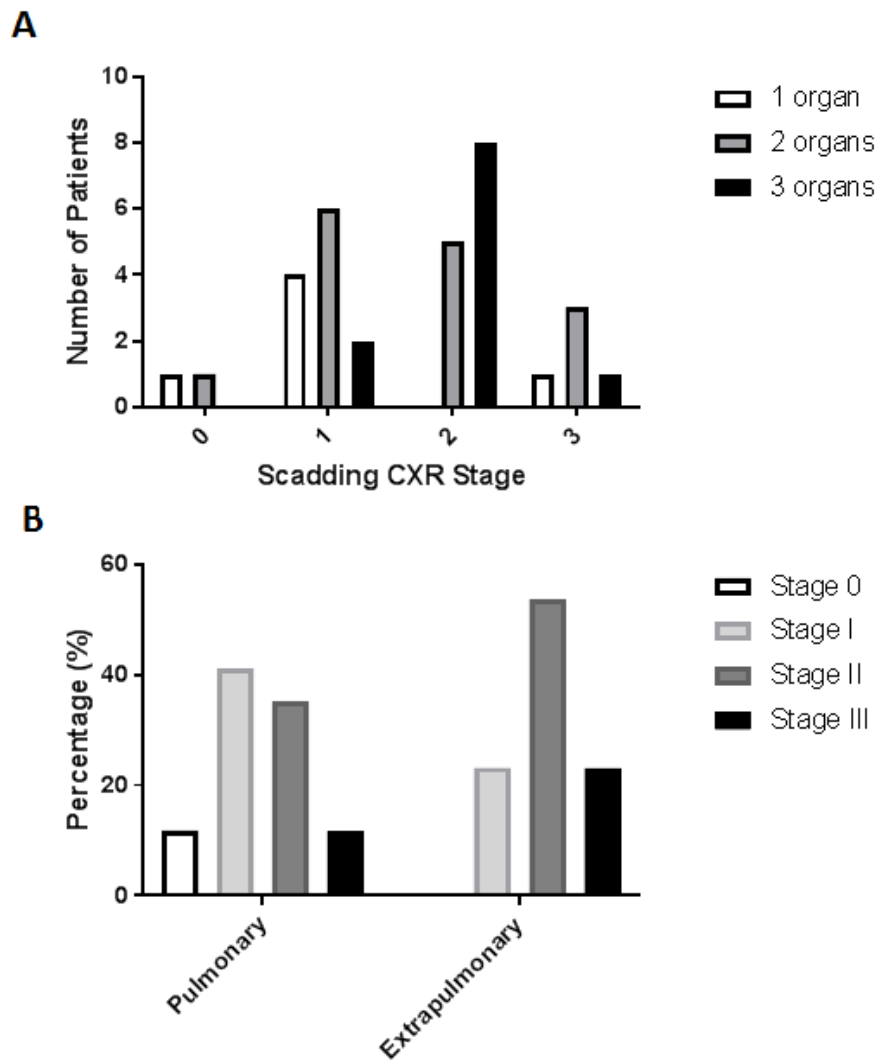
Figure 3.11: Correlation between the number and type of organ involvement and Scadding CXR stages in sarcoidosis patients.

A: The number of patients with a Scadding CXR stage and the number of organs involved within each stage. Patients displaying stage I would display predominantly 1 or 2 organs, whereas stage II was associated with 2 or 3 organs involved. **B:** The percentage of patients displaying specific Scadding CXR stages within pulmonary restricted or extrapulmonary presenting patients. 53.8% of patients with extrapulmonary symptoms were stage II, with significant numbers also belonging to stage III, 23.1%, compared with pulmonary restricted patients with 35.3% and 11.7% in stages II and III respectively.

The median serum ACE concentration of 84.5 U/L is above the normal reference range (8-65 U/L). The range (25-356 U/L) and the standard deviation (81.7 U/L) highlights that the cohort does not universally display elevated serum ACE when compared with healthy controls, 12 patients (40%) had a serum ACE within the 'normal' reference range. Female patients had a higher median serum ACE level (98.5 U/L, IQR 115) than male patients (82

U/L, IQR 59), though this was not statistically significant (Appendix A3.5, p= 0.22, Mann-Whitney U test). Age did not appear to have any statistically significant impact on serum ACE concentrations (Appendix A3.5, p = 0.869, R^2 = 0.009, linear regression).

Patients with a larger number of organs involved typically had a higher serum ACE concentration and higher serum ACE was associated with higher Scadding stages, though stage II patients had a higher median ACE level than stage III patients, though none of the results were statistically significant (Figure 3.12, p > 0.05, Kruskal-Wallis Test). Extrapulmonary organ involvement was associated with increased serum ACE levels (Figure 3.13). Patients with pulmonary restricted organ involvement had a lower median sACE concentration of 55.5 U/L (IQR 43.5) than those with extrapulmonary involvement, who had a median of 112 U/L (IQR 121), with all but 1 individual in the latter group displaying a concentration above the normal reference limit (p= 0.01, Mann-Whitney U test).
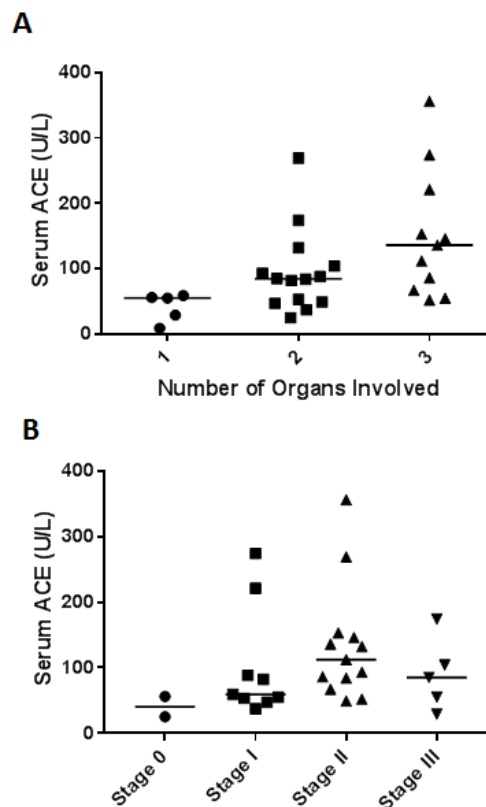
Figure 3.12: Serum ACE levels in patients with sarcoidosis in different Scadding CXR stages and with different numbers of organs involved.

A: A larger number of organs involved would display a higher median serum ACE level in the blood, though this was not statistically significant (p > 0.05), determined using Kruskal-Wallis Test.

B: The Scadding CXR stage II patients had a higher median serum ACE level within the blood. This was not statistically significant (p > 0.05), determined using Kruskal-Wallis Test. Data presented with median (black lines).
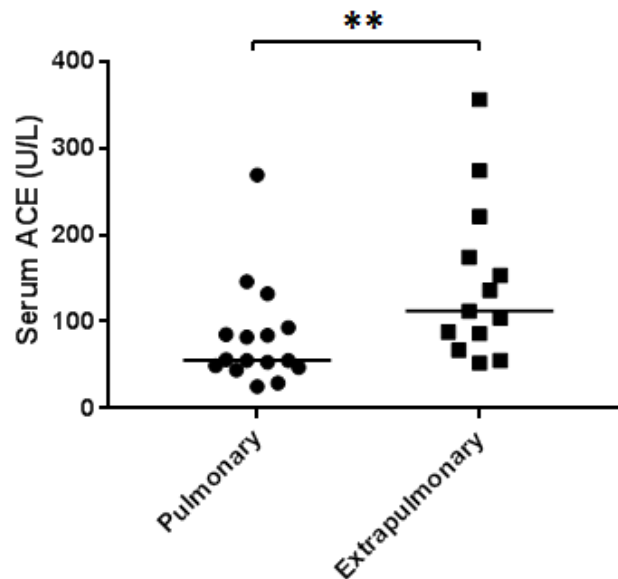
Figure 3.13: Serum ACE levels in sarcoidosis patients with pulmonary restricted or extrapulmonary organ involvement.

Patients with extrapulmonary organ involvement tended to display a higher sACE level than pulmonary restricted patients, median 112 U/L compared to 55.5 U/L respectively. Data presented with median (black lines). ** p = 0.01, Mann-Whitney U test.

The median CRP and PV concentrations were within the normal ranges (Table 3.1), though the patient range shows that a proportion had values above normal for both of these markers. 26% of patients have an elevated CRP concentration and 27% had an elevated PV level in the blood. There was a correlation between the two markers directly (Appendix A3.6, p = 0.026, R^2 = 0.024), though there was not a strong association between CRP and ACE concentrations (Appendix A3.7, p = 0.264, R^2 < 0.001). The median CRP was higher in patients with a larger number of organs involved, 6.7 mg/L (IQR 8.26) compared with 3.4 mg/L (IQR 7.13) for patients with 3 or 2 organs respectively. Patients with extrapulmonary organ involvement also had a higher median CRP level, 7.2 mg/L (IQR 10.26), compared with pulmonary restricted patients, 2.65 mg/L (IQR 4.53). However, there was no statistically significant difference in these associations with CRP and organ involvement (Appendix A3.8). CRP levels were also increased in higher Scadding stages, from 1.95 mg/L (IQR 4.83) in stage I to 6.4 mg/L (IQR 5.9) and 10.2 mg/L (IQR 48.88) in stages II and III respectively (Appendix A3.9). Female and male patients had a similar median level of CRP, 3.3 mg/L (IQR 10.15) and 3.9 mg/L (IQR 5.7), with a broader

range of values in women between 0.7-64 mg/L (Appendix A3.10). Increasing age does appear to correlate to a low degree with CRP levels in patients (Appendix A3.10). PV (measured in milliPascal seconds, m.Pas.s) showed a weak association with ACE (Appendix A3.7, p = 0.361, R^2 = 0.0033, linear regression) and patients in increasing radiographic stages had higher PV levels, median 1.72 m.Pas.s (IQR 0.25) and 1.71 m.Pas.s (IQR 0.18) in stages III and II compared with 1.59 (IQR 0.14) in stage I patients (Appendix A3.9). Patients with more than 1 organ involved have an increased median CRP level compare with those with only 1 system effected, 1.68 m.Pas.s (IQR 0.29) and 1.71 m.Pas.s (IQR 0.12) with 3 and 2 organs respectively compared with 1.58 m.Pas.s (IQR 0.11) with 1 organ (Appendix A3.8). Patients with extrapulmonary organ involvement had a significantly higher concentration of PV in their blood compared with pulmonary restricted patients, 1.72 m.Pas.s (IQR 0.26) compared to 1.65 m.Pas.s (IQR 0.16) respectively (p = 0.03, Mann-Whitney U test) (Appendix A3.8). The PV in female patients, 1.67 m.Pas.s (IQR 0.11), was not significantly different from male patients at 1.71 m.Pas.s (IQR 0.27), though the range for male patients was broader, 1.51-1.93 m.Pas.s compared with 1.54-1.74 m.Pas.s in female patients (Appendix A3.10). Age was not a significant factor in PV levels in patients (p= 0.765, R^2 = 0.009, linear regression, see Appendix A3.10). Overall, age did not appear to be a major factor in most of the clinical features characterised in this patient cohort. Gender specific differences were more significant however, as was the presence of extrapulmonary organ involvement.

3.3.3 Peripheral Blood Mononuclear Cell Number and Number of Leukocytes

Peripheral blood mononuclear cells (PBMCs) from fresh whole blood were isolated and the number of cells determined using the method previously described (section 3.2.2 - Calculation of Peripheral Blood Mononuclear Cell Concentration from the Blood). Total undifferentiated PBMCs were significantly lower in number in patients with sarcoidosis compared with healthy controls (Figure 3.14), 1.21×10^6 cells/ml (± SEM 0.08) compared with 2.03×10^6 cells/ml (± SEM 0.10) in healthy controls (p < 0.0001, Student's T test). Latent TB patients were comparable to healthy controls, with 1.97×10^6 cells/ml (± SEM 0.35). The ratio of populations were determined and the total population numbers calculated using these ratios.
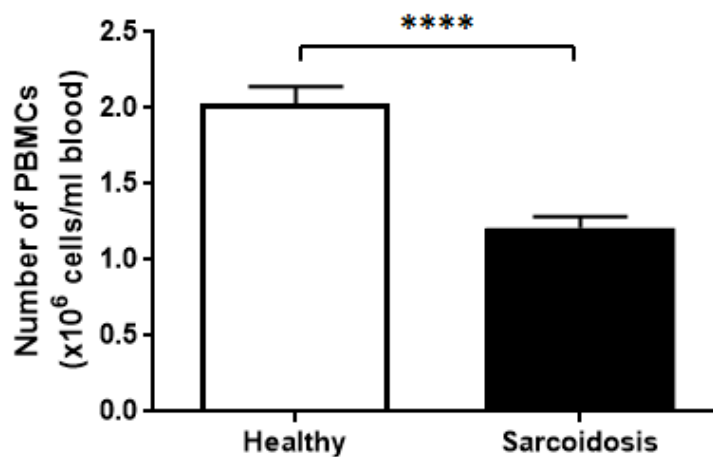
Figure 3.14: Peripheral blood mononuclear cell (PBMC) number in the blood of patients with sarcoidosis and healthy controls.

Patients presented with significantly lower numbers of PBMCs per ml of blood (1.21×10^6 cells/ml) compared with healthy controls (2.03×10^6 cells/ml). Data presented as the mean with the standard error of the mean. **** $p < 0.0001$, Student's T test.
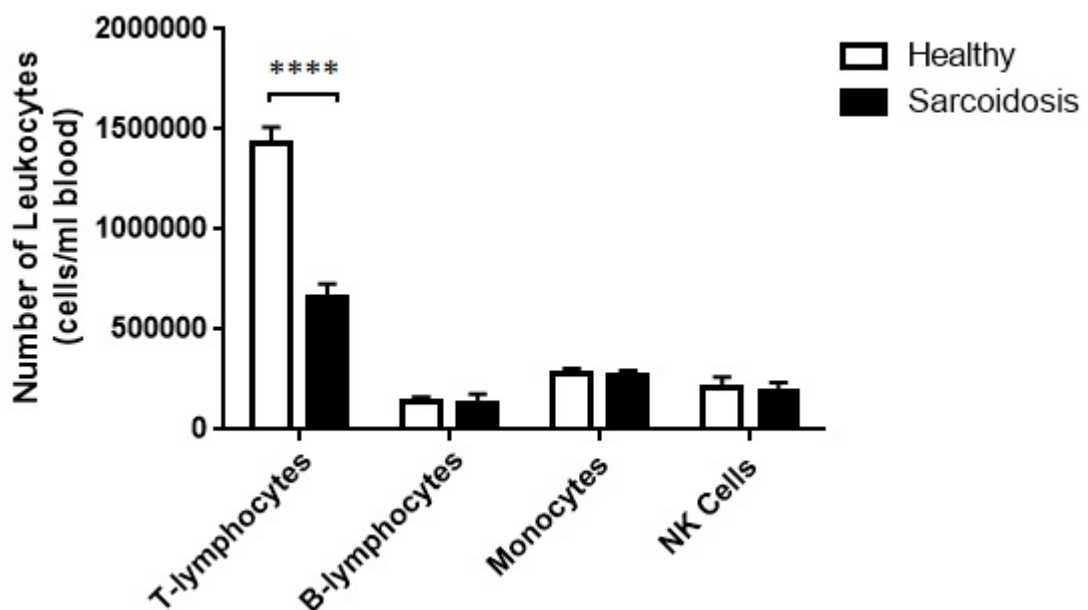
Figure 3.15: PBMC subsets present in the blood of patients with sarcoidosis and healthy controls.

There were significantly fewer circulating T-lymphocytes in sarcoidosis patients compared with controls. T-lymphocytes ($CD3^+$), B-lymphocytes ($CD19^+$), Monocytes ($CD14^+$) and Natural Killer Cells ($CD3^-$ $CD56^+$ lymphocyte gated) were determined by flow cytometry combined with total PBMC counts. Data presented as mean ± SEM. **** $P < 0.0001$, as determined by Two-way ANOVA (with Sidak's Multiple Comparisons Test). Healthy controls, T-lymphocytes and monocytes n = 25. Healthy controls, B-lymphocytes and NK cells n = 10. Sarcoidosis patients, T-lymphocytes and monocytes, n = 22. Sarcoidosis patients, B-lymphocytes and NK cells n = 5.

In order to compare the proportions of the major subsets of leukocytes, assessment by flow cytometry of the cell populations were completed. A range of cell surface markers could be used to differentiate different cell populations, though not all markers were used in this study. T-lymphocytes (CD3$^+$), Monocytes (CD14$^+$), B-lymphocytes (CD19$^+$) and Natural Killer Cells (CD3$^-$ CD56$^+$) were assessed (Figure 3.15). There was no statistically significant difference in the total number of monocytes, B-lymphocytes or NK cells between patients with sarcoidosis and healthy controls. There was a clear T cell lymphocytopenia in sarcoidosis patients, the mean number of T-lymphocytes was over 2 fold lower at 0.66×10^6 cells/ml ($\pm$SEM 0.07×10^6) compared with 1.43×10^6 cells/ml ($\pm$SEM 0.08×10^6) in healthy controls ($p < 0.0001$, Two-way ANOVA). 86% of patients had a T-lymphocyte number below the minimum range of the healthy control cohort (0.84×10^6 cells/ml), which is defined in this study as T cell lymphocytopenia. Based upon this, it can be concluded that the lower PBMC number observed in sarcoidosis blood is due to a T-lymphocyte reduction in the blood of sarcoidosis patients.

The lower T-lymphocyte number is reflected in a reduced CD3:CD14 ratio (Table 3.2). The number of T-lymphocytes in patients did not significantly correlate with the ACE level in patients ($p = 0.76$, $R^2 = 0.005$), nor did the T-lymphocyte counts correlate with age in a statistically significant way in patients ($p = 0.13$, $R^2 = 0.11$) or healthy controls ($p = 0.44$, $R^2 = 0.03$). In both sarcoidosis patients and healthy controls, gender did not appear to significantly affect the number of T-lymphocytes (Appendix A3.11, adjusted p value = 1.00 for both healthy and sarcoidosis, Two-way ANOVA). There was no significant difference in T cell number between patients with pulmonary restricted (mean 0.70×10^6 cells/ml $\pm$SEM 0.10×10^6) and extrapulmonary organ involvement (mean 0.59×10^6 cells/ml $\pm$SEM 0.09×10^6), despite the difference in the mean cell number ($p = 0.57$, Mann-Whitney U test). Scadding stages and the number of organs involved also did not correlate with this reduction in T-lymphocyte number to a statistically significant way (Appendix A3.12). Latent TB patients also displayed a reduced number of T-lymphocytes relative to healthy controls, 0.99×10^6 cells/ml ($\pm$ SEM 0.17×10^6), though this was not statistically significant when compared with either sarcoidosis patients or healthy controls ($p = 0.17$ compared with sarcoidosis patients, One-way ANOVA). The reduced number of patients in this cohort means the value obtained is open to greater variance than is present in other cohorts.

Table 3.2: Leukocyte populations in subjects with sarcoidosis and healthy controls.

Results are presented as mean ±SD; Student's T-test and Mann-Whitney U test. Healthy controls n= min 5, max 21, sarcoidosis patients n= min 5, max 19.

Leukocyte Population	Healthy	Sarcoidosis	P value
PBMC number (x10^6 cells/ml)	2.03 (0.60)	1.21 (0.40)	< 0.0001
Percentage of monocytes in PBMCs	15.1 (4.84)	22.2 (8.49)	0.001
Lymphocyte:monocyte ratio	4.67 (2.58)	2.34 (1.50)	< 0.0001
Percentage of CD3$^+$ T lymphocytes in total lymphocytes	72.6 (6.42)	54.4 (15.00)	< 0.0001
Ratio of CD4$^+$:CD8$^+$ T lymphocytes	1.93 (0.99)	2.28 (2.24)	0.561
Ratio of naïve to memory T lymphocytes (CD45RA:RO)	0.86 (0.63)	0.58 (0.50)	0.185
Percentage of CD19$^+$ B lymphocytes in PBMCs	8.53 (4.40)	11.6 (8.80)	0.679
Percentage of CD3$^-$CD56$^+$ Natural Killer cells in PBMCs	11.9 (5.46)	18.5 (6.75)	0.778
Percentage of CD3$^+$CD56$^+$ NK-like T lymphocytes in T lymphocytes	7.70 (3.01)	5.24 (3.39)	0.190
Percentage of CD25$^+$ activated T lymphocytes in T lymphocytes	22.2 (6.49)	28.8 (11.0)	0.025

3.3.4 Lymphocyte Subsets

T-lymphocytes can be further characterised as naïve (CD45RA$^+$), memory (CD45RO$^+$) and activated (CD25$^+$), as well as T-helper cell subsets (CD4$^+$) and cytotoxic T-lymphocytes (CD8$^+$). These were calculated using the appropriate markers in combination with CD3. The similar CD4:CD8 ratio between healthy controls and patients with sarcoidosis indicates that both subsets are reduced in sarcoidosis, therefore this reduction is not entirely specific to T-helper or cytotoxic cells (Figure 3.16, Table 3.2). Using a boundary defining a lymphopenic CD4$^+$ T-lymphocyte count as below 0.3 x10^6 cells per ml of blood, based upon that established for idiopathic CD4$^+$ lymphocytopenia [702], 50% of the patient population could be defined as T-helper cell lymphopenic. It should be noted that 86% of the patient population displayed reduced T cell numbers below the minimum

number present in the healthy population. 38% of patients displayed a CD8$^+$ T-lymphocyte number below the minimum present in healthy controls, suggesting that, whilst CD4:CD8 ratios were not significantly altered, in the majority of cases CD4$^+$ but not CD8$^+$ T-lymphocytes were technically lymphopenic by strict definitions. The ratio of naïve to memory T-lymphocytes is also similar between the cohorts, suggesting that there is no peripheral increase in memory T cells in sarcoidosis (Table 3.2). The ratio of double positive CD45RA/RO to single CD45RA + single CD45RO in healthy controls (1.561 ±SEM 0.167) and sarcoidosis patients (1.395 ±SEM 0.162) was also not significantly different between the cohorts (p = 0.481, Student's T test).

CD25, a component of the IL-2 receptor, was used to indicate the proportion of 'active' T-lymphocytes. Sarcoidosis patients displayed a statistically significant increased percentage of CD25$^+$ cells and higher expression of CD25 within the T-lymphocyte population compared with healthy controls (Figure 3.17). Despite this, the total number of CD25$^+$ T-lymphocytes was still lower in patients with sarcoidosis due to the generalised T cell lymphocytopenia (Figure 3.17). The proportion of CD4$^+$ CD25^{high} lymphocytes within the T-lymphocyte population was lower in patients than healthy controls, though this was not statistically significant (p = 0.10, Student's T test). The lymphocytopenia did mean that the number of CD4$^+$ CD25^{high} lymphocytes was significantly reduced in patients however, 1.49×10^4 cells/ml (±SEM 0.19×10^4) in patients compared with 3.49×10^4 cells/ml (±SEM 0.42×10^4) in healthy controls (p = 0.03, Student's T test). Without additional differentiating cell markers, a positive identification of these CD25^{high} cells as T regulatory cells could not be made.

CD56 was observed on a proportion of both CD3$^+$ and CD3$^-$ cells (Figure 3.18). CD3$^-$ CD56$^+$ lymphocytes were broadly described as "Natural Killer cells", the number of these cells were not significantly different between healthy controls and sarcoidosis patients (Table 3.2). CD3$^+$ CD56$^+$ T-lymphocytes were assigned the name "Natural Killer T-lymphocytes" and were reduced as a percentage of the population in sarcoidosis, though not significantly (p = 0.19, Student's T test). There was a statistically significant reduction in number relative to healthy controls, due again to the global reduction in T cell number in sarcoidosis (Figure 3.18, p = 0.01, Student's T test).

As previously described, CD19^{+} lymphocytes, the "B-lymphocytes", were not significantly different in number between sarcoidosis patients and healthy controls, though these cells (along with NK cells) made up a greater proportion of the lymphocyte population in sarcoidosis due to the reduced T cell numbers. Overall, the data suggests that there may be specific lymphocyte subset differences between healthy controls and sarcoidosis patients, though the most significant alteration was the lymphocytopenia in T cells.
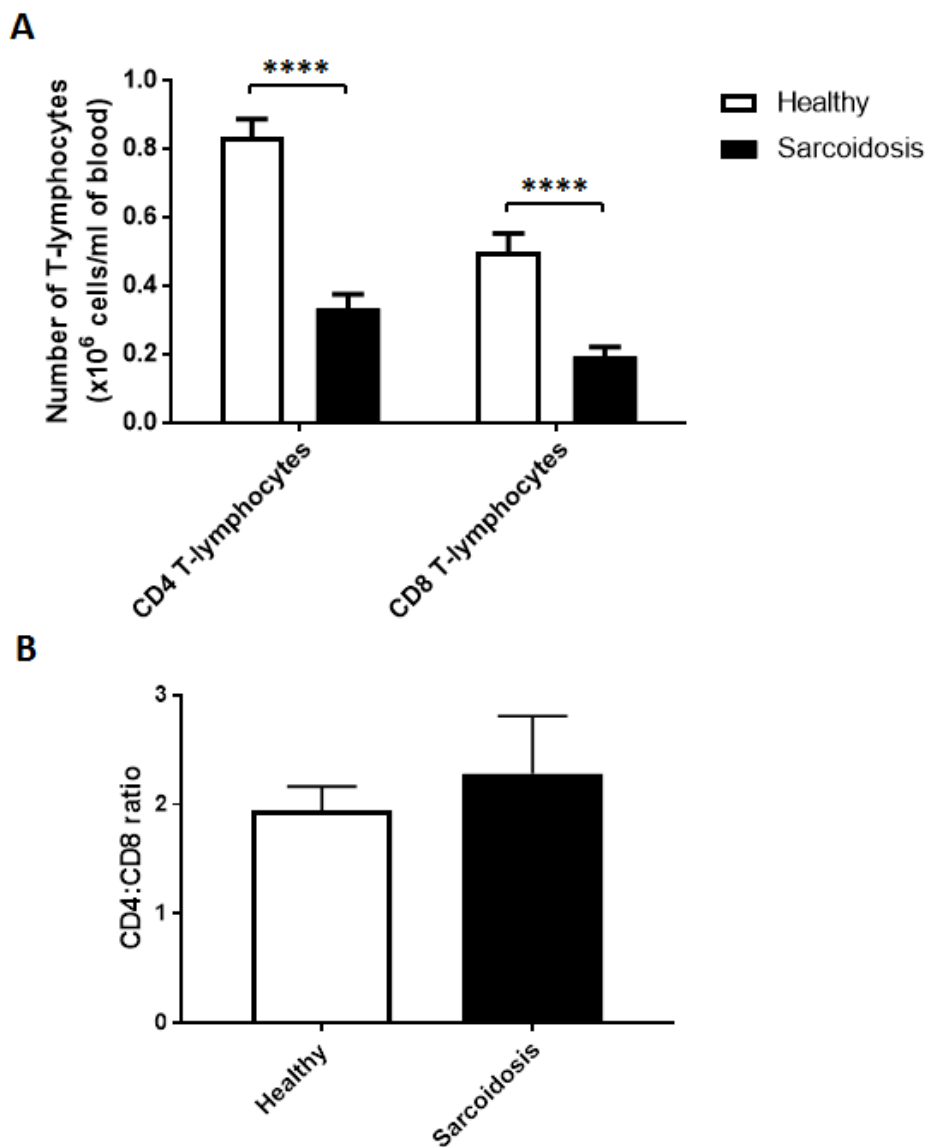
Figure 3.16: T-lymphocyte CD4 and CD8 subsets in PBMCs.

A: Number of CD4^{+} and CD8^{+} T-lymphocytes in PBMCs from healthy controls (n = 23 and 19 respectively) and patients with sarcoidosis (n = 22 and 18 respectively). Patients had a proportional reduction in both subsets of T-cells. Data presented as mean ± SEM. **** $p < 0.0001$, as determined by Two-way ANOVA (with Sidak's Multiple Comparisons Test). **B:** Ratio of CD4 to CD8 T-lymphocytes in patients with sarcoidosis (n = 18) compared with healthy controls (n = 19). Data presented as mean ± SEM, $p = 0.552$ determined by Student's T Test.
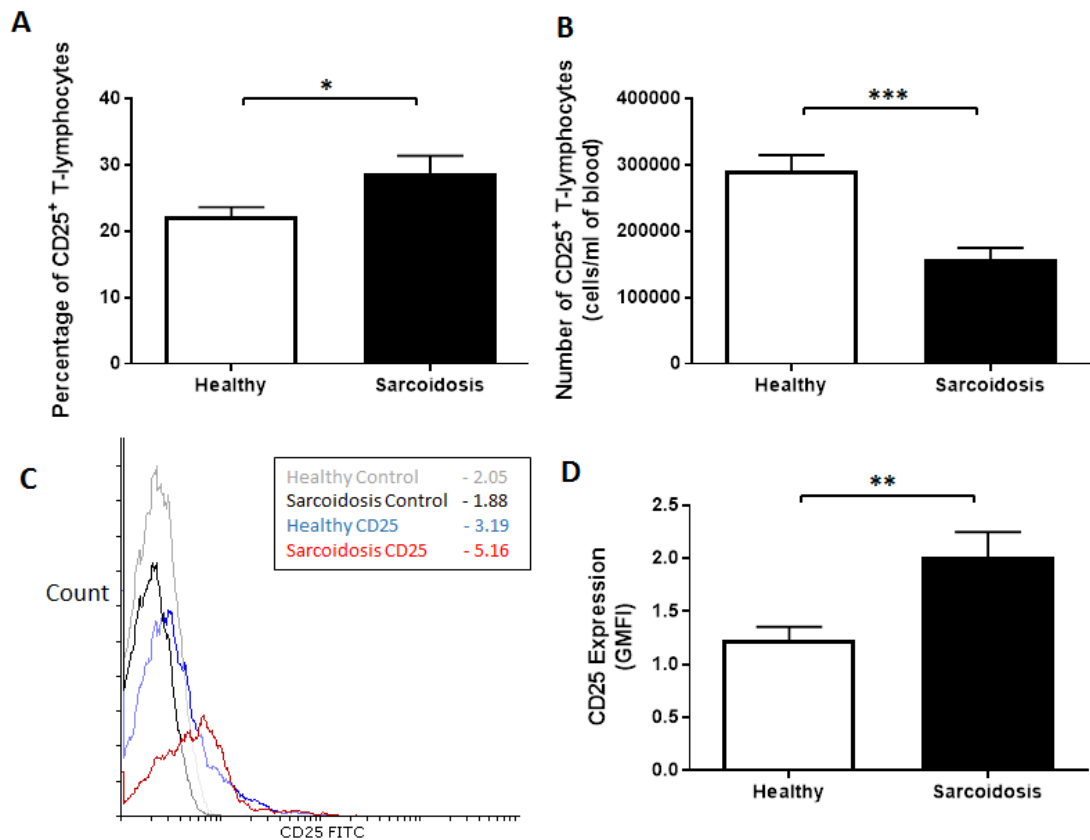
Figure 3.17: T-lymphocyte CD25 expression in sarcoidosis patients and healthy controls.

A: The percentage of the T-lymphocyte population expressing CD25 was greater in sarcoidosis compared with healthy controls. * p = 0.025, as determined by Student's T test. **B:** Number of CD25^{+} T-lymphocytes per ml of blood in patients with sarcoidosis is lower than in healthy controls. *** p = 0.0001, as determined by Student's T test. **C:** Representative histogram displaying the CD25 expression (FITC) in T-lymphocytes in healthy controls (blue line) and sarcoidosis patients (red line), with isotype control antibodies set on lymphocytes for both (grey and black lines respectively). Geometric mean fluorescent intensity (GMFI) values displayed in the box. **D:** Amount of CD25 expression on T-lymphocytes in healthy controls is lower than in sarcoidosis patients, as determined by flow cytometry GMFI. ** p = 0.010, as determined by Student's T test. Data presented as mean ± SEM (A, B and D), n = 23 and 17 for healthy controls and sarcoidosis patients respectively.
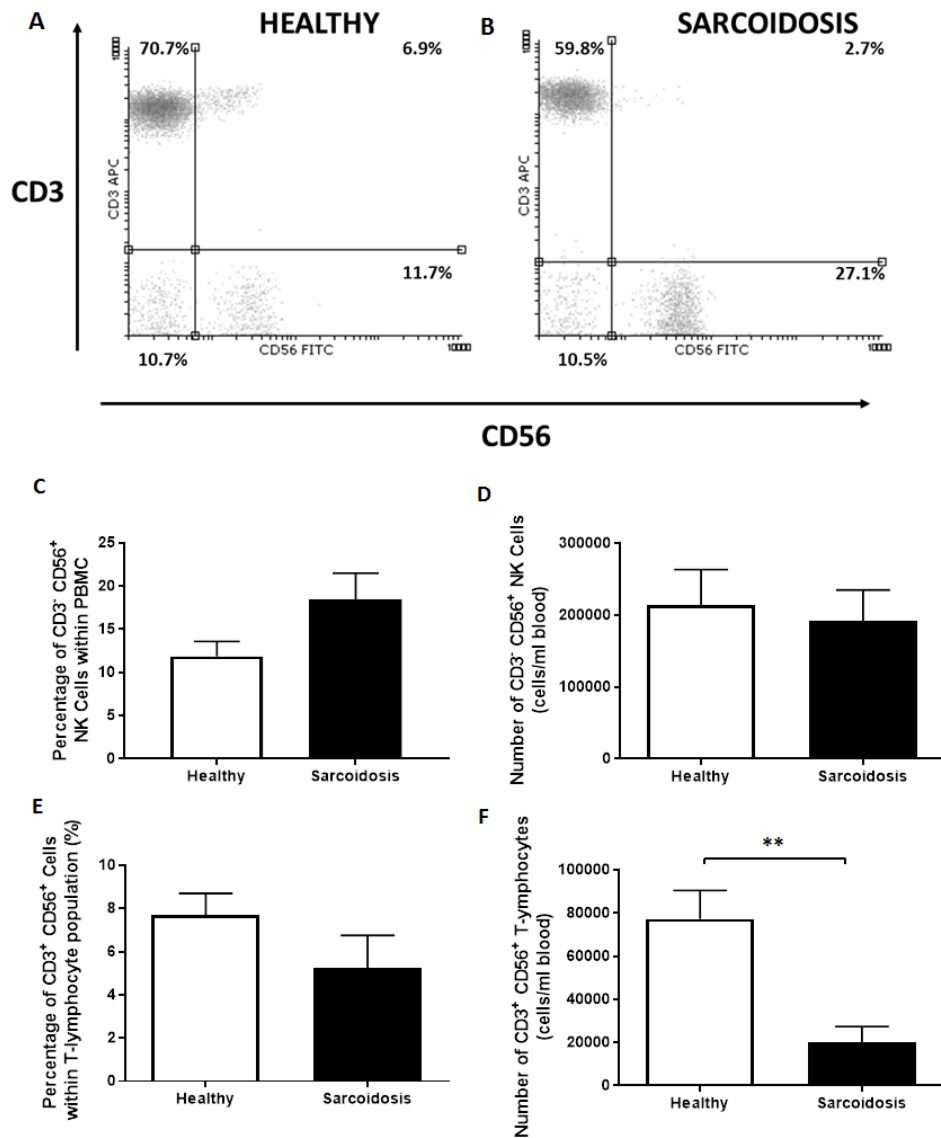
Figure 3.18: CD56 expression in PBMCs and the proportions of Natural Killer cells and NKT cells in sarcoidosis patients and healthy controls.

A and **B:** Representative density plots from analysis of CD56 and CD3 on PBMCs by flow cytometry in healthy controls and sarcoidosis patients. Population represented are lymphocyte gated cells. CD3$^+$ cells are represented in the top left and top right quadrants, CD3$^+$ CD56$^+$ T-lymphocytes (NKT cells) are represented in the top right quadrant. NK cells are present in the bottom right quadrant (CD3$^-$ CD56$^+$). Percentages represent proportion of total lymphocyte population. **C** and **D:** Natural Killer cells make up a greater proportion of the PBMCs in sarcoidosis, but there is no significant difference in the number of these cells between patients and controls (determined by Student's T test). **E:** The percentage of NKT cells within the T-lymphocyte population is reduced in patients with sarcoidosis, but is not statistically significant (Student's T test). **F:** The number of NKT cells within the blood is significantly reduced in sarcoidosis compared with healthy controls. ** p = 0.007, Student's T test. Data presented as mean ± SEM. Sarcoidosis patients n = 5, healthy controls n=9.

3.3.5 Monocyte Subsets

Traditionally, monocytes have been identified by the presence of the CD14 marker on their cell surface. There are subsets of monocytes in humans, which can be differentiated using a combination of the CD14 and CD16 markers. Monocytes can be divided into classical $CD14^{++}/CD16^{-}$, non-classical $CD14^{+}/CD16^{++}$, and intermediate $CD14^{++}/CD16^{+}$ cell populations [335].

Proportionally, most monocytes in healthy controls and sarcoidosis patients' blood were classical $CD14^{++}/CD16^{-}$ cells. However, there was a statistically significant reduction in classical monocytes in sarcoidosis compared with controls, 78.1% (±SEM 1.13) compared with 83.6% (±SEM 2.01) (P<0.01, Two-way ANOVA) (Figure 3.19). There was no significant difference in non-classical monocytes between the cohorts (7.9% ±SEM 1.51 vs 7.1% ±SEM 0.76). In sarcoidosis patients there was expansion of the intermediate monocyte population, 13.6% (±SEM 1.01) compared with 9.3% (±SEM 0.82) in healthy controls (p < 0.05, Two-way ANOVA).

There was a significant difference between patients with Scadding stage I and stage II (p = 0.04, Two-way ANOVA) in terms of the percentage of intermediate monocytes (Figure 3.20); stage II patients had a higher mean percentage of this subset (15.9% ±SEM 1.34) compared with stage I patients (10.8% ±SEM 0.49). There was a trend indicating a possible correlation between increased organ involvement and increased percentages of intermediate monocytes, though this was not significant. Nor was there a significant difference between the intermediate monocyte percentage in pulmonary restricted and extrapulmonary groups of patients or in terms of gender. Lastly, serum ACE levels also did not correlate with this monocyte population (Figure 3.20, p = 0.75, R^2 = 0.008). It is unknown what the significance of this population is in pathological terms, though the expansion of intermediate monocytes does not appear to correlate to a high degree with the majority of clinical findings previously assessed.

Monocytes also express CD25 (Figure 3.21), the mean expression of which was not significantly different between healthy controls and patients with sarcoidosis (p = 0.409, Student's T test) and the proportion of monocytes expressing CD25 were also similar

between the cohorts, 72.5% (±SEM 2.63) in healthy controls and 70.66% (±SEM 4.12) in patients with sarcoidosis (p = 0.691, Student's T test).
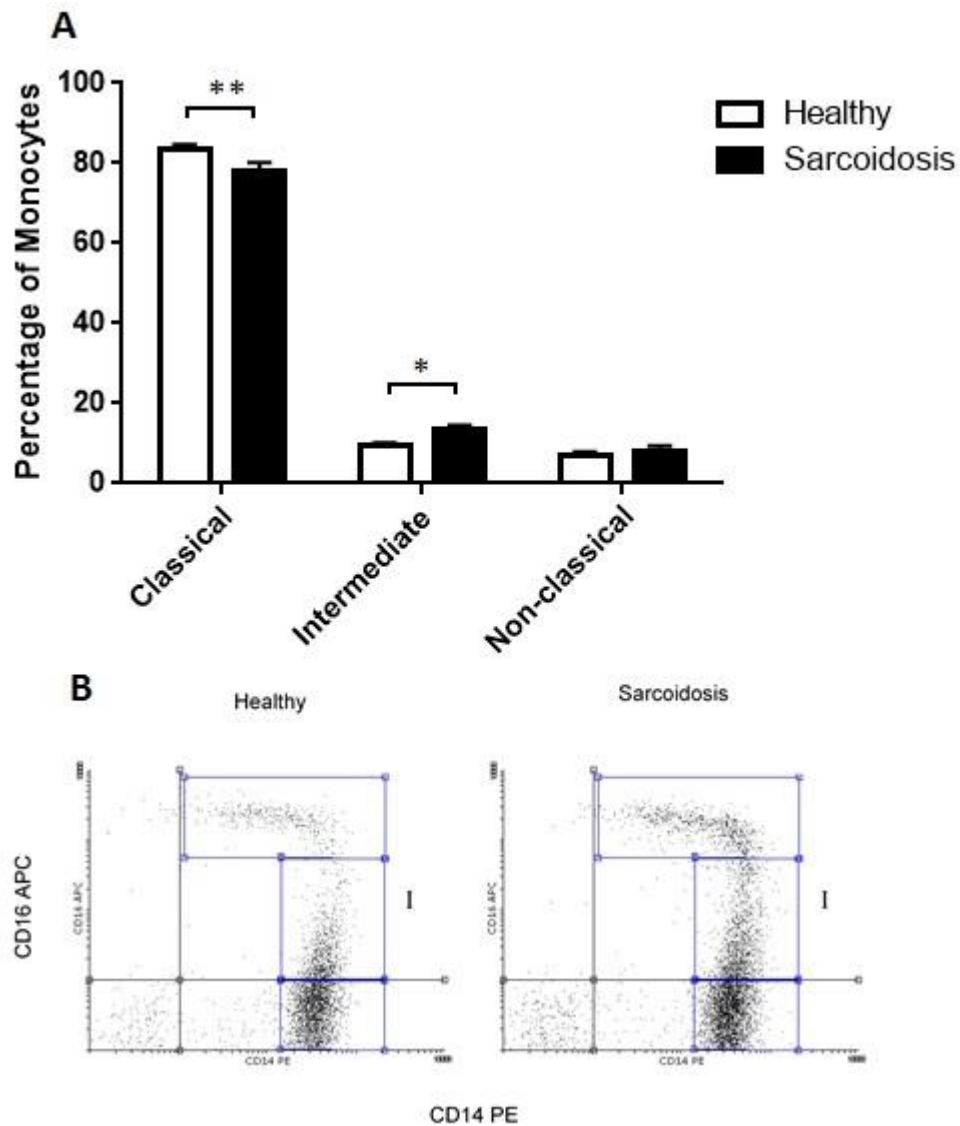
Figure 3.19: Monocyte subsets in blood determined by extracellular antibody staining for CD16 and CD14.

A: Mean percentages (±SEM) of CD14^{++}CD16$^+$ "intermediate" monocytes, CD14^{++}CD16$^-$ "classical" monocytes, and CD14$^+$CD16^{++} "non-classical" monocytes shows that classical monocytes are significantly reduced in sarcoidosis, whereas intermediate monocytes are significantly expanded in the patients with sarcoidosis. ** $p < 0.01$, * $p < 0.05$, as determined by Two-way ANOVA. Healthy n = 18, Sarcoidosis n=14. **(B)** Representative flow cytometry dot plots from healthy and sarcoidosis subjects with intermediate monocytes indicated (I).
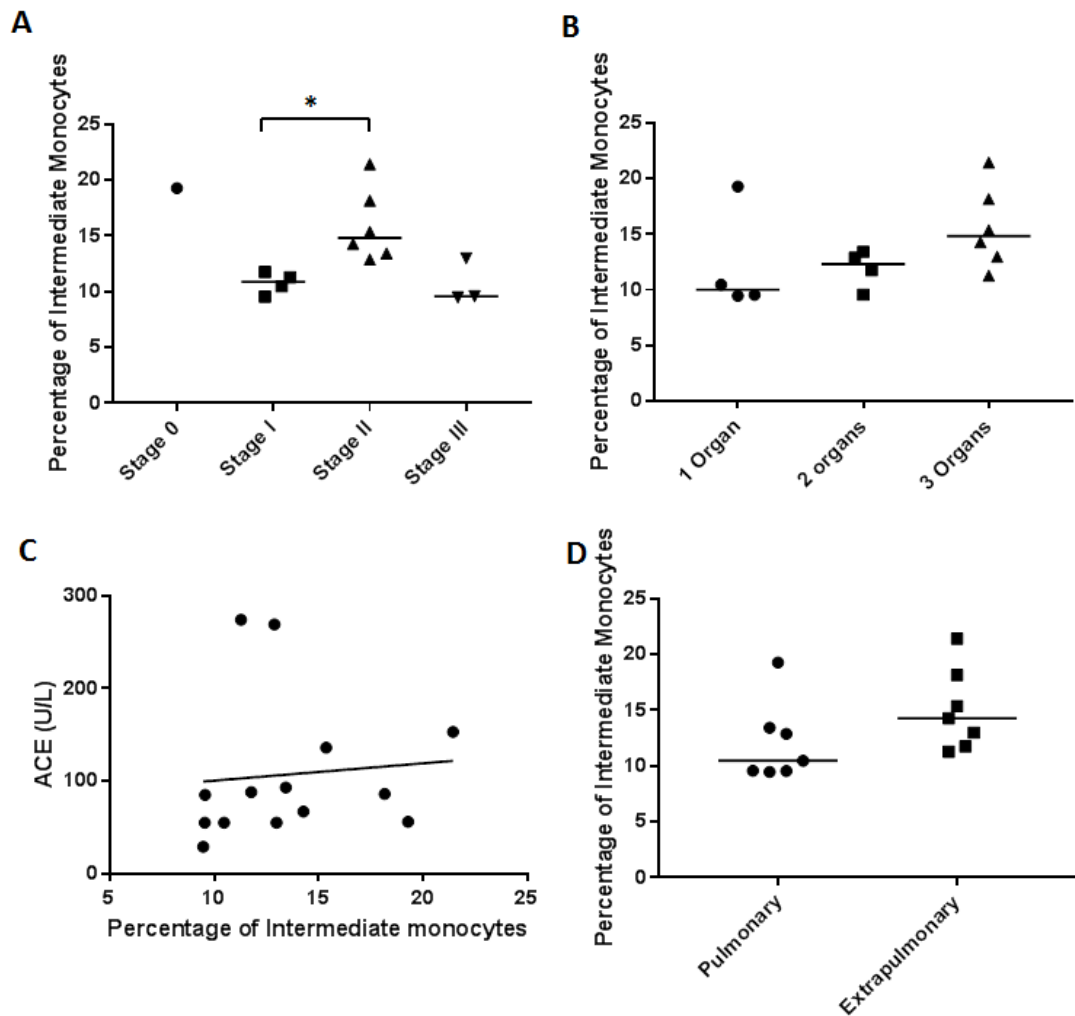
Figure 3.20: Correlation between the percentage of intermediate monocytes in patients with sarcoidosis and clinical features.

A: Correlation between intermediate monocytes and Scadding stages**.** Intermediate monocytes were significantly increased in Scadding stage II patients compared with stage I patients. * p = 0.044 as determined using One-way ANOVA. **B:** Correlation between number of organs involved and intermediate monocyte percentage. Not statistically significant, determined using One-way ANOVA. **C:** Serum ACE correlated with intermediate monocyte percentages. Not statistically significant (p = 0.75, R^2 = 0.008), determined using Spearman's Rank Correlation Test. Black line = linear regression. **D:** Differences in intermediate monocytes between patients with pulmonary restricted and extrapulmonary organ involvement. Not significant (p = 0.097), determined using Mann-Whitney U test. Data from A, B and D presented with median (black line).
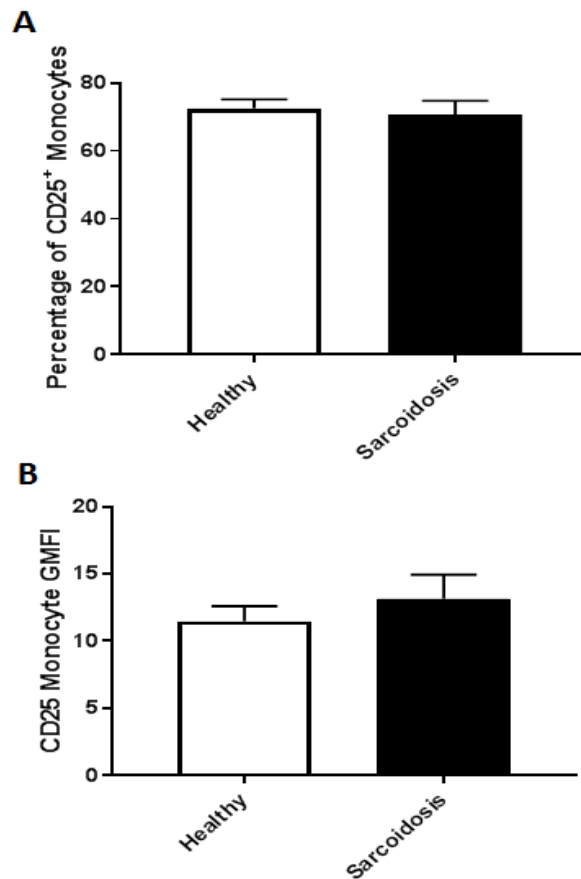
A: The percentage of monocytes expressing CD25 in healthy controls and patients with sarcoidosis. Not significant, (p = 0.691), as determined using Student's T test. **B:** The expression of CD25 on monocytes in healthy controls and patients with sarcoidosis. Not significant (p = 0.409), as determined using Student's T test. Data presented as mean ± SEM. Sarcoidosis patients n = 15, healthy controls n = 22.

3.3.6 Validation of the Whole Blood Assay

Previous reports have been contradictory as to whether PHA is able to induce a greater release of cytokines from whole blood compared against comparable isolated peripheral blood mononuclear cells [703-705]. In order to establish whether a whole blood assay would be preferred to isolated PBMCs, blood samples from healthy controls were treated with varying concentrations of PHA for 16 hours alongside PBMCs re-suspended at the same concentration from the same donors. The plasma was subsequently analysed for cytokine release.

Whole blood is shown to release comparable levels of pro-inflammatory cytokines as PBMCs at lower concentrations of PHA stimulation, but significantly greater concentrations of cytokines are released at and above 10 µg/ml of PHA stimulation (Figure 3.22). This is particularly significant for IFN-γ, TNF-α and IL-6, though the differences are less significant and more variable for IL-10 release. The response to PHA was over 5 fold greater in whole blood compared with isolated PBMCs for IFN-γ at some concentrations, confirming previously reported findings of higher release from whole blood [703].

Given that the use of whole blood would perturb the system to a lesser degree than isolation of PBMCs, it was decided that an *ex vivo* whole blood assay would be used in favour of PBMC assays for further work. It also benefited from retaining the variety of other factors in blood that might otherwise be removed in PBMC preparation.
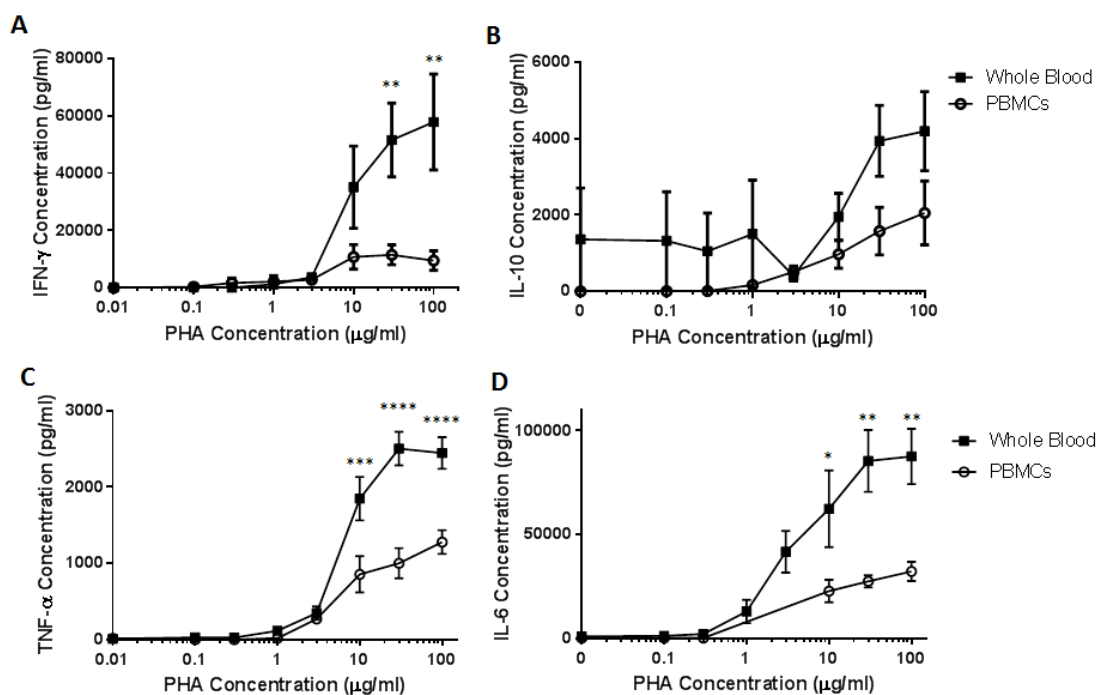
Figure 3.22: Comparison between whole blood and matched isolated PBMC cytokine release in response to Phytohaemagglutinin (PHA) in healthy controls.

Whole blood and PBMCs re-suspended in RPMI media and stimulated with PHA for 16 hours had cytokine release analysed by ELISA for **A:** IFN-γ, **B:** IL-10, **C:** TNF-α and **D:** IL-6. There was higher cytokine release in whole blood compared with PBMCs. Data presented with mean ± SEM and analysed by Two-way ANOVA. IL-6 and IFN-γ n = 6, TNF-α and IL-10 n = 5. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.
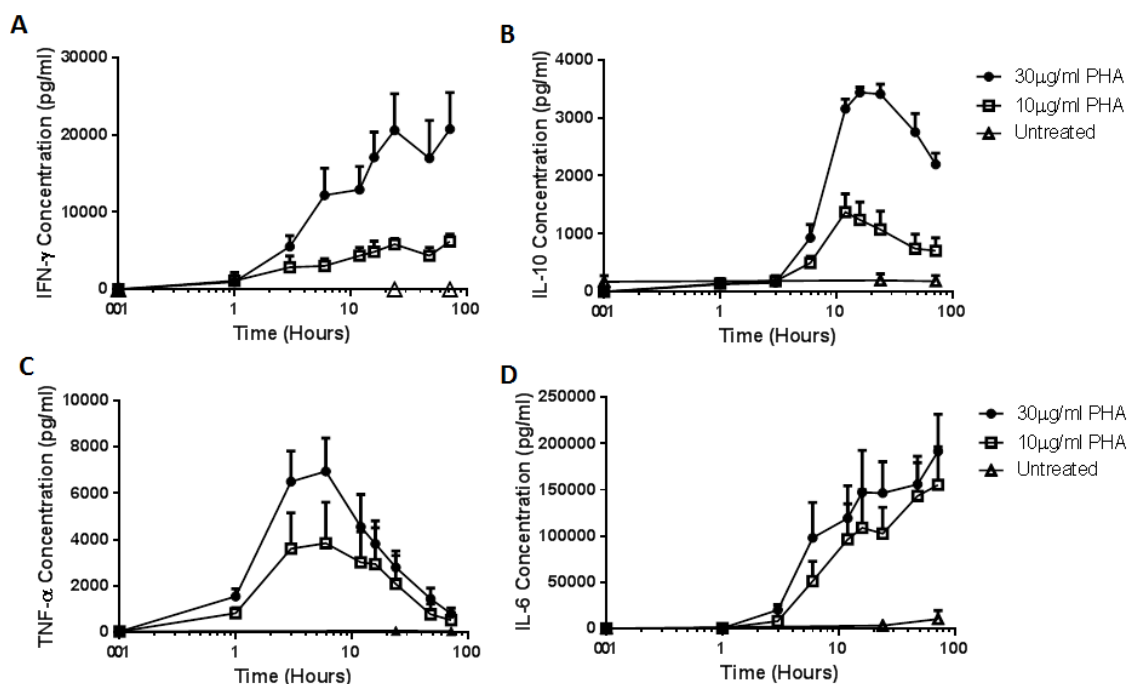
Figure 3.23: Timecourse of whole blood cytokine release in response to PHA in healthy controls.

Whole blood was treated with 30 µg/ml or 10 µg/ml of PHA (or a PBS control) for times between 1-72 hours. Plasma was analysed by ELISA for **A:** IFN-γ, **B:** IL-10, **C:** TNF-α and **D:** IL-6. Peak cytokine release differed between the cytokines, 6 hours for TNF-α but continual rises in IL-6. Both IFN-γ and IL-10 peak release is between 16-24hours. Data presented with mean ± SEM (one sided error bars). All PHA, n = 4. All untreated, n = 3.

3.3.7 Time Kinetics of PHA Stimulation of Whole Blood in Healthy Controls

In order to select an appropriate time point for subsequent experiments, a timecourse of cytokine release would need to be performed on whole blood in healthy controls to assess the kinetic profile of PHA release in this system. A single time point would need to be selected that would best accommodate the peak release of the majority of cytokines under investigation. Blood was stimulated with 2 sub-optimal concentrations of PHA, 10 µg/ml and 30 µg/ml. Plasma concentrations of the Th-1-like inflammatory cytokines IFN-γ, IL-12(p70) and TNF-α, the pleiotropic inflammatory IL-6, the Th-2-like cytokine IL-4 and the regulatory cytokine IL-10 were analysed over the course of 72 hours (Figure 3.23). Maximal release of IFN-γ occurred at 24 hours, whereas peak release by IL-10 was between 16-24 hours. The reduction in IL-10 after 24 hours coincides with a continual

increase in IL-6 over the 72 hour period. IL-12(p70) was not detected at the minimum range over the period, and IL-4 release was subject to minimal release in response to PHA (data not shown). TNF-α was rapidly secreted between 3-6 hours, before diminishing at later time points. Subsequent experiments would be standardised at 16 hours, though it is acknowledged that the TNF-α release as an early activated cytokine would not be optimally accounted for with these assays, though would still be detectable at 16 hours.

3.3.8 Cytokine Release by Whole Blood in Response to Phytohaemagglutinin

PHA is typically described as a T cell mitogen, capable of stimulating T-lymphocyte proliferation and cytokine release [696, 706]. As such, the hypothesis was that the T cell lymphocytopenia observed in sarcoidosis might negatively impact the peripheral immune responses of patients and would thus lead to a reduction in pro-inflammatory cytokine release from whole blood in response to PHA. Whole blood was stimulated for 16 hours with varying concentrations of PHA and the plasma cytokine concentrations subsequently analysed. TNF-α, IL-6, IFN-γ, IL-8, IL-12(p70) and IL-4 were all quantified from healthy controls and sarcoidosis patients. A limited number of latent TB patients were also assessed for specific cytokines (IFN-γ, IL-4, IL-12(p70) and IL-10).

Higher concentrations of secreted pro-inflammatory cytokines were found in sarcoidosis samples in response to high concentrations of PHA. At 100μg/ml of PHA, there was a significantly increased release of TNF-α in patients with sarcoidosis compared to healthy controls, mean concentration of 4408 pg/ml (±SEM 906) compared with 2832 pg/ml (±SEM 453) respectively (Figure 3.24, $p < 0.05$, Two-way ANOVA). Whilst the mean TNF-α release was also higher at 10 and 30 μg/ml of PHA stimulation in patient samples, this was not statistically significant. Whereas the peak release for healthy controls was reached upon stimulation with 30 μg/ml, patients with sarcoidosis had increased release beyond this PHA concentration.

At PHA concentrations of 30 and 100 μg/ml, healthy controls showed a reduced rate of increase of IL-6, appearing to peak at 10 μg/ml (Figure 3.24). Above this concentration, sarcoidosis patients had a significantly increased concentration of IL-6 in the blood after

stimulation, continuing to rise above that of healthy controls. After stimulation with 100 µg/ml of PHA, patients with sarcoidosis displayed a mean IL-6 concentration of 165531 pg/ml (±SEM 24451), compared with only 92594 pg/ml (±SEM 12993) in healthy controls, which was highly significant ($p < 0.001$, Two-way ANOVA). Whilst less significant ($p < 0.05$, Two-way ANOVA), there was still a difference observed at 30 µg/ml of PHA in patients as well, suggesting that there was a robust response to PHA from sarcoidosis leukocytes. IFN-γ release was not significantly different between healthy controls and sarcoidosis patients, though the mean concentration of IFN-γ in blood after the addition of 100 µg/ml of PHA was higher in patient samples, 72259 pg/ml (±SEM 18100) in sarcoidosis compared with 59170 pg/ml (±SEM 10943) in controls (Figure 3.25). Latent TB patients had a lower mean IFN-γ release in response to PHA than either patients with sarcoidosis or healthy controls, though the low number of donors (n=5) and high degree of variation between individuals makes it difficult to accurately determine whether this would be representative of latent TB populations as a whole (Appendix A3.13) .

IL-8 was not statistically different between healthy controls and patients with sarcoidosis, although the mean release of the chemokine was higher in patients above 10 µg/ml of PHA stimulation (Figure 3.25). There was a significant variability in release between patients that was not as pronounced in healthy controls.

IL-12(p70) was not detectable after 16 hours in healthy donors. In sarcoidosis, at 100µg/ml of PHA the majority of donors did not secrete any IL-12(p70), aside from a single donor (data not shown). IL-4 did not increase in a dose dependent manner in response to PHA and the baseline cytokine level varied between individuals. Latent TB patients also did not show any significant IL-4 or IL-12(p70) cytokine release in response to PHA stimulation. IL-10 release increased above 3 µg/ml of PHA stimulation, though there was no statistically significant difference in the concentrations of this cytokine between healthy controls and patients with sarcoidosis (Figure 3.25). The mean values were higher in healthy controls, though there was only a modest difference, 938 pg/ml was the largest difference at 30 µg/ml of PHA stimulation. As with IFN-γ release, the donor variability between latent TB patients was significant, though the mean IL-10 release was lower than healthy controls but higher than sarcoidosis patients (Appendix A3.13)
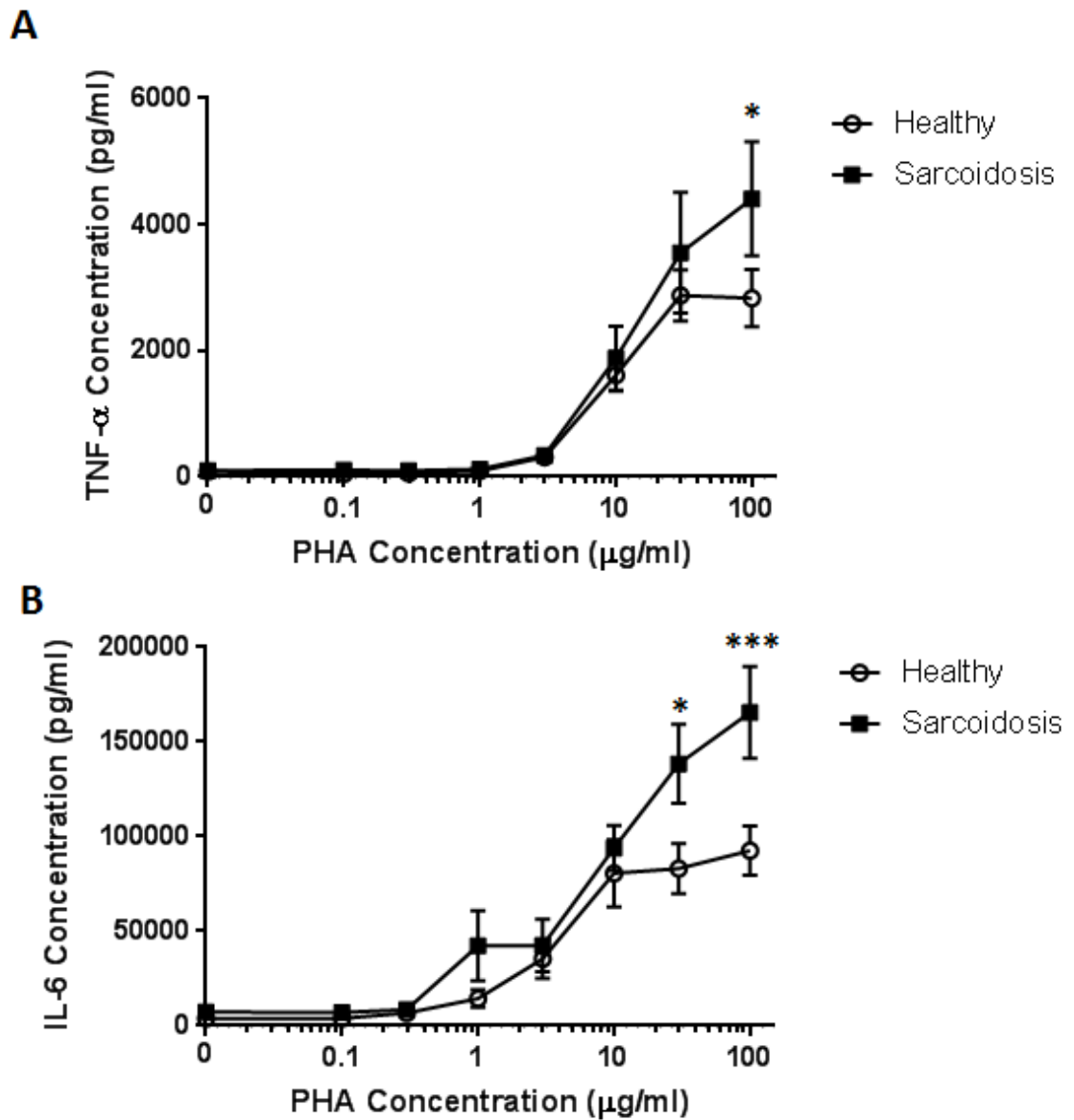
Figure 3.24: Whole blood TNF-α and IL-6 cytokine release in response to PHA in patients with sarcoidosis compared with healthy controls.

A dose response to PHA was undertaken for 16 hours at concentrations of 0 µg/ml to 100 µg/ml of PHA in patient and control whole blood. Plasma was analysed by ELISA for **A:** TNF-α **,** and **B:** IL-6. There was significantly increased IL-6 and TNF-α release in patient blood upon treatment with 100 µg/ml of PHA. Stimulation with 30 µg/ml of PHA also led to significantly higher IL-6 release in sarcoidosis patients. Data presented with mean ± SEM and analysed by Two-way ANOVA. * $p < 0.05$, ** *** $p < 0.001$. TNF-α n = 14 and n= 12 for controls and patients respectively. IL-6 n = 17 for both patients and controls.
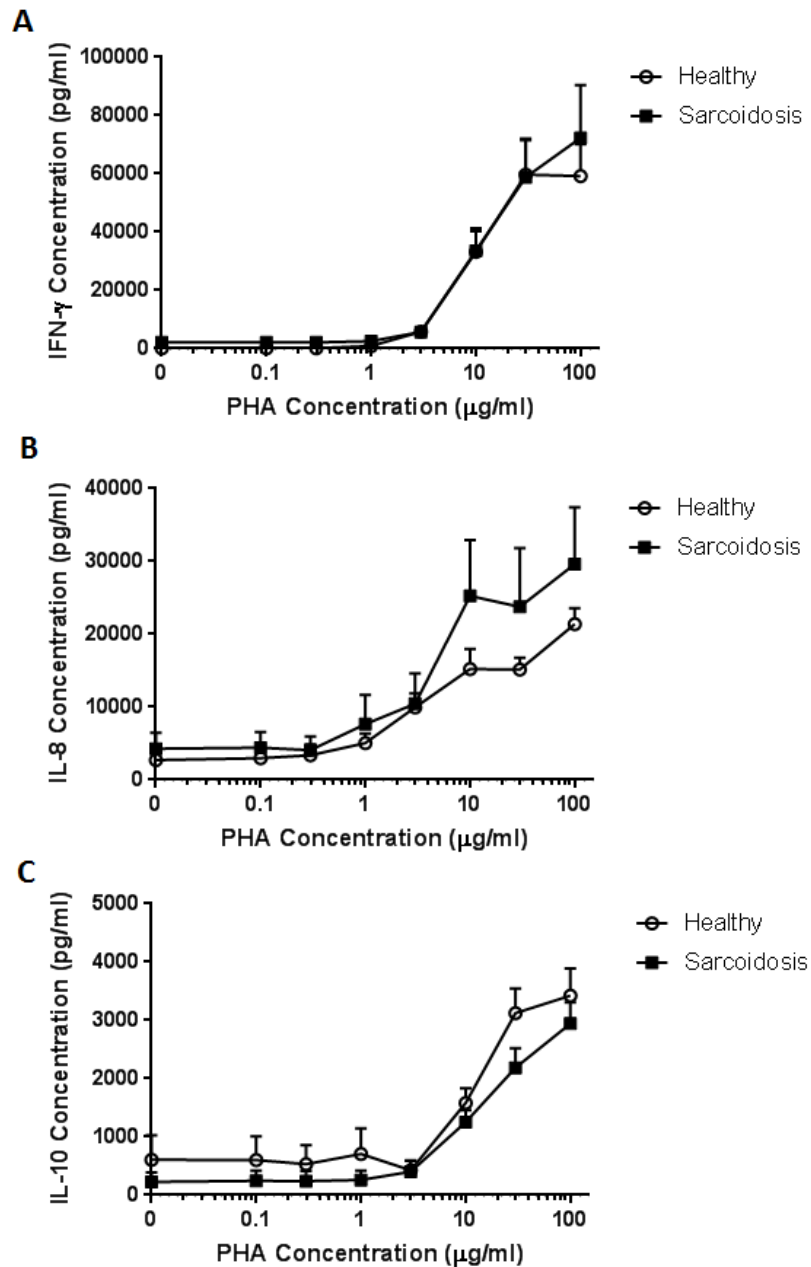
Figure 3.25: Whole blood IFN-γ, IL-8 and IL-10 cytokine release in response to PHA in patients with sarcoidosis compared with healthy controls.

A dose response to PHA was undertaken for 16 hours at concentrations of 0 µg/ml to 100 µg/ml of PHA in patient and control whole blood. Plasma was analysed by ELISA for **A:** IFN-γ**, B:** IL-8 and **C:** IL-10. There was no statistically significant difference in any of the cytokines tested at any concentration of PHA stimulation. Data presented with mean ± SEM (one sided) and analysed by Two-way ANOVA. IFN-γ n = 21 and n= 18, IL-8 n = 8 and n = 7 and IL-10 n = 17 and n = 11 for healthy controls and patients with sarcoidosis respectively.

Overall, there was a significantly increased pro-inflammatory cytokine response observed in patients with sarcoidosis in response to PHA, specifically in TNF-α and IL-6 release. There was not the expected reduction in cytokine release attributable to the T cell lymphocytopenia observed in these patients, even IFN-γ release was not reduced relative to heathy controls. Nor was the regulatory cytokine IL-10 significantly lower in patients to account for this elevated cytokine release.

3.3.9 Correlation between Cytokines Released in Response to PHA

An individual cytokine does not exist in a vacuum, it is part of the milieu of factors released in response to a stimuli such as PHA. As a result, it was important to assess whether the increase in a cytokine observed in the whole blood assay correlated either positively or negatively with others previously studied. The association between TNF-α, IL-6 and IFN-γ were of particular interest, for their role in sarcoidosis and the previously highlighted differences observed in the assay. Data for 100 µg/ml of PHA stimulation from the whole blood assays was used to assess the correlation between cytokines, though donor usage for specific cytokines meant that this could present data lower in number than those present in the dose responses.

Figure 3.26 displays the correlations between TNF-α, IL-6 and IFN-γ for both healthy and sarcoidosis patients. Although there was a tendency for individuals with higher IL-6 release to also have higher TNF-α release in healthy controls, this was not statistically significant ($p = 0.438$, $R^2 = 0.113$). In contrast to healthy controls, patients with sarcoidosis displayed a strong correlation between the release of IL-6 and TNF-α ($p = 0.001$, $R^2 = 0.56$). When correlating TNF-α with IFN-γ, sarcoidosis patients displayed no significant association between these cytokines ($p = 0.457$, $R^2 = 0.01$), whereas healthy controls showed a strong correlation exists between the cytokines in this cohort ($p = 0.0002$, $R^2 = 0.61$). There was little apparent correlation between IL-6 and IFN-γ in either patients with sarcoidosis ($p = 0.585$, $R^2 < 0.01$) or healthy controls ($p = 0.264$, $R^2 = 0.01$).

In other combinations (Appendix A3.14), the cytokines from the whole blood assay usually did not correlate either positively or negatively in healthy controls or patients with

sarcoidosis. For example, IFN-γ and IL-10 were not associated with each other in controls (p = 0.152, R^2 = 0.07) or patients (p = 0.94, R^2 < 0.01).

The data highlights specific differences in the correlation between cytokines released in response to PHA in patients with sarcoidosis compared with healthy controls. Ultimately, the correlations do not suggest a direct association, thus it is necessary to ascertain why patients respond more to this stimuli than healthy controls.
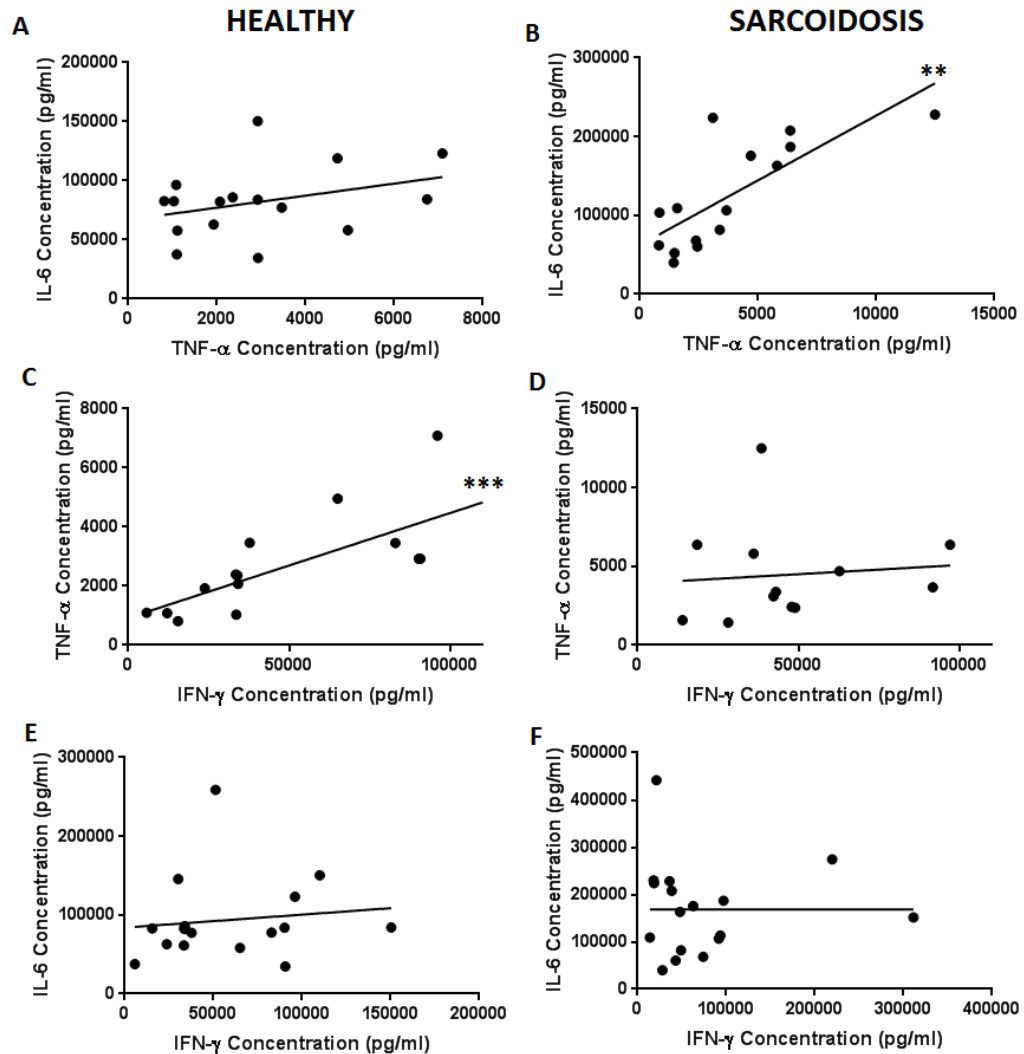
Figure 3.26: Correlation between pro-inflammatory cytokines released from PHA stimulated whole blood in patients with sarcoidosis and healthy controls.

IFN-γ, TNF-α and IL-6 release were correlated for healthy controls (A, C and E) and sarcoidosis patients (B, D and F). **A** and **B:** Correlation between TNF-α and IL-6, healthy controls (p = 0.438, R^2 = 0.113), sarcoidosis patients (** p = 0.001, R^2 = 0.56). **C** and **D:** TNF-α correlation with IFN-γ, healthy controls (** p = 0.0002, R^2 = 0.61), sarcoidosis patients (p = 0.457, R^2 = 0.01). **E** and **F:** IL-6 correlation with IFN-γ, healthy controls (p = 0.264, R^2 = 0.01), sarcoidosis patients (p = 0.585, R^2 < 0.01). Black line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.

3.3.10 Time Kinetics of PHA Stimulation of Whole Blood in Patients with Sarcoidosis

The elevated cytokine release in patients with sarcoidosis in spite of the reduced T-lymphocyte number in the blood may be related to the dynamic multi-cell system in the blood. The time kinetics in healthy blood revealed that the TNF-α concentration within plasma fell after 6 hours, IL-10 peaked and fell after 24 hours. This could be due to other cells internalising and degrading the soluble cytokines, therefore it was necessary to perform a timecourse experiment with sarcoidosis blood to observe whether different kinetics might explain the elevated PHA induced IL-6 and TNF-α concentrations observed (Figure 3.27).
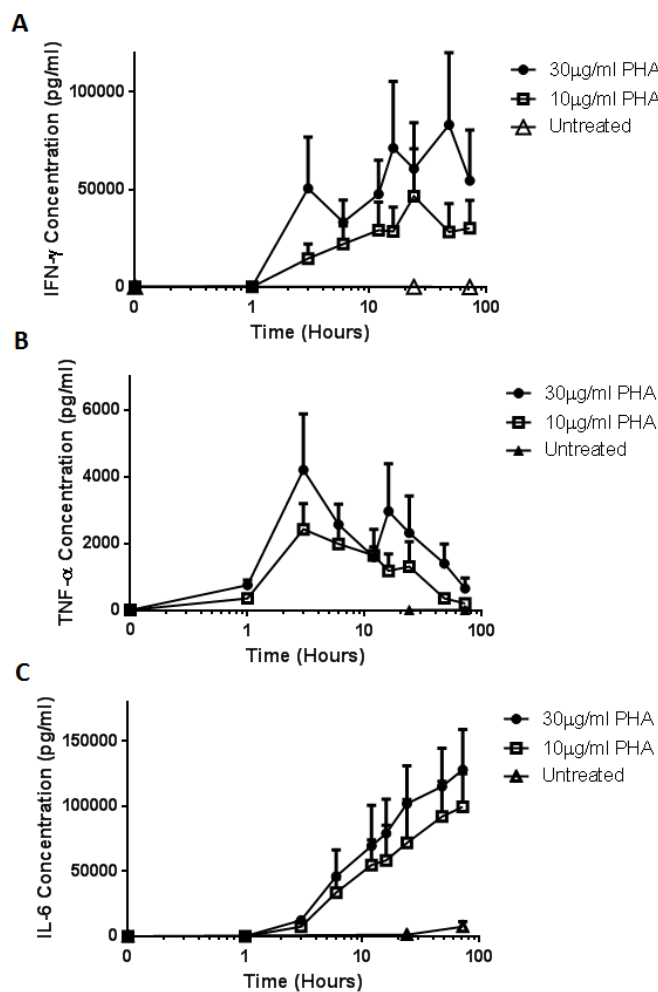
Figure 3.27: Timecourse of whole blood cytokine release in response to PHA in patients with sarcoidosis.

Whole blood was treated with 30 μg/ml or 10 μg/ml of PHA (or a PBS control) for times between 1-72 hours. Plasma was analysed by ELISA for **A:** IFN-γ, **B:** TNF-α and **C:** IL-6. Data presented with mean ± SEM (one sided error bars). All PHA, n = 4. All untreated, n = 4.

There was a large degree of variation between individuals, but the patterns observed with the limited number of patients showed a similar kinetic profile for IL-6 release as had been seen from healthy controls, where concentrations continued to rise over the 72 hour period. TNF-α release peaked at 3 hours post-stimulation, though appeared to rise slightly at 16 hours. This was not consistent for all donors, thus it is difficult to determine whether this is significant given the limited numbers of patients used and the high variability. IFN-γ in patients was again, variable, but not dissimilar to healthy controls at 10 μg/ml. 30 μg/ml appeared to lead to 'peaks and troughs' in cytokine concentration, particularly a reduction at 6 hours. There were clearly differences in some of the kinetics, though it was unlikely that the elevated TNF-α and IL-6 was related to reduced uptake of these cytokines by other leukocytes, as the patterns were similar but not identical to those seen in controls.

3.3.11 Cytokine Production by Lymphocytes and Monocytes in Response to PHA

Whole blood is a mixture of a large number of different leukocytes and other cell types, such as erythrocytes. In order to ascertain whether monocytes or lymphocytes may be responsible for the elevated TNF-α and IL-6 observed in the *ex vivo* experiments, PHA stimulation of PBMCs was undertaken *in vitro* and the cytokine production quantified over time using intracellular flow cytometry. Brefeldin A, a Golgi transport inhibitor [707], was added to PBMCs alongside PHA to prevent extracellular secretion of cytokines and allow the accumulation of the proteins inside the cells for detection. The secondary aim was to determine the direct impact of PHA on leukocyte pro-inflammatory cytokine production, thus elucidating the early activation events involved in PHA stimulation. Preventing exogenous cytokine release (as confounding co-stimulatory signals) whilst allowing cell to cell interactions to remain using surface molecules (for possible accessory interactions that may be required) perturbs later activation events.

TNF-α was not detectable significantly in lymphocytes upon PHA stimulation over the course of 16 hours in response to either 1 or 10 μg/ml of PHA, but increased over time in monocytes (Figure 3.28). The accumulation of TNF-α was greater in PBMCs in sarcoidosis patients than healthy controls at both 6 and 12 hours in response to 10 μg/ml of PHA (p =

0.01 and 0.05 respectively, Two-way ANOVA), resulting from an initial drop in intracellular TNF-α levels in healthy monocytes between 3-6 hours. At a lower concentration of PHA (1 μg/ml), the monocytes in sarcoidosis patients were more responsive, accumulating significantly more TNF-α by 16 hours than healthy controls (Figure 3.28, $p < 0.001$, Two-way ANOVA).
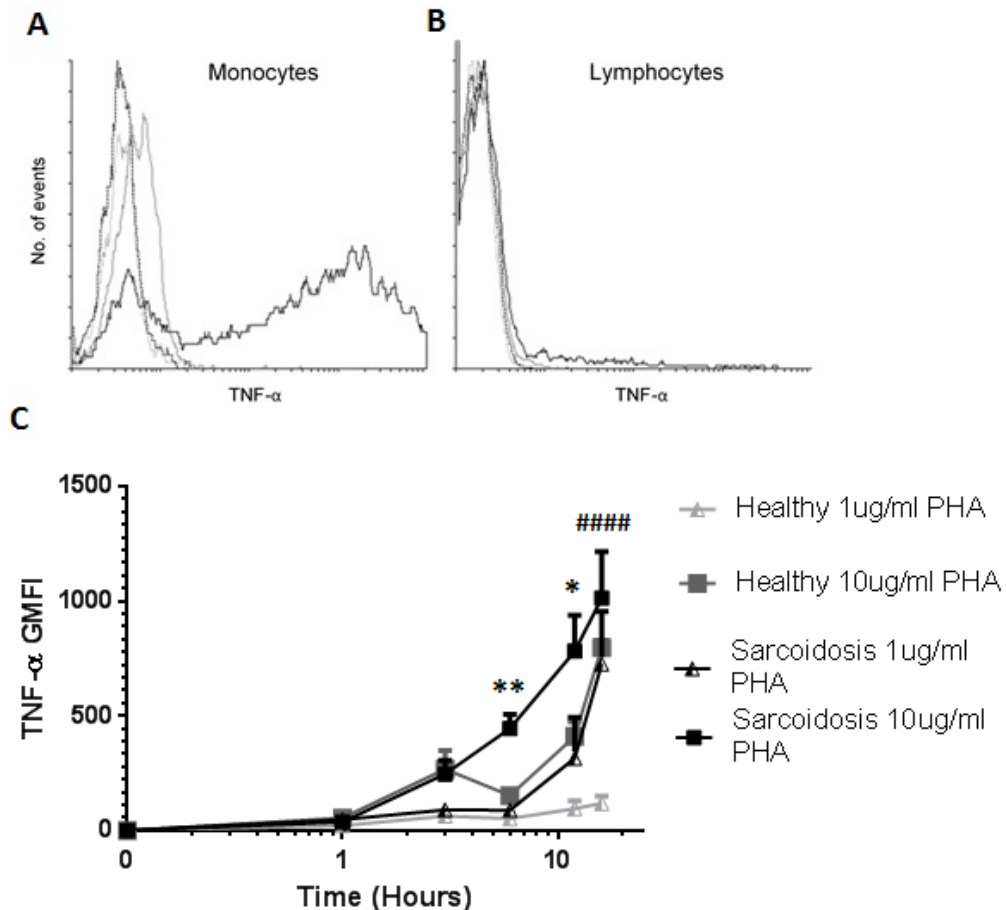
Figure 3.28: Intracellular TNF-α accumulation in response to PHA treatment in healthy controls and patients with sarcoidosis.

PBMCs were treated with Brefeldin A to allow for cytokine accumulation inside the cell. **A:** Monocyte intracellular TNF-α accumulation after incubation with PHA for 16 hours. Non-stimulated = 7.5% positive, PHA stimulated = 84.7% positive. **B:** Lymphocyte intracellular TNF-α accumulation after incubation with PHA for 16 hours. Non-stimulated = 0.5% positive, PHA stimulated = 4.9% positive. Dark grey, unstimulated isotype Ab; light grey, unstimulated TNF-α; solid black, PHA stimulated TNF-α. There was no significant difference in stimulated and non-stimulated isotype controls. **C:** Intracellular TNF-α accumulation in monocytes in response to PHA (1 and 10μg/ml) in sarcoidosis patients and controls. Data presented as mean ± SEM. * $p < 0.05$ and ** $p < 0.01$ between patients and controls for 10 μg/ml of PHA (n=3). #### $p < 0.0001$ between patients and controls for 1 μg/ml of PHA (n = 2 and n = 3 respectively).
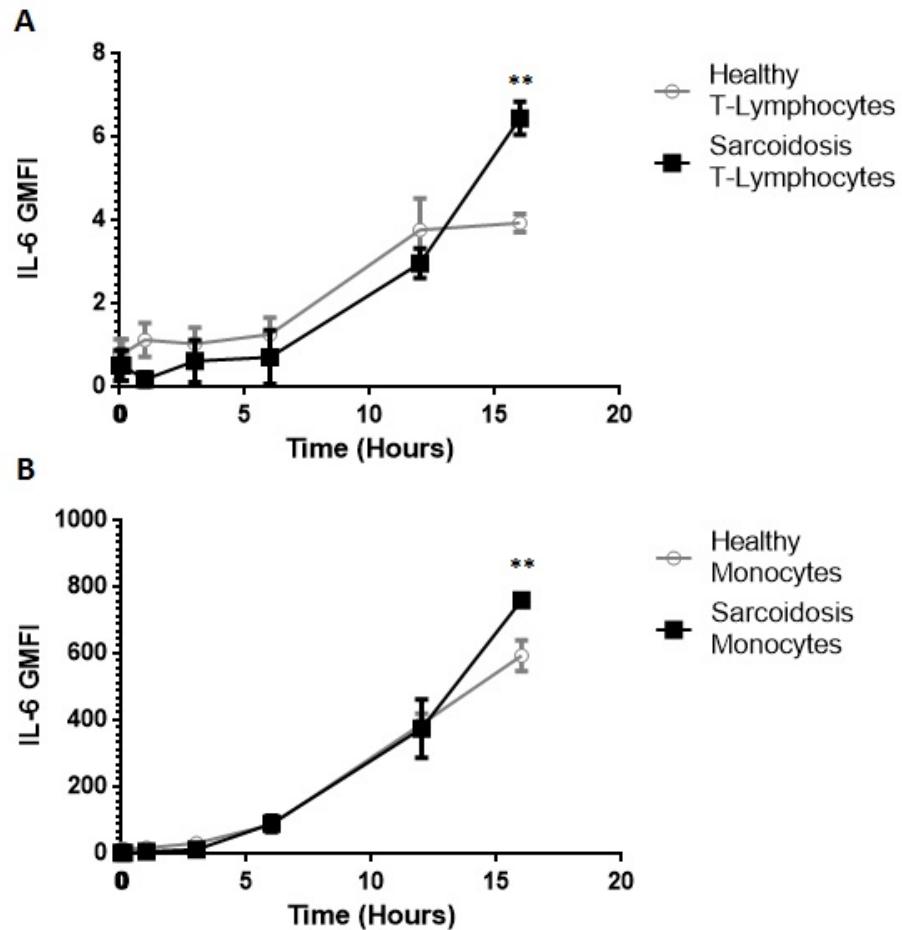
Figure 3.29: Intracellular IL-6 accumulation in response to Brefeldin A treated PHA stimulated PBMCs in healthy controls and patients with sarcoidosis.

A: Intracellular IL-6 accumulation in T-lymphocytes in response to PHA (10 μg/ml) in sarcoidosis patients and controls. This is greater in patients after 16 hours. **B:** Intracellular IL-6 accumulation in monocytes in response to PHA (10 μg/ml) in sarcoidosis patients and controls shows greater accumulation than T-lymphocytes. This is also elevated in patient monocytes. Data presented as mean ± SEM. ** $p < 0.01$ between patients and controls for 10 μg/ml of PHA (n=3).

IL-6 was detectable in T-lymphocytes after PHA stimulation, rising after 6 hours of PHA stimulation (Figure 3.29). IL-6 levels were significantly higher in patients with sarcoidosis after 16 hours compared with healthy controls ($p < 0.01$, Two-way ANOVA). The relative amount of IL-6 as determined using fluorescence was greater in monocytes compared with T-lymphocytes, a corrected geometric fluorescence intensity maximum of 777 compared with 6.8 respectively, suggesting that monocytes produced more IL-6 in response to PHA. Much like with the T-lymphocytes, a statistically significant difference was observed in sarcoidosis patients after 16 hours, with monocytes accumulating more

intracellular IL-6 than in controls (p < 0.01, Two-way ANOVA). It is acknowledged that more donors would be required to determine this accurately however.

IFN-γ was not detectable in either lymphocytes or monocytes in response to PHA stimulation and Brefeldin A treatment in this assay (n=3 healthy controls, n=3 sarcoidosis patients, data not shown). IL-10 production by healthy monocytes was demonstrably low and rose a modest degree in the 16 hours after PHA stimulation (Appendix A3.15). T-lymphocyte IL-10 was not detectable after PHA stimulation.

This data indicates that early temporal events in the stimulation of leukocytes by PHA predominantly activates monocytes to produce significant levels of IL-6 and TNF-α, and that production of these pro-inflammatory cytokines are increased in monocytes in sarcoidosis patients in the absence of other external factors. It also shows that IFN-γ is not produced directly upon PHA stimulation on either monocytes or lymphocytes.

3.3.12 Correlation between Whole Blood Assay Cytokine Release and Monocyte/T-lymphocyte Cell Numbers

The data had indicated that TNF-α and IL-6 release to PHA was elevated in sarcoidosis and may be predominantly monocyte derived in nature. The previous findings of T cell lymphocytopenia in the disease was highly significant, whereas monocyte number was not significantly different between healthy controls and patients with sarcoidosis. To identify whether leukocyte number might be a factor in the release of the cytokines from PHA stimulated whole blood leukocytes, the results from assay were correlated with the leukocyte numbers calculated during the immunophenotyping of patients and healthy controls. Additionally, the percentage of different monocyte subsets were compared to IL-6 and TNF-α release from patients to identify whether this may account for the increase in these cytokines in sarcoidosis.

As shown in Figure 3.30, the number of T-lymphocytes appeared to not correlate with IFN-γ, TNF-α or IL-6 release in response to PHA in whole blood. This applied to both

healthy controls and patients, suggesting that the lower T-lymphocyte number in sarcoidosis patients is not a significant factor in PHA induced responses.

TNF-α release (Figure 3.31) was not associated with increased monocyte number in either sarcoidosis patients (p = 0.209, R^2 = 0.088) or healthy controls (p = 0.604, R^2 < 0.001). Conversely, monocyte number in healthy controls correlated positively with increased PHA induced IL-6 (p = 0.005, R^2 = 0.212). Patients with sarcoidosis also displayed a similar trend in monocyte number and IL-6 release, though the results were not statistically significant (p = 0.059, R^2 = 0.389). Despite this apparent correlation, the numbers of monocytes in sarcoidosis patients and healthy controls for those assessed for IL-6 in the whole blood assay was not significantly different (p = 0.482, Mann-Whitney U test). Indeed, as there was a skew towards patients displaying lower monocyte numbers (median 0.192 $x10^6$ cells/ml IQR 0.156 $x10^6$) compared with healthy controls (median 0.293 $x10^6$ cells/ml IQR 0.157 $x10^6$), the elevated IL-6 in sarcoidosis was unlikely to be associated with monocyte number. There was not particular association between the raw CD3:CD14 ratio and IFN-γ, TNF-α or IL-6 release in patients with sarcoidosis or healthy controls (Appendix A3.16).

The CD4:CD8 ratio correlated in specific ways with the pro-inflammatory cytokine release observed (Figure 3.32). Lower CD4:CD8 ratios were associated with elevated IFN-γ release in healthy controls (p = 0.031, R^2 = 0.349) but not sarcoidosis patients (p = 0.683, R^2 = 0.074). A similar correlation applies for IL-6, which is associated with CD4:CD8 in healthy controls (p = 0.021, R^2 = 0.177) but patients with sarcoidosis do not display a statistically significant correlation between these factors (p = 0.146, R^2 = 0.040). However, TNF-α release in sarcoidosis patients did correlate with a low CD4:CD8 (p = 0.026, R^2 = 0.181), whereas TNF-α in healthy controls did not significantly correlate with the ratio (p = 0.752, R^2 = 0.081).

Neither the percentage of CD25 expressing T-lymphocytes nor the relative expression of CD25 on T cells correlated significantly with IFN-γ release in response to PHA (Figure 3.33). TNF-α release was unusual when associated with T-lymphocyte CD25. Patients with sarcoidosis displayed a negative correlation between TNF-α and higher percentages of CD25+ T-lymphocytes in the bloods (Figure 3.33, p = 0.009, R^2 = 0.161), which was also

mirrored in CD25 expression, though this was not statistically significant. Healthy controls displayed a statistically significant positive correlation between CD25 expression and TNF-α release (p = 0.031, R^2 = 0.512). Associations between IL-6 release and CD25 expression were not statistically significant (Figure 3.33).

The increased percentage of intermediate monocytes in sarcoidosis patients did not appear to directly correlate with either IL-6 release (p = 0.105, R^2 = 0.101) or TNF-α release (p = 0.539, R^2 = 0.005) from PHA stimulated whole blood (Appendix A3.17). Nor did the percentage of non-classical monocytes correlate with increased pro-inflammatory cytokine release (Appendix A3.18). In healthy controls, despite the small numbers involved, there appeared to be a negative correlation between intermediate monocytes and IL-6 release (p = 0.022, R^2 = 0.468), though no association was found between TNF-α release and the percentage of intermediate monocytes (p = 0.783, R^2 < 0.001). There was an inverse correlation between TNF-α release in healthy controls and the percentage of classical monocytes (p = 0.034, R^2 = 0.705) which was not found in patients with sarcoidosis (p = 0.924, R^2 = 0.005), shown in Figure 3.34. IL-6 release did not correlate with the percentage of classical monocytes (Appendix A3.19). No other positive or negative correlations were observed between cytokines and the monocyte subsets. PHA induced pro-inflammatory cytokine release does not appear to directly correlate with T-lymphocyte number, neither is the altered CD3:CD14 ratio a predictive factor in the release of these cytokines in the *ex vivo* assay performed. The relationship between monocytes and TNF-α and IL-6 release in response to PHA in this system is more complex however, suggesting that within cohorts both monocyte number and the subset can influence the release of these cytokines to some extent.
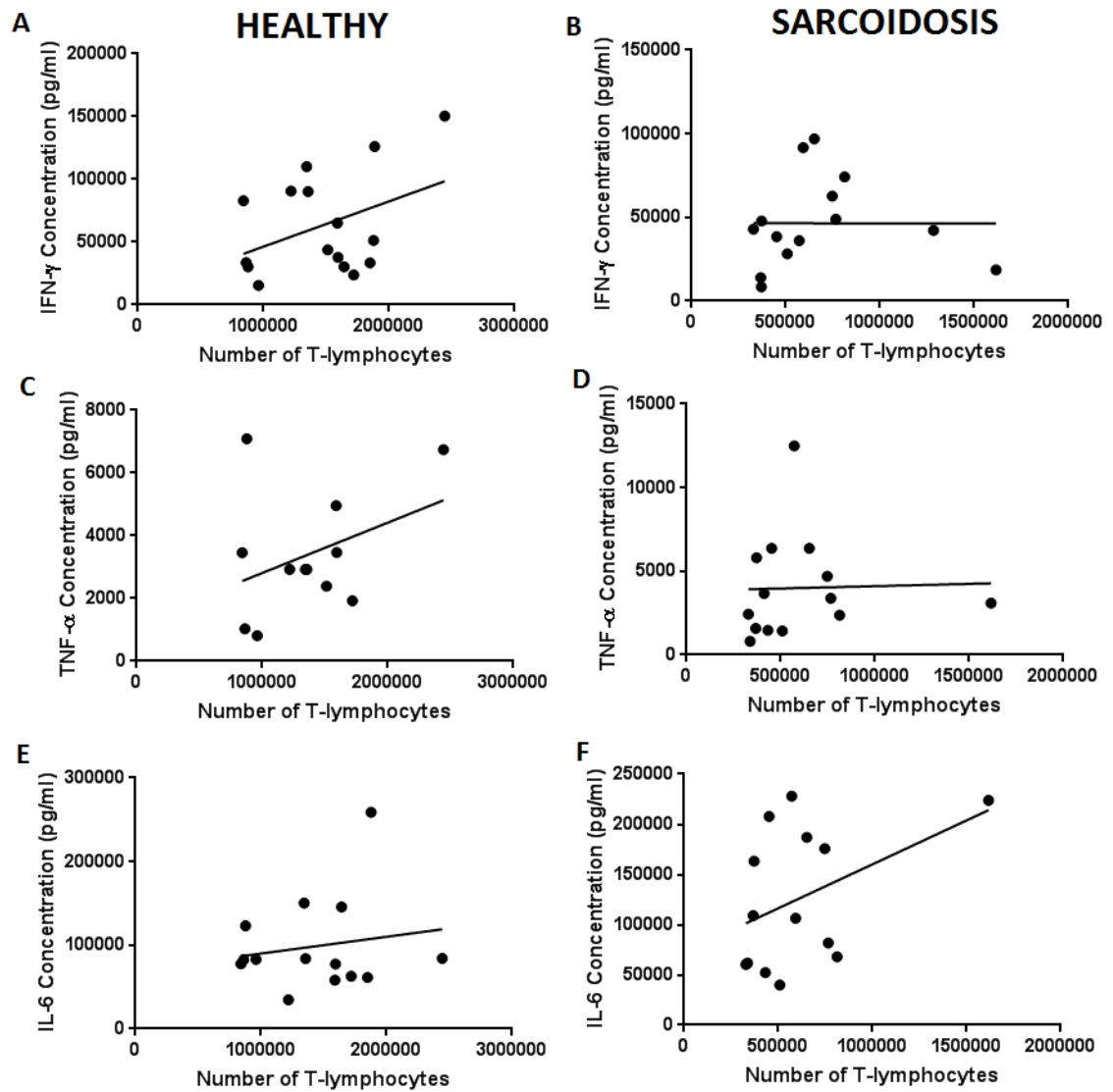
Figure 3.30: Correlation between pro-inflammatory cytokine release from PHA stimulated whole blood and T-lymphocyte number in patients with sarcoidosis and healthy controls.

IFN-γ, TNF-α and IL-6 release were correlated for healthy controls (**A, C** and **E** respectively) and sarcoidosis patients (**B, D** and **F** respectively). **A** and **B:** Correlation with IFN-γ, healthy controls ($p = 0.450$, $R^2 = 0.160$), sarcoidosis patients ($p = 0.221$, $R^2 < 0.001$). **C** and **D:** Correlation with TNF-α, healthy controls ($p = 0.543$, $R^2 = 0.140$), sarcoidosis patients ($p = 0.325$, $R^2 < 0.001$). **E** and **F:** IL-6 correlation, healthy controls ($p = 0.693$, $R^2 = 0.027$), sarcoidosis patients ($p = 0.181$, $R^2 = 0.179$). Black line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.
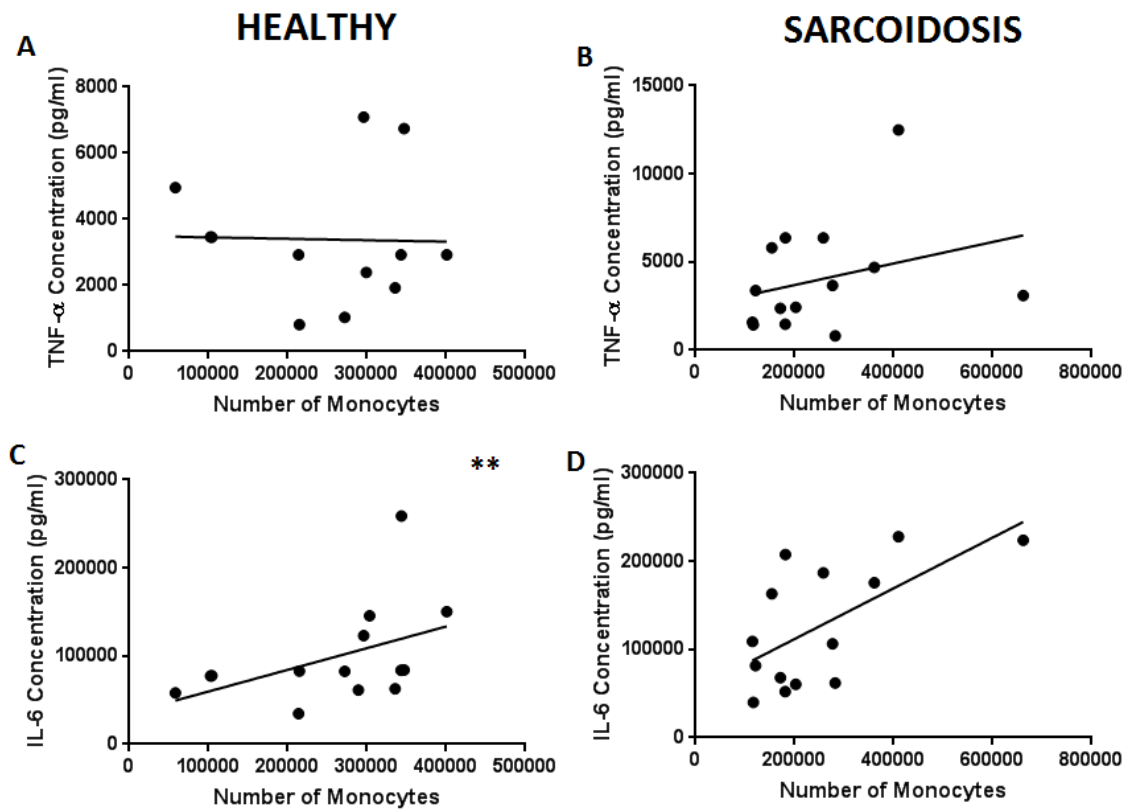
Figure 3.31: Correlation between the number of monocytes and the PHA stimulated whole blood release of TNF-α and IL-6 in patients with sarcoidosis and healthy controls.

TNF-α and IL-6 release were correlated for healthy controls (**A** and **C**) and sarcoidosis patients (**B** and **D** respectively). **A** and **B:** Correlation with TNF-α, healthy controls (p = 0.604, R^2 < 0.001), sarcoidosis patients (p = 0.209, R^2 = 0.088). **E** and **F:** IL-6 correlation, healthy controls (** p = 0.005, R^2 = 0.212), sarcoidosis patients (p = 0.059, R^2 = 0.389). Black line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.
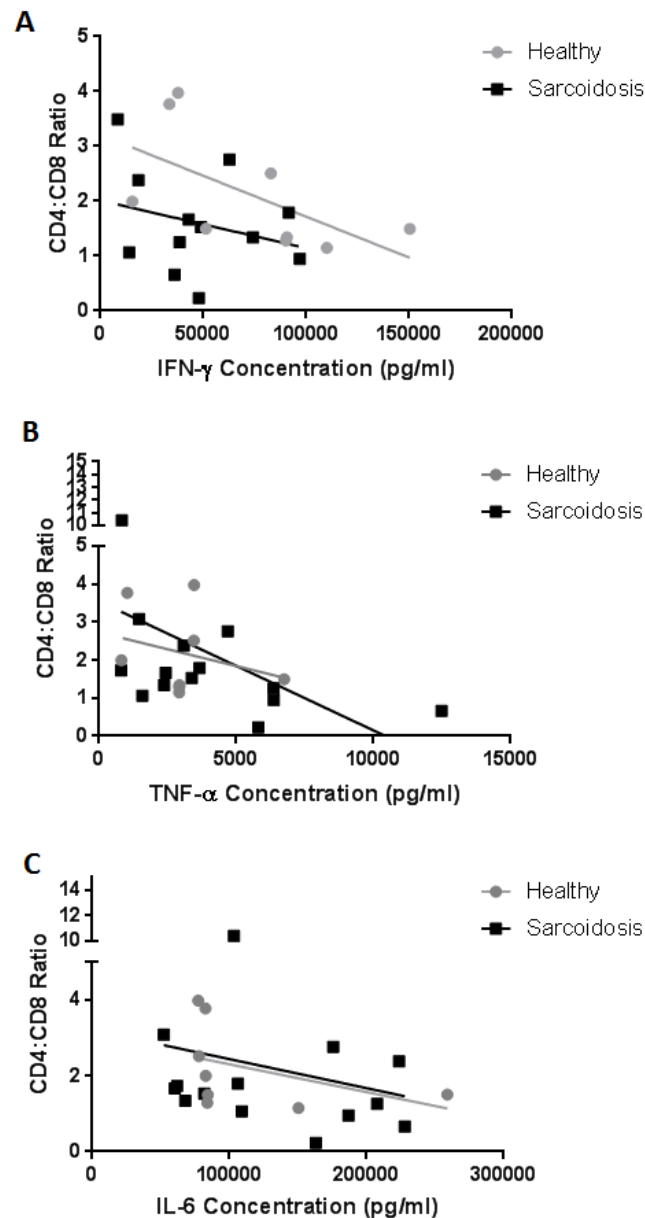
Figure 3.32: Correlation between IFN-γ, TNF-α and IL-6 release from PHA stimulated whole blood and the CD4:CD8 T-lymphocyte ratio in patients with sarcoidosis and healthy controls. IFN-γ, TNF-α and IL-6 release (**A, B** and **C** respectively) were correlated for healthy controls (grey circles) and sarcoidosis patients (black squares). **A:** Correlation with IFN-γ, healthy controls (* p = 0.031, R^2 = 0.349), sarcoidosis patients (p = 0.683, R^2 = 0.074). **B:** Correlation with TNF-α, healthy controls (p = 0.752, R^2 = 0.081), sarcoidosis patients (* p = 0.026, R^2 = 0.181). **C:** IL-6 correlation, healthy controls (*p = 0.021, R^2 = 0.177), sarcoidosis patients (p = 0.146, R^2 = 0.040). Black/Grey line = linear regression in sarcoidosis and healthy respectively, statistical significance determined using Spearman's Rank Correlation Test.
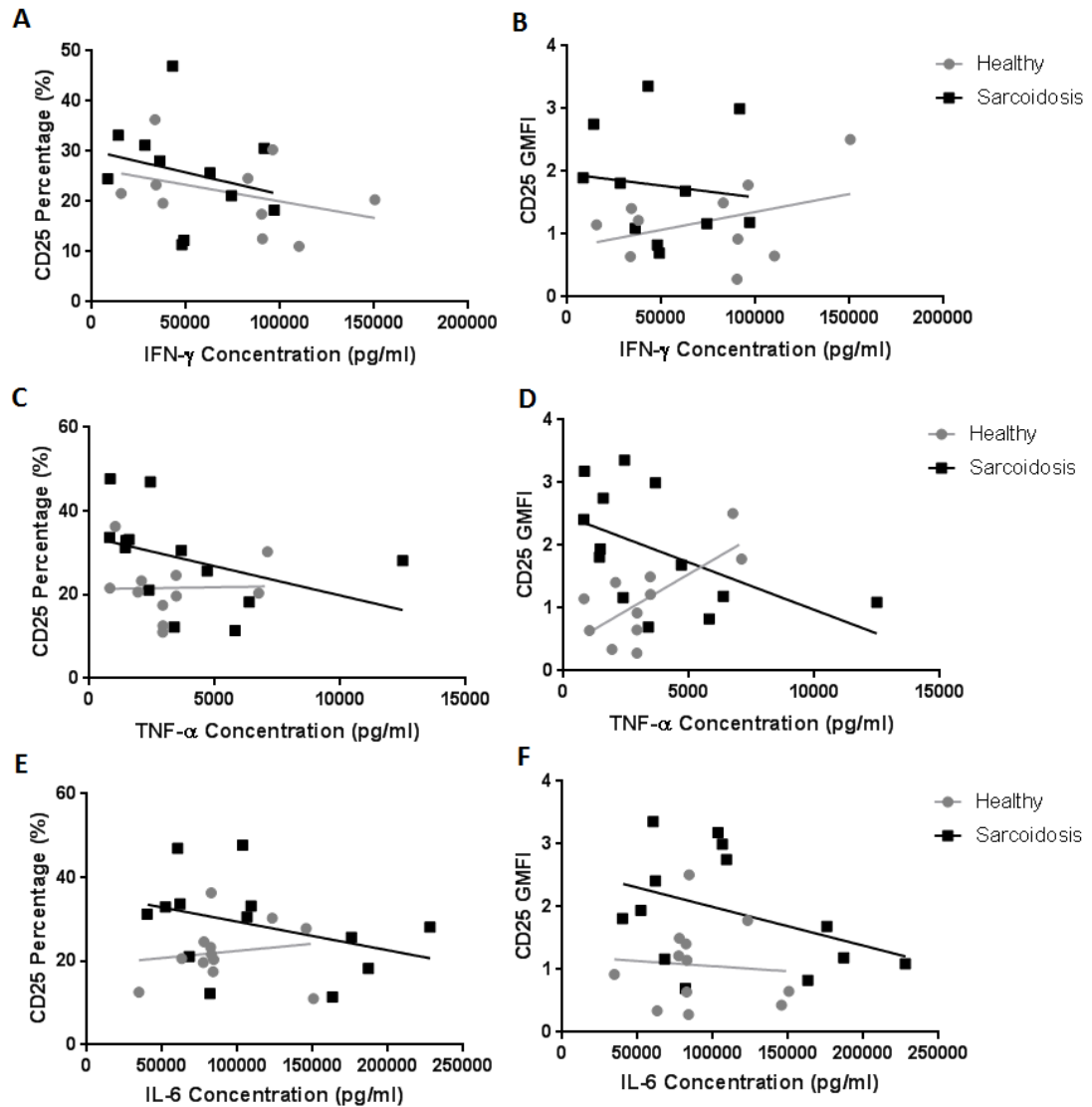
Figure 3.33: Correlation between pro-inflammatory cytokine release from PHA stimulated whole blood and T-lymphocyte CD25 in patients with sarcoidosis and healthy controls.

The percentage of CD25^{+} T-lymphocytes (A, C and E) and the level of CD25 expression on T-cells (B, D and F) were correlated with IFN-γ, TNF-α and IL-6 release in healthy controls (grey circles) and patients with sarcoidosis (black squares). **A:** IFN-γ in healthy controls (p = 0.204, R^2 = 0.135) and sarcoidosis patients (p = 0.225, R^2 = 0.062). **B:** IFN-γ in healthy controls (p = 0.407, R^2 = 0.142) and sarcoidosis patients (p = 0.521, R^2 = 0.014). **C:** TNF-α in healthy controls (p = 0.714, R^2 = 0.001) and sarcoidosis patients (** p = 0.009, R^2 = 0.161). **D:** TNF-α in healthy controls (* p = 0.031, R^2 = 0.512) and sarcoidosis patients (p = 0.061, R^2 = 0.277). **E:** IL-6 in healthy controls (p = 0.716, R^2 = 0.026) and sarcoidosis patients (p = 0.120, R^2 = 0.131). **F:** IL-6 in healthy controls (p = 0.939, R^2 = 0.001) and sarcoidosis patients (p = 0.224, R^2 = 0.156). Black/grey line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.
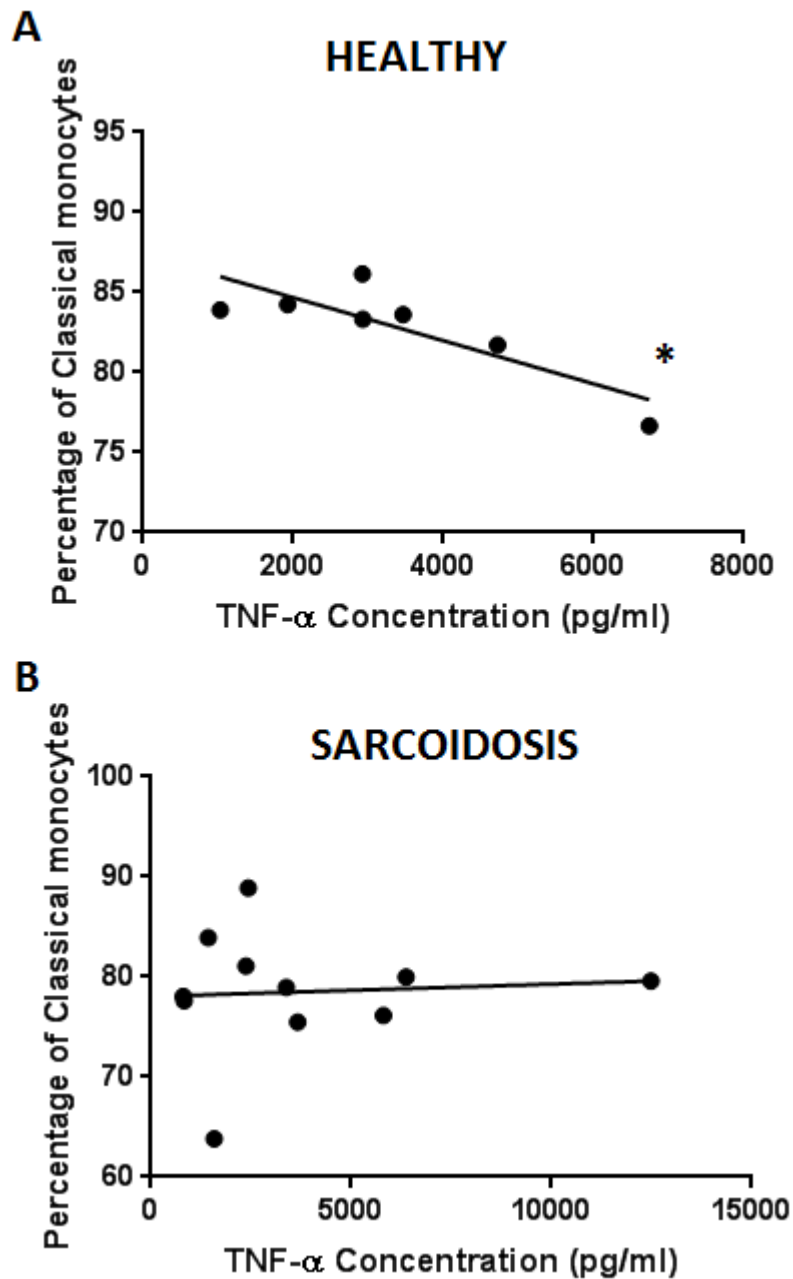
Figure 3.34: Correlation between TNF-α release and the percentage of classical

monocytes within the population.

A: Healthy controls, (* p = 0.034, R^2 = 0.705). **B:** Patients with sarcoidosis, (p = 0.924, R^2 = 0.005). Black line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.

3.3.13 Whole Blood Leukocyte Response to the Superantigen Staphylococcal Enterotoxin A (SEA)

Sarcoidosis patients had responded to PHA, typically considered a T cell activator, with enhanced responses despite a reduced T-lymphocyte number in their blood. The lectin has been described as activating T cell receptor signalling as a mode of action [705]. To be able to identify whether T-lymphocytes were capable of enhanced activity in sarcoidosis in a TCR mediated activation mechanism, a 'superantigen', Staphylococcal Enterotoxin A (SEA) was used that requires the direct interaction of TCRs with an accessory cell MHC to elicit a response [700, 701]. This was completed at 16 hours to compare and contrast responses in whole blood with those of PHA, using varying concentrations of the stimulant.
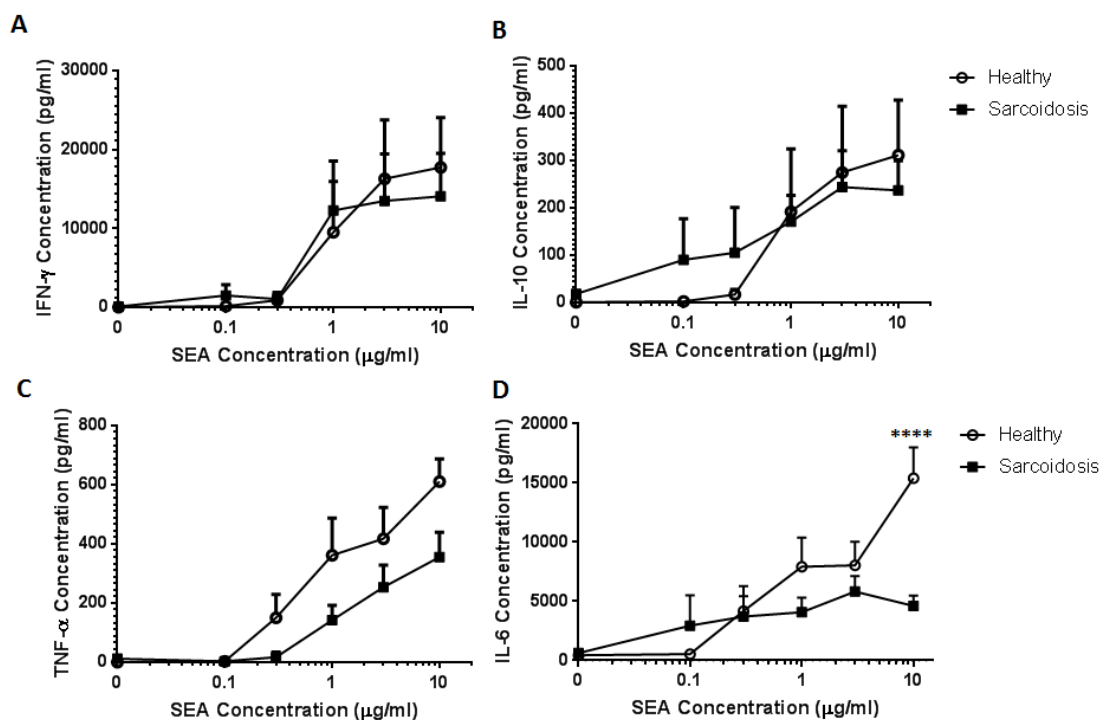
Figure 3.35: Cytokine release from whole blood stimulated with Staphylococcal Enterotoxin A (SEA) in patients with sarcoidosis and healthy controls.

A dose response to SEA was undertaken for 16 hours at concentrations of 0 µg/ml to 10 µg/ml in patient and control whole blood. Plasma was analysed by ELISA for **A:** IFN-γ**, B:** IL-10, **C:** TNF-α and **D:** IL-6. There was no statistically significant difference in IFN-γ**,** IL-10 or TNF-α tested at any concentration of SEA stimulation. IL-6 at 10 µg/ml in healthy controls was higher than patients with sarcoidosis, p < 0.0001, as determined by Two-way ANOVA. Data presented with mean ± SEM (one sided). IFN-γ n = 7 and n= 7, IL-10 n = 6 and n = 5, TNF-α n = 9 and n = 6 and IL-6 n = 8 and n = 7 for healthy controls and patients with sarcoidosis respectively.

Figure 3.35 displays the dose response to SEA for IFN-γ, TNF-α, IL-6 and IL-10 in whole blood for both patients with sarcoidosis and healthy controls. The mean concentration of IL-6 release was greater in healthy controls when compared with sarcoidosis patients, and was statistically significant at 10 µg/ml of SEA stimulation ($p < 0.0001$, Two-way ANOVA). The mean TNF-α release was consistently lower in patient samples, though this was not statistically significant. There was also no significant difference in IFN-γ and IL-10 release. IL-4 and IL-12(p70) did not significantly increase in response to SEA stimulation, the latter was not detected after 16 hours (data not shown). When the data for both cohorts were combined, there was a statistically significant correlation between IL-6 release in response to SEA and the number of T-lymphocytes in the blood (Figure 3.36, $p = 0.003$, $R^2 = 0.619$), however, this appeared to be the case in healthy controls more than patients with sarcoidosis. TNF-α and IFN-γ release in response to SEA were not correlated with the number of T cells in donors.

IL-6 release in response to SEA correlated positively with the number of T-lymphocytes in the blood (Figure 3.36, $p = 0.0029$, $R^2 = 0.619$), though the cohorts did not appear to display this correlation individually. TNF-α release did not correlate with the number of T-lymphocytes in the blood ($p = 0.375$, $R^2 = 0.002$), nor did IFN-γ release ($p = 0.532$, $R^2 < 0.001$), either in combined cohorts or individual patient and control groups (Appendix A3.20). IL-6 release was also positively correlated with the number of monocytes in healthy controls (Figure 3.36, $p = 0.034$, $R^2 = 0.275$), but not sarcoidosis patients ($p = 0.906$, $R^2 < 0.001$). There was no statically significant correlation between monocyte number and either TNF-α or IFN-γ release in response to SEA (Appendix A3.21). This was also reflected when assessing the CD3:CD14 ratio, where TNF-α and IFN-γ did not correlate significantly with the ratio of T cells to monocytes (Appendix A3.22). IL-6 showed a limited correlation to CD3:CD14, though this was not statically significant ($p = 0.067$, $R^2 = 0.075$). Correlating with specific T-lymphocyte subsets, the CD4:CD8 ratio did not correlate with pro-inflammatory cytokine release in response to SEA to a statistically significant degree (Appendix A3.23).

IFN-γ release in response to SEA was not associated with either the percentage of CD25^{+} T-lymphocytes present in the blood or the number of CD25^{+} expressing T-lymphocytes in total (Appendix A3.24). By contrast, there was a statistically significant positive

correlation between the number of CD25$^+$ expressing T-lymphocytes and TNF-α release in sarcoidosis patients (Appendix A3.24, p = 0.012, R^2 = 0.510), though this was not observed in healthy controls. Neither the number of CD25$^+$ T cells nor the percentage of CD25$^+$ T cells within the population correlated with IL-6 release (Appendix A3.24).

In summary, this suggests that the SEA induced response was more significant in healthy controls, the inverse of that observed in PHA induced mechanisms. Increased IL-6 release in healthy controls is associated with the higher T-lymphocyte number present in this cohort and can be elevated in those individuals with higher monocyte numbers also, though this is not the case for sarcoidosis patients.
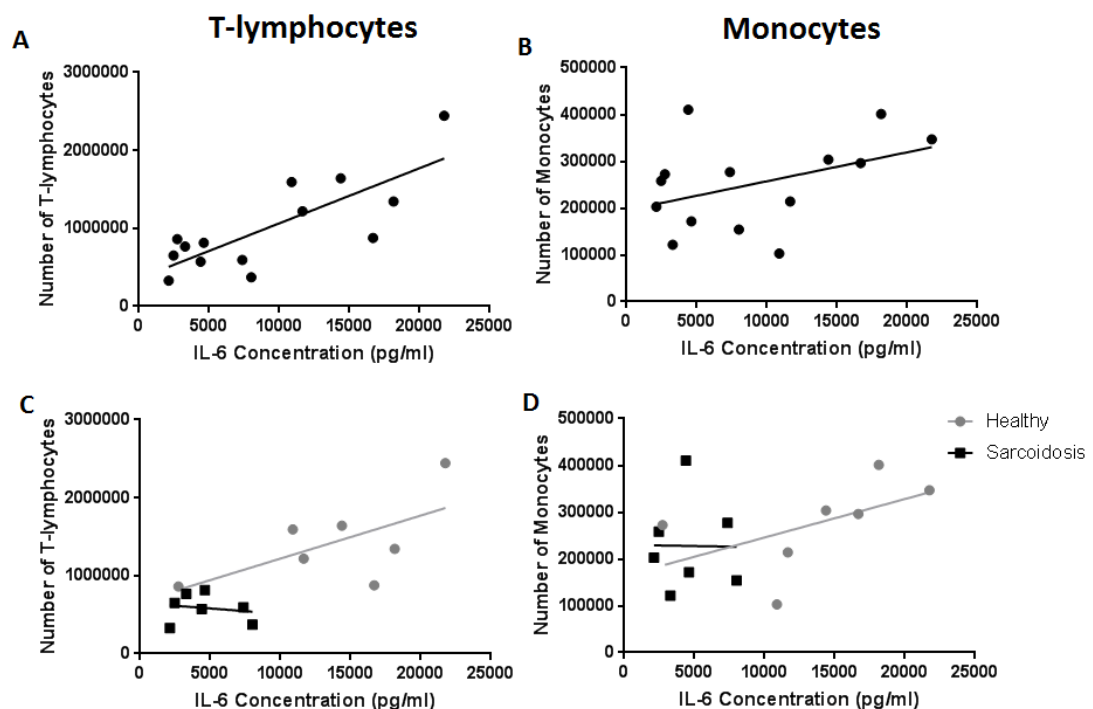
Figure 3.36: Correlation between IL-6 release from SEA stimulated whole blood and leukocyte numbers in patients with sarcoidosis and healthy controls.

T-lymphocyte numbers (A, C) and monocytes numbers (B, D) were correlated with IL-6 release in healthy controls (grey circles), patients with sarcoidosis (black squares) or pooled together for analysis (A, B). **A:** Pooled data from both healthy controls and sarcoidosis patients IL-6 release correlated with T-cell number. ** p = 0.003, R^2 = 0.619. **B:** Pooled data from both healthy controls and sarcoidosis patients IL-6 release correlated with monocyte number. p = 0.155, R^2 = 0.174. **C:** IL-6 correlated with T-lymphocyte number in healthy controls (p = 0.236, R^2 = 0.387) and sarcoidosis patients (p = 0.906, R^2 = 0.030). **D:** IL-6 correlated with monocyte number in healthy controls (* p = 0.034, R^2 = 0.275) and sarcoidosis patients (p = 0.906, R^2 < 0.001. Black/grey line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.

3.3.14 Whole Blood Leukocyte Response to the Lectin Wheat Germ Agglutinin (WGA)

Patients with sarcoidosis had a reduced peripheral blood immune response to SEA when compared with healthy controls. There were elevated responses to PHA and evidence that monocytes may be important in this mechanism of cytokine release. To examine whether there was a generalised hyperactivity present in monocytes, or whether there was a specific feature of PHA stimulation that was responsible for the elevated response, a monocyte/macrophage binding non-mitogenic lectin wheat germ agglutinin (WGA) was used within the whole blood assay. This had different stimulatory properties to PHA, so would be used to see if monocytes were naturally more active in patient blood.

WGA stimulated cytokine release in healthy controls were typically lower (at comparable concentrations) than SEA or PHA induced activation, the latter typically significantly greater than either of the other stimuli (Figure 3.37). WGA induced activation occurred at concentrations of 10 µg/ml and above, though the IFN-γ and IL-10 release was modest, even at higher concentrations. IL-6 and TNF-α release at 100 µg/ml of WGA was significantly higher than at lower concentrations of WGA. Thus, concentrations of 10, 30 and 100 µg/ml would be assessed principally in sarcoidosis patients.

As shown in Figure 3.38, there was no statistically significant difference between patients with sarcoidosis and healthy controls for any of the cytokines released in response to the three concentrations of WGA used. There was a high degree of variability in response within the cohorts, though none of the pro-inflammatory cytokines were significantly elevated in patient samples. Whether or not this would be consistent in a larger sample size is not known, but it appears that the responses to WGA indicate that the observed PHA induced increase in IL-6 and TNF-α results from a specific interaction and not a generalised monocyte hyperactivity to stimuli.
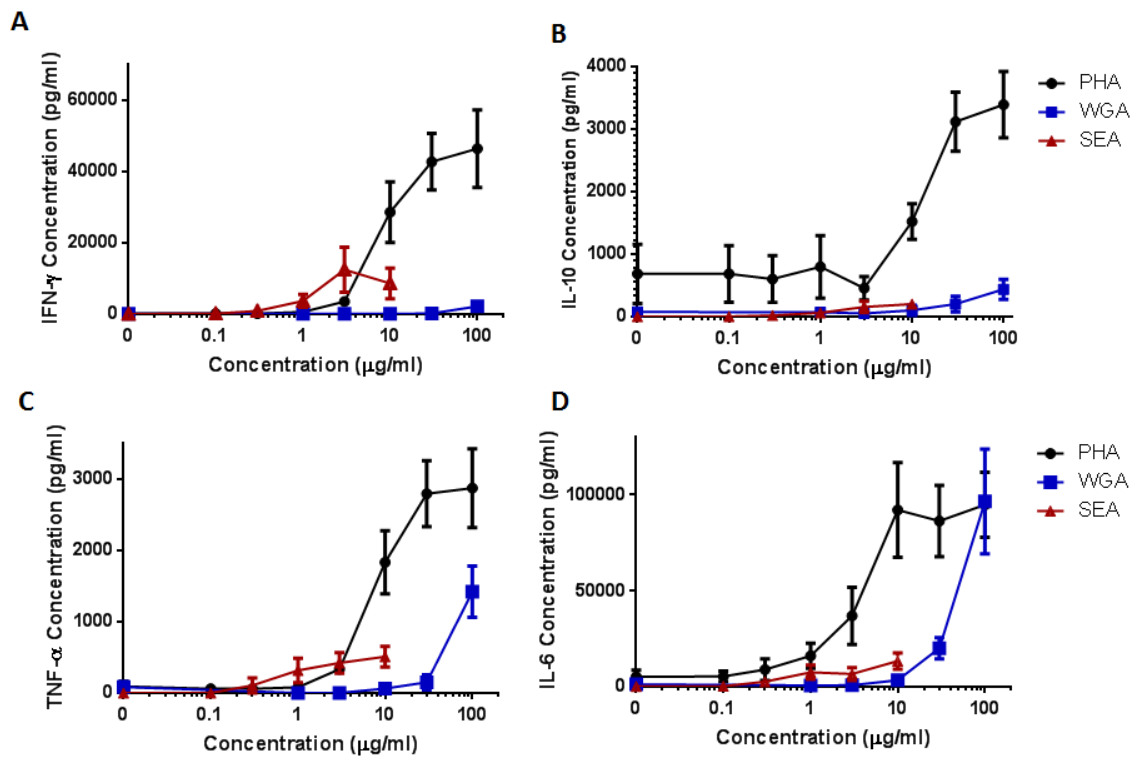
Figure 3.37: Comparison between immune activator cytokine release in selected healthy controls at the concentration range used in whole blood assays.

Phytohaemagglutinin (PHA), Wheat Germ Agglutinin (WGA) and Staphylococcal Enterotoxin A (SEA), represented in black, blue and red respectively, display different dose responses at the concentrations used. **A:** IFN-γ**, B:** IL-10, **C:** TNF-α and **D:** IL-6. Data presented with mean ± SEM.
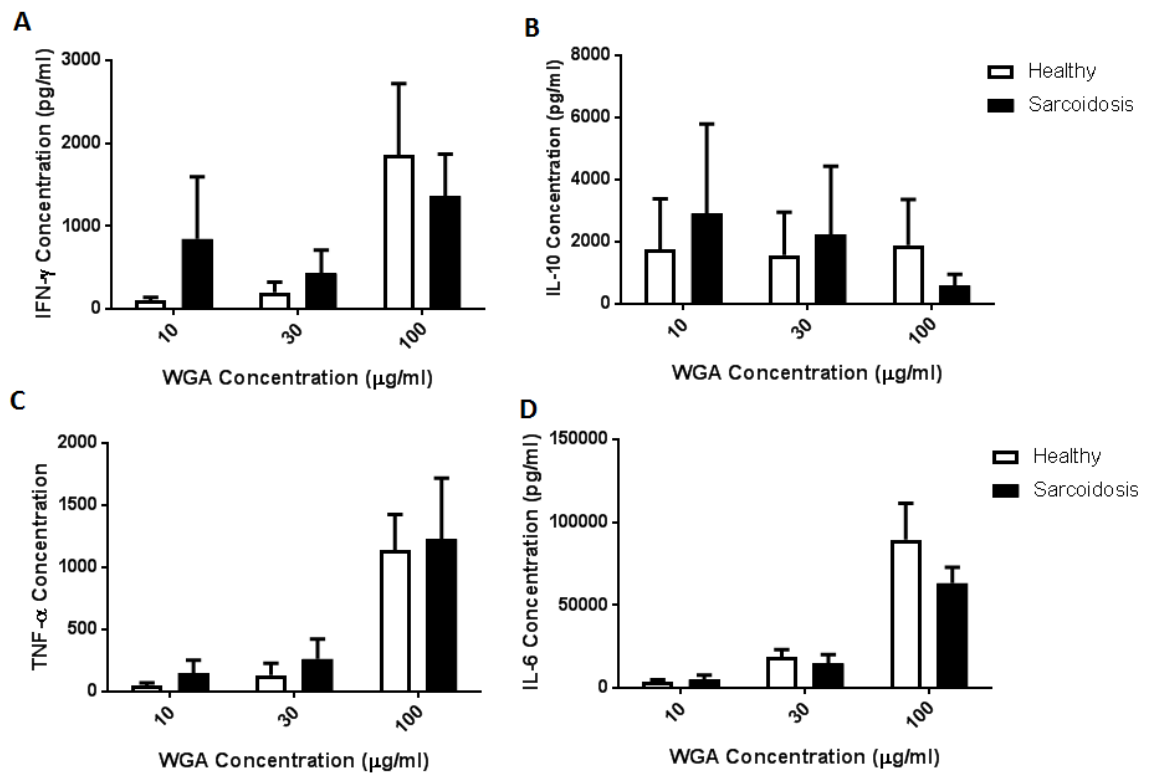
Figure 3.38: Cytokine release from whole blood in response to different concentrations of wheat germ agglutinin (WGA) stimulation from patients with sarcoidosis and healthy controls.

WGA concentrations used were 10, 30 and 100 µg/ml. Healthy controls (white bars) and patients with sarcoidosis (black bars) were analysed for release of **A:** IFN-γ (n = 12 and 8 for controls and patients respectively), **B:** IL-10 (n = 9 and 5), **C:** TNF-α (n = 11 and 8) and **D:** IL-6 (n = 10 and 8). Data presented with mean ± SEM. No statistical significance ($p > 0.05$) in any cytokine or concentration of WGA between controls and patients, as determined using a Two-way ANOVA.

3.3.15 Association between Clinical Features of Sarcoidosis Patients and PHA Whole Blood Assay Cytokine Responses

Patients with sarcoidosis had shown an elevated responsiveness to PHA in whole blood, though it was important to place these results in context with features used currently to define the status of a patient clinically. Though many of these, such as the Scadding CXR stages and serum ACE, have been used to categorise patients for severity, they are often not considered effective in defining the course of the disease. Correlating the assay results with these features would indicate whether these results were strongly associated with the existing methods used to stratify patients, or represented an alternative mechanism to categorise patient subsets. Given the significantly different responses to TNF-α and IL-6, these cytokines would be assessed rather than utilising IFN-γ or IL-10 associations

In healthy controls, increased age was not associated with increased TNF-α (p = 0.877, R^2 < 0.001) nor increased IL-6 release (p = 0.488, R^2 = 0.044) in response to PHA (Appendix A3.25). Gender was not a significant factor in controls either, with IL-6 (p = 0.884, Mann-Whitney U test) and TNF-α (p = 0.635, Mann-Whitney U test) release comparable between females and males (Appendix A3.25). In sarcoidosis patients, there was no statistically significant correlation between age and either TNF-α release (p = 0.398, R^2 = 0.028) or IL-6 release (p = 0.880, R^2 = 0.003) in response to PHA stimulation (Appendix A3.26). TNF-α release was not statistically different between female patients (mean 3163 pg/ml ±SEM 962) and male patients (mean 4096 pg/ml ±SEM 1108), despite the mean difference observed (p = 0.953, Mann-Whitney U test). The mean IL-6 release was also not significantly different between male and female patients (p = 0.536, Mann-Whitney U test). This suggests that neither gender nor age were major factors in determining the response to PHA induced TNF-α or IL-6 release.

When correlating the results of the assay with serum ACE levels, it was found that there was no statistically significant association between TNF-α or IL-6 cytokine release and the sACE levels of patients (Figure 3.39). This lack of correlation also applied for IFN-γ and IL-10 (Appendix A3.27).
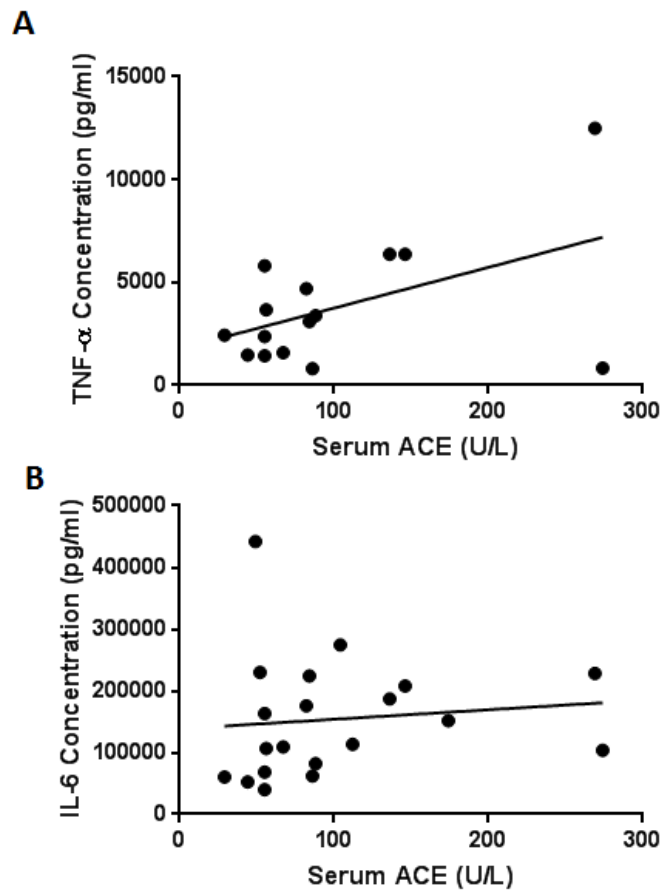
Figure 3.39: Correlation between TNF-α and IL-6 release from PHA stimulated whole blood and serum ACE levels from patients with sarcoidosis.

A: There was no statistically significant correlation between TNF-α release and serum ACE from patients, p = 0.299, R^2 = 0.239. **B:** There was also no statistically significant correlation between IL-6 release and serum ACE from patients, p = 0.262, R^2 = 0.012. Black line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.

IL-6 release in response to PHA (Figure 3.40) was greater in patients with 2 organs involved rather than 1 (p = 0.047, Kruskal-Wallis test) and also greater in those with stage II compared with stage I chest X-rays (p = 0.049, Kruskal-Wallis test). There was not a statistically significant difference in the mean IL-6 concentration between patients with extrapulmonary and pulmonary restricted organ involvement, mean 147501 pg/ml (±SEM 21349) compared with 160373 pg/ml (±SEM 39153) respectively (p = 0.739, Mann-Whitney U test). By contrast, there was no significant correlation between TNF-α release and the number of organs a patient had involved or the Scadding stage (Figure 3.40). The mean TNF-α observed in patients with extrapulmonary organ involvement (3127 pg/ml

±SEM 1014) and pulmonary restricted involvement (4223 pg/ml ±SEM 1159) was also not significantly different (p = 0.607, Mann-Whitney U test).
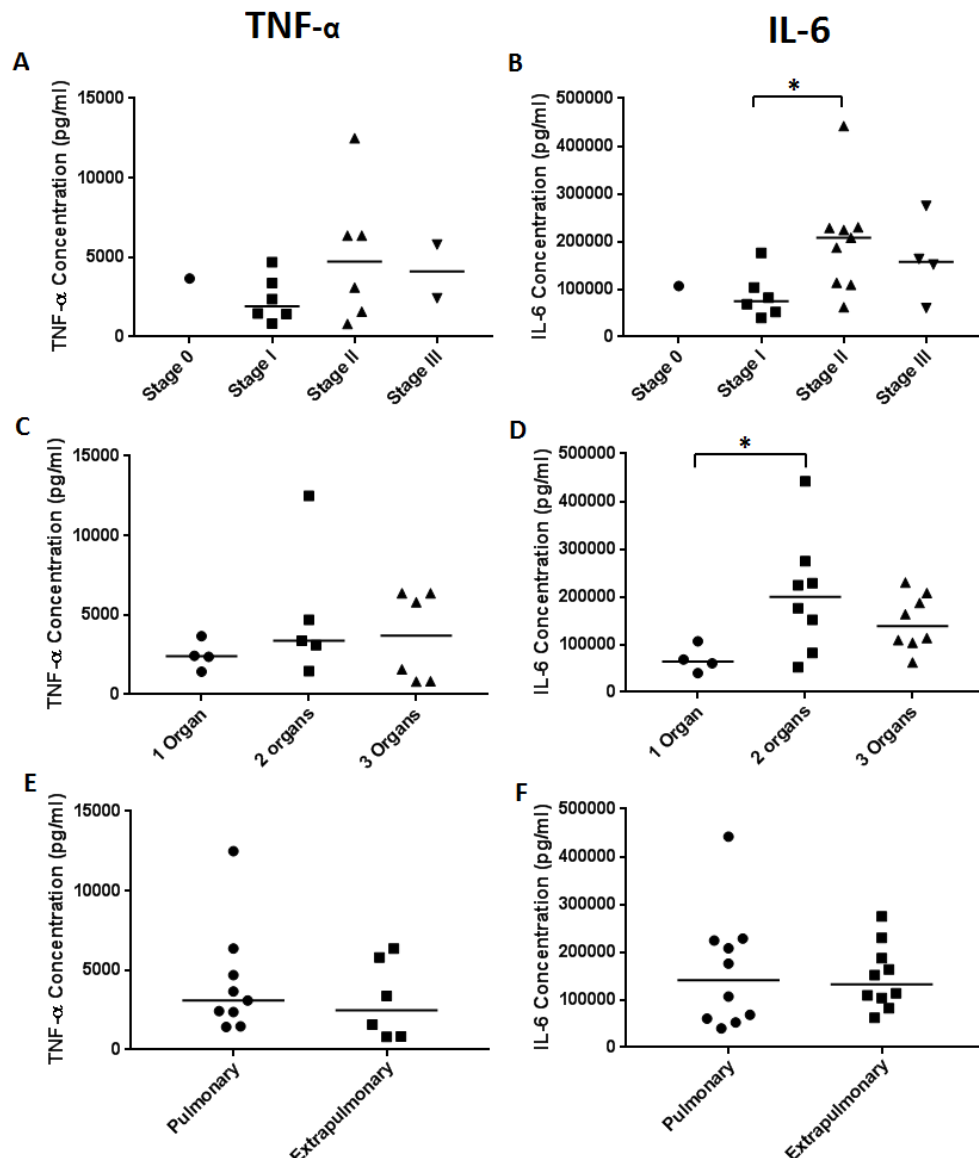
Figure 3.40: Correlation between TNF-α and IL-6 release from PHA stimulated whole blood and the organ involvement/Scadding CXR stage in patients with sarcoidosis.

TNF-α release (**A, C** and **E**) and IL-6 release (**B, D** and **F**) from whole blood assays were correlated with the Scadding CXR stage (**A** and **B**), number of organs involved (**C** and **D**) and pulmonary restricted versus extrapulmonary organ involvement (**E** and **F**). There was no statistical significance unless otherwise indicated, as determined using Kruskal-Wallis tests (**A-D**) and Mann-Whitney U tests (**E** and **F**). Data presented with median (black line). **B:** * p = 0.049 between stage I and stage II. **D:** * p = 0.047 between 1 organ and 2 organs involved.

Overall, the data suggests that the PHA induced pro-inflammatory cytokine release assay is not directly associated with serum ACE levels, age, gender or the presence of extrapulmonary symptoms observed in patients. Patients with more organs involved or stage II radiographic disease appear to be capable of enhanced IL-6 release in response to PHA, though these factors are not associated with increased TNF-α release.

3.4 Discussion

The issue of immune responses in the peripheral system in sarcoidosis is complex, the term 'paradox' has been applied to the disease to highlight the apparent contradictions between isolated BAL cell hyperactivity and suspected peripheral blood cell 'anergy'[23]. The concept of a 'sarcoidosis peripheral anergy' is based upon early work identifying a reduced response of peripheral leukocytes to tuberculin and other antigenic challenges [21], though the immunology of the disease is more complex [452]. This study has added to the ongoing questions regarding the immunopathogenesis of sarcoidosis and supports a differentiated approach to the description of the peripheral immune responses. At high concentrations, PHA induces a greater release of inflammatory cytokines in sarcoidosis from whole blood leukocytes than controls. In stark contrast, the superantigen staphylococcal enterotoxin A induced lower concentrations of IL-6 release in patients with sarcoidosis. Taken together, this suggests that peripheral immune responses are not subject to generalised anergy and are capable of enhanced inflammatory activity in response to stimulation of a, yet to be determined, receptor or pathway.

3.4.1 Cohort Composition and Clinical Features of Patients with Sarcoidosis

3.4.1.1 Recruitment, Age and Gender

Sarcoidosis is a heterogeneous disease, a feature that presents within the cohort of patients recruited for this study. A range of ages were present in the cohort, as well as variable manifestation of symptoms in patients. Despite this, the multi-disciplinary team

assessment used in the diagnosis of the disease, based upon guidelines set out by the joint statement on sarcoidosis [1], allows a consistency in establishing who are considered to have the condition and confidence in the diagnosis. A total of 30 patients with sarcoidosis were included in this study, recruited over the course of approximately 3 years. Whilst this is a significantly smaller number than those obtained from studies such as ACCESS [47, 372, 503], the demographic of the local area and the exclusion criteria meant that recruitment of large numbers of patients would be problematic. As all patients were Caucasian, the number available for the study was lower than if other racial groups were included [48]. Patients and healthy controls were excluded if they were on immunomodulatory medication, mainly corticosteroids, which again reduces the potential pool of individuals who could be recruited. With an estimated incidence of 5 per 100,000 in the UK as a whole [382], sarcoidosis is an uncommon disease; thus recruiting 30 patients was considered a good overall number for this preliminary study into the condition.

This introduced a few limitations that have to be acknowledged. Unlike other larger, multi-centre studies, this research focussed on a narrower patient demographic and was subject to a degree of referral bias more pronounced than may have been present in epidemiological studies, as the majority of patients were recruited through the respiratory department. Referrals were taken from other departments however, though it is common for patients suspected of having sarcoidosis to undergo a respiratory assessment, as this is the most common organ system involved [47]. It is not as common to have every other organ system examined for the presence of granulomata unless a patient has a symptomatic complaint. As this was an immune study, the decision to exclude individuals taking corticosteroid therapy would invariably exclude patients with severe inflammatory symptoms or progressive disease. As this research was not principally epidemiological in nature, these limitations were considered acceptable for a preliminary study of this kind.

The notable difference in the age between the two primary cohorts, a median difference of 10 years (41 years old in healthy controls compared with 51 years old in sarcoidosis patients) was accounted for by correlating subsequent data with age. Whilst not ideal, this was necessary to ensure the results were not adversely influenced by this skew in

age. The recruitment of healthy donors used the epidemiological findings in other studies to inform recruitment strategy, which suggested that the age of patients might cluster around age 30-49 [47, 95, 382]; though it is acknowledged that this relates to age of incidence rather than age of patients within a cohort. The age matching was a limitation of this study, though the correlations would provide a reasonable validation required for the results. Recruiting 'healthy' controls was met with specific problems in strategy, as it was more common for women to be willing to donate blood than men during the study and older controls were more likely to suffer from a medical condition (such as diabetes) or be on medication (such as corticosteroids) that would likely exclude them from the study. Smokers were also excluded from the study, again reducing the potential pool of donors available. Despite this, the epidemiological studies suggested that there is an expected modest skew towards women in a cohort of this type in sarcoidosis [47, 95, 382], thus the 58% female healthy control gender divide was broadly in line with these findings. The fact that patients were actually only 47% female could be the result of recruitment strategy (willingness to participate), though it is also possible this results from the exclusion of patients on medication who may present with more severe sarcoidosis. Female patients have demonstrably shown a tendency towards more severe disease outcomes [47, 78, 95], thus it is possible that more of this group were excluded from consideration than males. It is equally possible that this occurred by chance, as recruitment for this study was not all-inclusive; only those willing to participate were used and therefore this is not a definitive assessment as to the actual cohort of patients with sarcoidosis in the region. None of the patients recruited smoked, which aligns with research suggesting that the incidence of sarcoidosis amongst smokers was lower than those who did not smoke tobacco [91, 394].

3.4.1.2 Organ Involvement

All the patients included in this study had some form of pulmonary involvement, be it parenchymal lung or mediastinal lymph node inflammation. Given that the major source of patients for recruitment was through the respiratory department, this was not unexpected. The ACCESS study found that 95% of the patients included had lung involvement [47], so the fact that this was the predominant organ system involved

supports previous findings. No patients with Scadding CXR stage IV disease were included, this may have been related to the exclusion of patients undergoing corticosteroid therapy from this study. As stage IV has previously been observed in approximately 1 in 20 patients with the diseases suggests that this would have been uncommon in a cohort of this size [47], even if there was not exclusion of these patients. The proportions of other stages were approximately similar in proportion to those seen in the ACCESS study [47], with the exception that the cohort in this study comprised more stage III patients as a proportion (17% compared with 9.8%) and there were more stage II than stage I patients in this study (the inverse was true in ACCESS) [47]. There were 2 patients with stage 0 disease based upon chest X-rays, yet these individuals were classified as having lung involvement based upon further investigation with CT scans, reinforcing evidence that Scadding staging by X-ray has limitations [73, 74, 79]. It is acknowledged that one limitation of the Scadding CXR staging is that there can be variability in assigning a stage depending on the observing radiologist [76]. Diagnosis of sarcoidosis made for the cohort in these studies rely on a multi-disciplinary team (MDT) assessment, which includes the radiologists responsible for determining the staging. Whilst there are more than 1 radiologist responsible for these assessments, there is a degree of moderation that arises from regular meetings by the team. Even though inter-observer variability cannot be excluded entirely in this context, the MDT meetings and assessments aim to keep the subjectivity in radiographic scoring to a minimum.

Details of any pulmonary function tests patients may have undertaken were not included in this present study. Patients were not asked to perform spirometry for this particular pilot study and the details of any previous tests were not included in the data as there would be a wide degree of variation in the length of time between these tests and sampling. This is acknowledged as a limitation in assessing the status of the patients in this current study. Any future work should include lung function within the data to aid in determining the severity of the pulmonary symptoms of the patients in the cohort.

Studies approach the issue of organ involvement differently when reporting their findings; whether the lungs and mediastinal/pulmonary-associated lymph nodes constitute their own organ systems or treat them as 2 separate organ systems. The Scadding CXR staging allows for distinction between those with or without involvement in

each of these, therefore for this report thoracic lymph node involvement was treated as separate from 'lung' involvement. Drawing parallels with other studies would take this into account; the ACCESS study treated these as a single organ system and suggested that 48.1% of patients only had pulmonary involvement [47]. By contrast, in the cohort presented in this work, 57% were termed 'pulmonary restricted', with 43% showing some form of extrapulmonary organ involvement. The term 'pulmonary restricted' comes with a caveat however, as a patient sample was taken once and did not follow patient progress over time in a longitudinal study, providing what is termed as a 'temporal snapshot' of the disease. It is therefore not strictly accurate to assume that patients are exclusively 'pulmonary restricted' as a group, as other organs might be involved but not investigated/symptomatic or might become involved as the disease progresses [48, 59]. It is, however, the best way to describe these patients at the time of sampling. It is notable that female patients were more likely to have a greater number of organs involved and extrapulmonary organ involvement compared to male patients, whereas stage III Scadding radiographic findings were more common in males, both of which are supported by previous findings in the larger ACCESS epidemiological study [47, 48]. The data indicates that there are gender specific differences in organ involvement, as has been observed by other reports, and that gender should be considered when studying the disease itself.

3.4.1.3 Serum Factors

Markers of inflammation, C-reactive protein (CRP) and plasma viscosity (PV), were analysed as part of a patient's clinical care and were obtained to determine whether these were associated with any of the findings presented as part of this research. CRP and PV are part of the 'acute-phase response', short term indicators of inflammation and damage in tissue that can be used to indicate the presence of an active disease profile [584, 585]. Overall, neither CRP nor PV were definitive diagnostic indicators of the presence of sarcoidosis within the cohort, as the majority of patients were within the normal reference range for these. Approximately a quarter of patients had elevation in these two factors, which correlated well with each other but were not associated with the majority of other features of the disease, barring the observation that extrapulmonary

organ involvement appeared to be associated with elevated PV in patients. Trends were observed with both of these factors, associating higher CRP and PV with more organ involvement and higher Scadding stages, though without a larger sampling set, nothing definitive can be concluded as regards these inflammatory markers.

Whereas only 26% of the patient cohort presented in this work had elevated CRP levels and this was not significantly associated with many of the features of disease activity, a different report had a cohort of patients in which 36% of patients had elevated CRP and this was associated with serum ACE and multi-organ involvement [586]. The authors of that study suggested CRP was an indicator of worse prognosis in patients, though that study included Black patients alongside Caucasian patients, the former presenting with higher baseline CRP [586], which contrasts with the present study in which only Caucasian patients were included. Another found a poor correlation of CRP with classical features of the disease in patients, such as Scadding stages, but the protein has been demonstrably higher in patients with fatigue [708]. With only 27% of patients displaying elevated PV, this marker also appears to have limited utility as a diagnostic marker and in a study with Crohn's Disease, only 52% of patients with active disease showed elevated PV [709]. The same group also found that PV correlated well with CRP, as was found in this present study, and was possibly related to disease activity, but lacked sufficient sensitivity necessary for accurate determinations [709].

Serum angiotensin converting enzyme, serum ACE (sACE), has long been associated with sarcoidosis and it is routine to analyse levels of this protein for patients with the disease [1, 228, 232]. Serum ACE levels were taken for patients within this study, again providing a temporal snapshot of the disease in the patients rather than employed as a mechanism for tracking progress. 60% of patients had elevated sACE levels above the normal reference range, which is in keeping with previously reported results from other studies [231, 232, 237]. This supports the conclusion that, whilst useful in diagnosis of the condition, low serum ACE cannot be a primary finding for exclusion of sarcoidosis in diagnosis when 4 out of 10 patients with the disease have 'normal' levels of the enzyme in the blood. Although the levels of the enzyme tended to be higher in female patients, gender did not appear to be a major feature influencing sACE in this cohort. Neither was age associated with sACE levels to a high degree. Although not statistically significant, the

tendency for serum ACE to be elevated with increasing numbers of organs involved, coupled with the highest median levels of sACE being present in patients with stage II rather than stage I or III Scadding scores, suggests sACE may relate to the degree of the granulomatous inflammation in an individual. In other words, having more organs involved, as observed in stage II disease compared with I and III, can be indicated by the levels of serum ACE. This is further reinforced when looking at extrapulmonary organ involvement, where a highly significant proportion of these patients have an increased sACE level. It suggests that sACE relates more to how extensive the granulomatous inflammation is, perhaps also indicating that patients with more systemic symptoms are more likely to have elevated levels of the enzyme. Others have noted that sACE relates to the extent of granulomatous burden [232, 242-244]; importantly, the work presented here supports the research suggesting that sACE is a possible indicator of extrapulmonary organ involvement [244]. The lack of any strong association with C-reactive protein levels or plasma viscosity also suggests that the 'activity' of the disease is not a feature that can be accounted for using sACE levels. The fact that patients with extrapulmonary organ involvement have elevated sACE and increased PV indicates that these patients are distinct from those with a pulmonary restricted form, in both the extent of involvement and possibly in the activity of the disease profile. As extrapulmonary organ involvement has often been associated with a worse outcome for patients with sarcoidosis [47, 48, 53-55], the presence of elevated sACE coupled with elevated PV should merit further investigation in patients based on these findings. It is clear that sACE and other features have value clinically in the disease, though they still remain insufficient for either diagnosis or prognosis when used in exclusion of other factors.

3.4.2 Peripheral Blood Leukocyte Populations

One of the main findings of the immunophenotyping of blood leukocytes was that patients with sarcoidosis were T cell lymphocytopenic; healthy controls had twice the number of T-lymphocytes on average when compared with patients. As a result, the total peripheral blood mononuclear cell number was significantly reduced in sarcoidosis compared with healthy controls, attributed to the T-lymphocytes rather than monocytes. Within this, there was a global reduction in the both $CD4^+$ and $CD8^+$ T-lymphocytes,

whereas B-lymphocytes and Natural Killer Cells were not significantly different between patients and controls. One limitation of the current study relates to the method used to determine the cell numbers, relying on isolated PBMCs rather than a complete white cell count from routine haematology. Whilst the method employed in this research was consistent, future research would benefit from a haematological cell count for determining the broad leukocyte groups and using flow cytometry to calculate the proportion of individual subsets.

T cell lymphocytopenia has been noted in numerous other reports [257, 258, 710-715], though some findings have suggested that a more global lymphopenia exists [712] and another reports finding no significant difference in specific-lymphocyte subsets, such as T-helper inducer or T-suppressor cytotoxic T-lymphocytes [716]. In this study, although characterising only a limited number patients and controls for presence of CD19 and CD56, B-lymphocytes and NK cells appeared to both be similar in number between healthy controls and sarcoidosis patients, suggesting that the lymphopenia observed is attributable to T-lymphocytes. Where some authors have identified B-lymphocyte reductions in patients [712], others have found no evidence of B-cell lymphopenia [713, 714]. It should be acknowledged that it appeared that CD4$^+$ T-helper cells were significantly reduced, perhaps to a greater degree than CD8$^+$ cells. Ultimately, the CD4:CD8 ratio was not significantly different between controls and patients, therefore a global T cell lymphocytopenia appears to be a feature of the disease. The majority of T-lymphocytes in granulomata are CD45RO$^+$ rather than naïve CD45RA$^+$ cells [717], though based on the findings that patient and control ratios of naïve:memory were not significantly different, this would suggest that the polarising of T-lymphocytes to activated and memory subsets likely occurs locally rather than in the peripheral blood.

3.4.2.1 T cell Lymphocytopenia

The significance of this reduction in peripheral T-lymphocyte number is a matter of some debate, whether it arises from the translocation of peripheral cells to the site of granulomata in tissue (sequestration) or through ablation or immune suppression peripherally. The latter theory is supported by reports of expanded T regulatory cells in

the peripheral blood and their activity [23]. Research by Crouser *et al* [718] offers an alternative explanation, as patients appeared to respond favourably to infliximab, an anti-TNF-α therapeutic, with lymphocyte numbers increasing with this therapy but not under alternative existing medication. The authors suggest that this indicates T cell lymphopenia is dependent on TNF-α specifically and the effect it has on Treg cells [718], in which the cytokine inhibits proliferation of Tregs but not activation [719]. As existing therapies did not correct the T-lymphocyte depletion despite improving symptoms, this would suggest that sequestration is not the reason for T cell reduction peripherally. Alternatively, some associations exist between reduced T cell number in the blood and elevated BAL T-lymphocyte numbers from patients [256] and others have correlated worse symptoms with reduced lymphocyte numbers [712], both of which would indicate that T cell reduction is the result of lymphocytes being sequestered to the tissue from blood. Despite this, the current study found little correlation between T-lymphocyte number and clinical features associated with the disease, such as the number of organs involved or serum ACE levels. This does not necessary invalidate the concept of T cell sequestration as a cause of the lymphocytopenia observed, though this report cannot confirm that this is the reason for the observed results. There is evidence for the importance of peripheral T-lymphocytes in granulomatous inflammation in support of this concept of sequestration, which comes from patients who have sarcoidosis and HIV infection, where the granulomatous inflammation requires a CD4$^+$ T-lymphocyte count of over 200 cells/µl of blood to develop and progress [720]. This is not surprising, as the disease has been notable for being characterised by the accumulation of T-helper cells in local tissue which promote the inflammatory responses [279]. This could therefore be considered a feature of the chronic state of the inflammatory disease, whereby T-lymphocytes are transitioning from peripheral blood to the site of inflammation. In tuberculosis, the earliest stage of the disease presents with elevated numbers of T-lymphocytes (T-lymphocytosis) [721], but typical presentation in patients after this initiation of infection is either of normal T-lymphocyte number [721] or reduced numbers of circulating T cells [722, 723]. There appears to be some variation in tuberculosis reports, with either specific CD4$^+$ T-lymphocytes being reduced [723] or T-lymphocyte and B-lymphocyte populations both being reduced in the disease [722]. It should also be noted that monocyte numbers have been elevated in a number of TB patients (monocytosis) [722, 724]. The numbers of B-lymphocytes, NK cells and monocytes were not significantly different between

sarcoidosis patients and controls in this study, suggesting that there are haematological differences between tuberculosis and sarcoidosis.

This T cell lymphocytopenia in patients with sarcoidosis had wide ranging impact on the peripheral leukocyte system. The expression of the IL-2 receptor (as determined by CD25) was significantly increased in sarcoidosis lymphocytes as a proportion of the population of T cells; other 'activation markers' such as CD69 previously have been reported to be more heavily expressed in this cohort [725]. However, the number of circulating T-lymphocytes which were CD25 positive were significantly reduced compared with healthy controls. Using a crude measure of T regulatory cells based on $CD4^+ CD25^{high}$ T cells, the population of Tregs was not expanded as a proportion of the population and was reduced in real terms due to the lymphocytopenia. This would require further analysis using T regulatory cell specific markers (such as FOXP3) to be confident of these findings, as they contradict previously reported expansion of this population in peripheral blood [23], though are supported by findings from a different study in which Tregs were reduced in sarcoidosis [726]. Despite this, the overall expression of CD25 was also increased on T-lymphocytes, suggesting that cells may be more 'active' in patients with sarcoidosis compared with controls, as CD25 is an activation marker on T cells [727].

Although the number of NK cells were similar between patients and healthy controls, the higher percentage of $CD56^+$ T-lymphocytes (so called NKT cells) in healthy controls meant that, when coupled with the reduced T cell number in patients with sarcoidosis, there was significantly fewer of these cells in patients. Whilst this is supported by a study in which a subset of NKT cells was also diminished in sarcoidosis peripheral blood [728], a different report by Katchar *et al* found increased NKT cell numbers in the blood of patients with sarcoidosis [354]. The limited numbers of donors studied for CD56 limits the impact of the findings for the current study, therefore this would need confirming in a larger number of patients and controls.

3.4.2.2 Monocytes

The other key finding relating to peripheral blood leukocyte populations was that intermediate CD16$^+$ CD14^{++} monocytes were higher as a percentage of the monocyte population in patients with sarcoidosis when compared with healthy monocytes. This supports findings from another study by Hijdra *et al*, where this subset of monocytes was also increased and that the TNFR1 receptor is most heavily expressed on intermediate monocytes [336]. Others have noted that monocytes expressing CD16 were also increased in sarcoidosis [338, 729]. Not all reports have identified a difference in intermediate monocytes in sarcoidosis; preliminary work shows CXCL9^{high} patients with sarcoidosis have lower numbers of non-classical monocytes compared with CXCL9^{low} patients, but no difference in other subsets [730]. The proportion of classical monocytes, CD16$^-$ CD14^{++}, were significantly reduced in patients with sarcoidosis, suggesting that there may be a transition from one population to the other. Research by Sugimoto *et al* in rhesus macaques indicates that classical monocytes can transition to become intermediate and then become non-classical monocytes [731].

This study did not characterise dendritic cell populations, which is a limitation in the current report. Circulating dendritic cells tend to be low in number in healthy individuals, making up less than 1% of the total leukocyte cell number [732]. There are considered to be three types circulating blood dendritic cells, two myeloid dendritic cell types and one plasmacytoid dendritic cell type [335]. There is an inherent issue with identifying dendritic cells, as there is a great deal of overlap in the markers these cells display and those of the monocyte subsets (including CD14 and CD16) [335, 731, 733]. Work has suggested that markers such as CD1b, CD1c (BDCA-1), CD123, CD141 (BDCA-3) and other possible markers in combination may be beneficial in differentiating these from each other and circulating monocyte populations [731, 733]. Future work should include these as differentiating markers to exclude those dendritic cells from monocyte subsets. Other work has previously highlighted that blood dendritic cells are not increased in number in patients with sarcoidosis and that these cells display anergic responses [24, 353]. Given that dendritic cells make up a small proportion of circulating mononuclear leukocytes, the absence of differentiation in this report is not considered a major failing, though a comprehensive analysis including these cells would be appropriate in future studies.

Individuals with higher intermediate monocyte numbers relative to other subsets were more likely to be Scadding stage II relative to stage I and tended to be those with more organs involved. The tentative association between this subset and individuals with extrapulmonary organ involvement suggests that the expansion of intermediate monocytes may relate to the disease, whether it is as a measure of disease activity or the extent of granulomatous burden. Very recent work by Hijdra *et al* [734] has supported the findings presented here regarding the lack of difference between the proportion of intermediate monocytes in different genders. It also expanded on previous work the group had completed, showing that the percentage of intermediate monocytes can act as a differentiating factor in establishing whether a patient will respond in infliximab TNF-α therapy, with responders displaying higher intermediate monocyte percentages [734]. Rheumatoid arthritis is another inflammatory disease where patients exhibit an expansion of the intermediate monocyte population and anti-TNF therapy has proven efficacy in this disease [735, 736]. This is important, as it might suggest that this particular subset is essential in TNF-α mediated processes in the disease, which has been demonstrably shown to be vital in the immunopathogenesis of the disease (see Section 1.4). Further work is required to investigate the role of this monocyte subset in sarcoidosis, which may be important in assessing disease activity or prognosis in patients.

3.4.3 Peripheral Blood Leukocyte Responses to PHA, SEA and WGA in Sarcoidosis

There are advantages and disadvantages to employing a whole blood assay (WBA) in immune cytokine release assays of the nature undertaken in this study. Unlike PBMCs, which requires multiple steps in isolation, WBAs minimally perturb the leukocytes involved in the experimentation, thus removing possible confounding factors in loss of cells or unintended activation of responses. It provides an effective way to analyse a more physiological relevant medium consisting of all of the cell types present in the blood; the results incorporate the sum total of the interactions present. Certainly, this is less specific than analysing a single cell type in exclusion, but usually more relevant to the system present *in vivo*. However, unwinding and delineating all of these complex interactions can be difficult and any treatment with a molecule of interest ignores the one key physiological issue: *in vivo* there would be a change in leukocyte number and makeup

present over time. New leukocyte populations are dynamically added to the blood *in vivo*; the exact makeup of the leukocyte population varies and the array of plasma proteins (including chemokines and cytokines) also changes to accommodate the ever changing conditions in a multicellular organism. Taking a sample of blood provides a 'temporal snapshot' of the organism at a given point; both an advantage and disadvantage that *in vitro* and *ex vivo* researchers are acutely aware of. Despite this, the method is a powerful tool for analysing overall immune responses in an individual, it simply requires complementary methods to attempt to understand the interactions occurring.

3.4.3.1 Whole Blood versus Peripheral Blood Mononuclear Cells

A whole blood assay approach was selected after an initial optimising step, where undiluted whole blood was stimulated with PHA and compared to PBMCs matching the concentration of cells present in the original sample. The more robust response in PHA induced cytokine release from whole blood compared with PBMCs confirms that WBAs are a good medium for this type of stimuli. Whether this is helped by the type of PHA used, PHA-P with leukoagglutinating and erythroagglutinating PHA isoforms, is unknown but a possibility. It has not always been shown to be the case that whole blood release is greater than PBMC; some studies actually suggest that this is cytokine dependent [704], though these other reports often differ in the methodology employed (diluting whole blood or using different PHA isoforms). As a result, based on the system employed here, whole blood appeared to be capable of a higher level of cytokine release than PBMCs and would benefit from the other features a whole blood assay has to offer. Based on the data obtained from analysing leukocyte subsets, the percentage of T-lymphocytes within PBMCs would be significantly different between healthy controls and sarcoidosis patients; much lower in patient samples. As a result, there is a significant difference in the composition of the PBMC between healthy controls and patients with sarcoidosis, which means that these preparations are not directly comparable either, despite removing other cell types and plasma factors. Even adjusting the concentration of PBMCs so that they are equivalent in number ($1x\ 10^6$ cells per ml for example) between the cohorts would artificially increase the number of monocytes, B-lymphocytes and NK cells in patient samples beyond that of healthy controls, which would not be representative of

physiological conditions. As PHA has been shown to be most effective with the presence of accessory cells (such as monocytes) or their products [737, 738], separating the individual cell types for PHA stimulation would also be of limited value in this kind of study.

3.4.3.2 Peripheral Leukocyte Responses to PHA

The results of the immunophenotyping and leukocyte cell quantification revealed a significantly reduced number of T-lymphocytes were present in the blood of patients with sarcoidosis. As PHA is considered to be principally a T cell mitogen, with activation occurring via the T cell receptor (TCR) [696, 706], a reasonable hypothesis would be that patients with sarcoidosis should display reduced responses to this stimuli. Indeed, it has previously been reported that leukocyte PHA response in peripheral blood cells is reduced in patients when compared with healthy controls [739-741].

Despite this, the principle finding of this study was that high concentrations of PHA induced a greater release of IL-6 and TNF-α from whole blood in patients with sarcoidosis compared with healthy controls. Additionally, the IFN-γ and IL-8 release from whole blood was similar between healthy controls and patients with sarcoidosis, not diminished in the latter; IL-10 release was also not significantly different between these two cohorts. Nor was this a generalised response, as the release of these cytokines from whole blood in response to stimuli with different modes of action, SEA and WGA, were not enhanced in patients with sarcoidosis. Indeed, SEA induced cytokine release was typically lower in patients than controls, in keeping with expectations based upon the T cell lymphocytopenia initially identified in these individuals. Intracellular TNF-α and IL-6 accumulation in Brefeldin A treated PBMCs revealed that monocytes in sarcoidosis were the principle source of these cytokines, at least in early phases of stimulation, and were significantly increased in monocytes from patients with sarcoidosis. Overall, this suggested that the mechanism of PHA induced cytokine release was not simply as a T cell activator, with accessory monocytes as a 'cheerleader cells', but that monocytes in patients with sarcoidosis may be capable of enhanced activity and are more responsive to PHA as a stimuli themselves.

Latent TB patients were included in a limited number after initial results were obtained in order to determine whether the elevated responses observed to PHA stimulation were specific to sarcoidosis. There is a question as to whether latent TB patients represent a reasonable disease comparison and there were challenges in obtaining individuals matched to the other two cohorts. The sample size was limited and variable, as it was not envisioned that latent TB patients would be a primary focus. It is possible that future work could expand on this if parallels are required to other conditions, but in the current research, this group of patients was not a fair comparison for the sarcoidosis cohort and was therefore not investigated further.

PHA is not highly specific in binding. PHA-L, the leukoagglutinating isoform, essentially binds carbohydrates containing mannose, N-Acetylglucosamine and galactose present on glycosylated cell surface proteins [742]. Whilst it is well established that this causes cross linking and activation in the TCR [696, 697, 706, 737, 743], it is not fully understood how PHA causes the necessary second signal or leads to IL-2 release in T cells. More recent research suggests that PHA can activate members of the Toll-like Receptor family [698, 699], which may be the source of activation on monocytes.

The time course of cytokine release in the whole blood assays showed that TNF-α release reached the maximum between 3-6 hours post treatment with PHA. Whether this PHA induced TNF-α is the result of *de novo* synthesis or release of stored protein is not clear, but the increased concentration of this cytokine at 16 hours in sarcoidosis, coupled with the results of the intracellular accumulation of this cytokine and time course assessment of patients, suggests that the monocytes are generating new TNF-α in response to PHA. The reduction of TNF-α after 6 hours in whole blood, but not in Brefeldin A treated monocytes, might point to the utilisation of the cytokine by other cells (including T-lymphocytes) in their own activation, or a reduction in release of TNF-α in response to increasing concentrations of another cytokine inhibiting production and release of the inflammatory protein. The mode of action of Brefeldin A treatment leads to the accumulation of intracellular proteins, in this case cytokines, by preventing translocation of proteins from the endoplasmic reticulum to the Golgi apparatus, thus preventing secretion of proteins out of the cell [707]. This might explain why TNF-α continues to increase over the 16 hours in the PHA stimulated and Brefeldin A treated cells, as no

secretion means the cytokine will not be internalised by other cells or effected by post-release regulation. Thus, excluding these factors, TNF-α production can increase unabated, whereas the lack of detectable IFN-γ in response to PHA suggests that neither T cells nor monocytes release this cytokine as an initial response to PHA in early events, but require the release of a factor (cytokine) or the translocation of newly PHA-generated cell surface proteins required for T cell activation. Despite early work suggesting alveolar macrophages spontaneously release IFN-γ [346], this has not been easily replicated by other groups, nor was it detected in response to PHA stimulation in monocytes in exclusion of other activating factors. IL-6 accumulation was detected in both T-lymphocytes and monocytes, though the production was greater in the latter cell type. IL-6 and TNF-α have traditionally been considered to be more associated with monocyte/macrophage cell types, in much the same way that IFN-γ is more associated with T-lymphocytes [318, 319]. The findings of this work support previously reported flow cytometry of PBMCs with PHA where TNF-α is associated more with monocytes, though that group were able to get modest T-lymphocyte TNF-α and IFN-γ accumulation, albeit with monensin treatment rather than Brefeldin A [744]. It has been demonstrated that monocyte/macrophage/dendritic cell derived factors are an absolute requirement for PHA activation of T-lymphocytes [737, 738, 745, 746], amongst these IL-6 is considered important [737]. This provides a mode of action of PHA reliant on early monocyte-derived activation and subsequent immune response.

It is acknowledged that a 16 hour time point for collection of whole blood assay plasma was not perfectly suited for TNF-α release. It did accommodate other cytokines well, including IL-6 (which continually rose over 72 hours) and IFN-γ (peak 16-24 hours), therefore it was a compromise related to the amount of blood being obtained and logistical considerations in running multiple labour intensive experiments over multiple times points with large numbers of samples. Similar kinetic profiles in PHA stimulation have been noted by De Groote *et al* previously [704]. For an accurate determination of the role of TNF-α in this PHA induced system, a timeframe of release between 3-6 hours in whole blood would need to be performed.

IL-6 release was elevated in response to PHA in sarcoidosis, with large differences in the amount released compared with heathy donors. This is significant, as both monocytes

and T-lymphocytes are demonstrably capable of releasing IL-6, and it has been shown that this cytokine is important in acting as an accessory signal in PHA induced activity [737]. It is suggested here that the increased release of IL-6 would enhance the activity of PHA, even if the cytokine has been traditionally associated with reducing IFN-γ release in supporting a Th-2 [747]. IL-6 is a pleiotropic cytokine with both anti-inflammatory and pro-inflammatory activity depending on the context and cytokine milieu present [748]. It is released from various cell types, therefore the issue becomes identifying the source of the elevated IL-6 from patients when employing a whole blood system. What is interesting is that IL-6 release can be induced by TNF-α, which can activate multiple signalling pathways (such as p38 MAPK and JAK/STAT3) to lead to the release of IL-6 [749]. Additionally, IL-6 has a suppressive effect on TNF-α release in PHA stimulated PBMCs, acting as a form of negative feedback [750]. This could explain why there is a marked reduction in TNF-α release after 6 hours in whole blood whilst IL-6 continues to rise, as TNF-α might be both stimulating greater IL-6 release from blood leukocytes whilst suppressing further TNF-α release. In such a system, if patients with sarcoidosis show enhanced response to PHA, this could be manifesting more in IL-6 release than TNF-α, as increases in the latter may be the source of elevated IL-6.

The intracellular flow cytometry IL-6 staining after PHA stimulation suggested that both monocytes and T-lymphocytes release the cytokine, though the former cell type produced more of it relative to T cells in this assessment of early stage signalling. However, as the concentration of the cytokine continues to rise over 72 hours (as observed in the whole blood assay time course experiments), it is possible T-lymphocyte derived IL-6 may be responsible for the differences observed. In patients with sarcoidosis, whole blood IL-6 release correlated with TNF-α release, whereas in healthy controls this was not statistically significant. For healthy controls, TNF-α and IFN-γ were associated. As TNF-α release was demonstrably elevated in PHA whole blood from patients, this association between IL-6 and TNF-α in patients is notable for its absence in healthy controls. The absence of correlation between the number of T-lymphocytes in blood and the concentrations of TNF-α, IL-6 or IFN-γ release in either cohort suggests that T-lymphocytes may not be the principle factor explaining the elevated responses in patients. By contrast, IL-6 release was associated more with the number of monocytes in individuals, suggesting that the difference in IL-6 may arise from monocyte release. It should be noted that whilst IL-6 release does relate to monocyte number, as patients with

sarcoidosis have similar numbers of monocytes to healthy controls, this is not likely to be the explanation for the elevated release observed in the whole blood assay. Another key finding is that healthy controls CD25 expression in T-lymphocytes related to increased TNF-α expression, as might be expected given that CD25 relates to activation, yet the inverse was true for patients with sarcoidosis. The significance of this finding is unknown, although monocyte CD25 did not appear to relate to the PHA induced cytokine release in either cohort. Also it is unlikely that this CD25 expression relates to those CD25^{high} cells in particular, as it has been shown in this study that there is no increase in the number of CD25^{high} cells in sarcoidosis. Also, as CD4:CD8 ratio is not significantly different in patients with sarcoidosis and the raw number of CD25^{+} T-lymphocytes is reduced in the disease, neither of these are likely to account for the elevated cytokine responses observed. Despite there being an expansion of intermediate monocytes in sarcoidosis, it could not be determined that there was an association between this and IL-6 or TNF-α release from PHA stimulated whole blood from patients. It would therefore be necessary to perform specific experiments testing whether PHA stimulation leads to greater TNF-α release in specific monocyte subsets, in particular the intermediate or non-classic monocytes. This was not undertaken in the current study, but could be investigated further in subsequent research in this area. Overall, it leaves a number of questions unanswered, though the data would seem to indicate that the likely source of the elevated TNF-α and IL-6 from leukocytes in sarcoidosis are monocytes rather than lymphocytes.

3.4.3.3 Comparing SEA and WGA with PHA responses

The results of the stimulation with SEA and WGA supports this idea of monocyte importance in PHA to some degree, as both of these stimuli have different modes of action from PHA and display different results. WGA appears to bind more exclusively to myeloid derived cell types [698], though no statistically significant differences were observed in sarcoidosis. SEA is a polyclonal activator of T cells with broad specificity for TCR activation [700, 701]. A combination of T-lymphocyte and accessory cells interaction are required for T-lymphocyte inflammatory action [700]. As such, the observation that IL-6 is reduced in SEA stimulation in patients with sarcoidosis suggests that this stimuli, which acts through classical TCR signalling and is dependent on T-lymphocyte activation,

is reflecting the T cell lymphocytopenia present in patients in terms of the cytokine release. This is supported by the fact that it is more acutely affected by the number of T-lymphocytes and even monocyte number in healthy controls, yet does not show a similar elevation in cytokine release as has been observed with PHA. The conclusion would be that the differences in patients with sarcoidosis seen with PHA stimulation likely occur via a different mechanism than that typically described for PHA-TCR interactions [696, 706]. This is supported by previous findings that anti-CD3 anti-CD28 stimulation with beads, which are specific to activating the TCR complex on T-lymphocytes, showed diminished responses in patients with sarcoidosis [751]. To put this in a wider context, PHA is the positive control used within the QuantiFERON TB assays for latent TB infection [752]; the necessary activator to assess immune competency for whole blood leukocytes. Within this whole blood assay, the number of false positives involving sarcoidosis are demonstrably low in some studies [753, 754], and positive is other analyses [755]. With reduced T-lymphocyte numbers observed in patients there would be an expectation that a low response would be obtained with the positive control, yet sarcoidosis patients must display positive responses to the mitogen control. The response to SEA and PHA in whole blood indicates that the purported anergy is not generalised, though T-lymphocyte dependent polyclonal activation, as determined by activation with the superantigen, is clearly affected by the reduced leukocyte number.

3.4.3.4 Association of PHA responses with Clinical Features in Patients

When applying these findings to the cohort of patients and their clinical features, the observed whole blood cytokine release appeared to be independent of serum ACE, yet IL-6 did appear to be elevated in stage II patients and those with a greater number of organ involvement. It did not, however, relate to extrapulmonary organ involvement in patients, which contrasts with the correlations observed in serum ACE. The significance of these findings is not known; whether the peripheral responses in this *ex vivo* system can be discriminatory enough to draw conclusions as to the status of patients is unlikely based on the sensitivity provided. It does highlight that patients with sarcoidosis are far from anergic in terms of their peripheral immune responses. Whether these findings are

predictive of prognostic outcomes or other features of the disease pathogenesis is unknown, and would likely require a longitudinal study in patients to observe whether those with elevated peripheral responses to PHA are clinically distinct within the patient cohort.

3.4.4 Conclusions

This research highlights a fundamental contradiction at the heart of peripheral blood immunological study in sarcoidosis; with patients displaying a profound T cell lymphocytopenia, yet presenting a robust response to stimulation with phytohaemagglutinin (PHA). Both the leukocyte populations and immune responses to non-specific lectins and mitogens are distinctly altered in the disease, suggesting that there is a systemic aspect to sarcoidosis reflected in the peripheral immune responses.

The patient cohort is characteristically heterogeneous, with individuals displaying a variety of organ involvement and degree of granulomatous burden. Using these traditional clinical features, patients could be separated into groupings based upon the Scadding CXR staging (pulmonary involvement), the number of organs (1-3+) and whether the organ involvement was restricted to the pulmonary system or featured extrapulmonary involvement, such as altered calcium metabolism, skin, eyes etc. What this showed was that this cohort comprised patients manifesting the disease in a variety of ways, in keeping with previous analyses [47], though all had some form of pulmonary involvement. Elevated serum ACE was associated with features indicating a patient was suffering from greater granulomatous burden, whereas inflammatory markers, such as plasma viscosity, appeared to only be elevated in approximated a quarter of patients. The utility of these serum based tests are limited however, as not all patients display elevation in these in the cohort and therefore are of questionable value either diagnostically or prognostically. The conclusions would be to apply these findings in a wider context, though it is clear there is a need for more effective diagnostic and prognostic tests for the disease.

This occurs against a backdrop of notable peripheral leukocyte population differences between patients with sarcoidosis and healthy controls. The vast majority of patients with sarcoidosis are T cell lymphocytopenic, they have a greatly reduced number of T-lymphocytes in the blood well below the number present in healthy controls. $CD4^+$ cells (T helper cells) appear the worst effected, though there is a global reduction that impacts on the numbers of $CD8^+$ T-lymphocytes (cytotoxic T-lymphocytes), $CD25^+$ T-lymphocytes (activated T-lymphocytes), $CD56^+$ T-lymphocytes (NKT cells), Naïve and memory T cells. This was specific to T cells; B-lymphocytes, NK cells and monocytes were similar in number between patients and healthy controls. Based on a wealth of research in this area, the likely reasons for this reduced circulating T-lymphocyte number is either an elevated regulatory response in the peripheral system specific to T-lymphocytes or a sequestration of T cells from the blood to the tissue, leading to deficiency in numbers. Notably, the proportion of intermediate monocytes, a subset of the population present in blood, is elevated in sarcoidosis relative to controls, though the implications of this are not fully understood.

Despite the often cited peripheral blood anergy in the disease, even to PHA stimulation [23, 739-741], the results of this study suggest that patients with sarcoidosis can display elevated IL-6 and TNF-α release in response to high concentrations of PHA compared with healthy controls. This is not universal however, as patients displayed a diminished response to different non-specific activators, such as SEA. Based upon the research performed here, monocytes appear to be the likely cause of this elevated response in patients, with enhanced TNF-α and IL-6 release attributed to this cell type. Whole blood assays are a complex, multicellular system in which PHA leads to downstream events in different leukocytes. A model for this is presented in Figure 4.22, which displays both the early events of stimulation and the subsequent activity that might contribute to the results seen in whole blood. The precise reason for the enhanced monocyte response to PHA is currently unknown; whether this results from enhanced activity upon PHA interaction with a specific immune receptor, such as TLR4, or a defective regulatory response is a question that requires investigation. As a result, subsequent work would focus on identifying whether more specific stimuli could lead to enhanced responses in whole blood systems and characterising the regulatory receptor-ligand mechanisms to address this question.

In summary, the data suggests that monocytes in sarcoidosis may be capable of responding more vigorously to specific stimuli, such as PHA, compared with controls; this occurring in a peripheral blood system notable for having diminished numbers of T-lymphocytes. Monocytes are not generally hyperactive, though it may be that peripheral blood leukocyte responses are defined by either a hyperactive response to a specific stimulus, be it an individual receptor or pathway, or a dysfunctional regulatory mechanism specific for an inflammatory pathway.

Chapter 4

Innate Immune Receptor Activity in Peripheral Blood

4.1 Introduction

Innate immune responses are vital for the function of both early activity and subsequent adaptive immunity [756-758]. There are a number of mechanisms present that constitute the innate immune system, perhaps the best studied are the pattern recognition receptors (PRRs). The main receptor groups belonging to these are the Toll-like Receptors (TLRs), the NOD-like receptors (NLRs), the C-type lectin receptors (CLRs) and the RIG-I-like receptors (RLRs) [758]. The Toll-like receptors are among the better understood and heavily researched of these PRRs, of which there are currently known to be 10 receptors (TLRs 1-10) present in humans [756]. The pattern recognition receptors bind to different ligands depending on their specificity; some are relatively specific such as TLR3 which binds to double stranded RNA (dsRNA), whereas others are more promiscuous in their binding, like TLR2 which binds to various bacterial, viral, fungal and mycoplasma constructs [758]. The activation of these innate immune receptors is reliant on the recognition of pathogen associated molecular patterns (PAMPs) and damage associated molecule patterns (DAMPs). PAMPs are features of pathogens that are typically conserved in evolution but are not present in mammalian systems under normal conditions, such as the dsRNA from viruses or lipopolysaccharide (LPS) from bacteria [758-760]. DAMPs are molecules/proteins that are released from either necrotic cells or secreted as a danger signal for damage and cell stress that would not normally be present (or be present in low concentrations) under normal physiological conditions [513, 758]. The classic example of a DAMP is HMGB1, a chromatin binding protein that in apoptosis is irreversibly bound to the histone, but in necrosis is 'free' and released into the extracellular matrix [761]. It can also be secreted by monocytes/macrophages and other leukocytes in response to cytokine signals, receptor activation and cell stress [762, 763]. PRRs can activate a number of downstream inflammatory pathways depending on the PRR in question; for example, the majority of TLRs can activate MYD88-dependent (Myeloid Differentiation Primary Response 88) pathways, leading to NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation and the transcription of genes for inflammatory proteins [764]. However, TLR3 activates TRIF (TIR-domain-containing adapter-inducing interferon-β) instead of MYD88, ultimately leading to NF-κB activation and the activation of IRFs (interferon regulatory factors) that lead to interferon release and activation of other anti-viral responses [765].

A number of these pattern recognition receptors have been investigated for their role in sarcoidosis, particularly the TLRs, such as TLR4 and TLR2 [266, 268, 269, 436, 437]. Other PRRs have also been looked at in sarcoidosis, including intracellular bacterial receptors NOD1 and NOD2 (Nucleotide-binding oligomerization domain-containing protein). NOD2 has been associated with Crohn's Disease [429, 430, 664, 665], early onset sarcoidosis and Blau Syndrome [431-434]; the structure of the protein is represented in Figure 4.1. NOD2 signalling is activated by the binding of a PAMP called MDP (muramyl dipepetide) present in bacterial peptidoglycan [266, 268, 269, 436, 437]. By contrast, NOD1 has fewer established links with the disease, though some associations have been highlighted between sarcoidosis and NOD1 polymorphisms in Japanese patients [766]. Another peptidoglycan product, IE-DAP (γ-D-Glu-mDAP/ gamma-D-glutamyl-meso-diaminopimelic acid) binds to NOD1 to activate downstream signals [767].

The receptor for advanced glycation end-products (RAGE) is a multi-ligand pattern recognition receptor responsible for signalling responses to a range of DAMPs [768-770]. Expressing 3 isoforms, shown in Figure 4.1, the receptor can signal through multiple pathways, including MAPK (mitogen-activated protein kinase) and JAK/STAT (janus kinase/signal transducer and activator of transcription) [771, 772]. The role of this receptor for danger/damage signals has not been heavily investigated for its' role in sarcoidosis; a study suggested that the receptor was expressed in granulomatous tissue in sarcoidosis [773]. Reduced levels of circulating sRAGE (soluble RAGE) have been reported in patients with Rheumatoid Arthritis (RA) [774] and increased RAGE expression on macrophages has been identified in RA patients [775]. There have also been associations with RAGE isoforms and cystic fibrosis [776], suggesting that the receptor may be important in lung pathologies.

Results of the whole blood assay had shown that patients with sarcoidosis released greater concentrations of inflammatory cytokines in response to PHA, but not a T cell receptor dependent activator SEA. Whilst PHA has demonstrably been shown to act on the TCR [696, 697, 706], it has also been demonstrated that the lectin binds to TLRs 2, 4, 5 and 6, all extracellular receptors [699]. Because SEA did not lead to elevated PHA-induced responses from patients with sarcoidosis, the hypothesis was that innate immune

receptor activation, rather than adaptive T cell receptor activation, might be a source of the increased responses. The aim was to undertake a preliminary exploratory study to attempt to identify whether an inflammatory innate immune receptor might be the source of the increased PHA responses observed previously. A similar whole blood system was used under the same conditions, except a range of innate immune ligands, PAMPs and DAMPs, were used as stimuli. The sarcoidosis relevant cytokines IFN-γ and TNF-α were also included to test whether patients with sarcoidosis were more sensitive to these cytokines *ex vivo*. Agonists of the TLRs that PHA can activate were included in this panel, though attempts were also made to include PAMPs from a variety of causative organisms, such as fungal and viral ligands alongside bacterial in order to cover other possible aetiologically causes of the disease or act as controls (Chapter 1). In addition to the TLRs, as a great deal of focus has previously been on this group of receptors rather than others in sarcoidosis, the receptors NOD2 and RAGE were selected to be interrogated further by staining leukocytes for expression of these in a small subset of patients with sarcoidosis and healthy controls. Ligands for NOD2 and RAGE were included in the panel for whole blood assays, including MDP for the former and a range of S100 proteins and HMGB1 for RAGE. IE-DAP, the ligand for NOD1, was also included as a comparison between the two intracellular receptors. The final panel of stimuli is outlined in Table 4.1 and a summary of the interactions of ligands with receptors is represented in Figure 4.2. To the authors' knowledge, this would be the first report to characterise the expression of RAGE on peripheral blood leukocytes in sarcoidosis. As a broad challenge with a range of stimuli, the strategy would be to focus further on a particular receptor and stimuli should significant differences be observed in response from the assays performed.
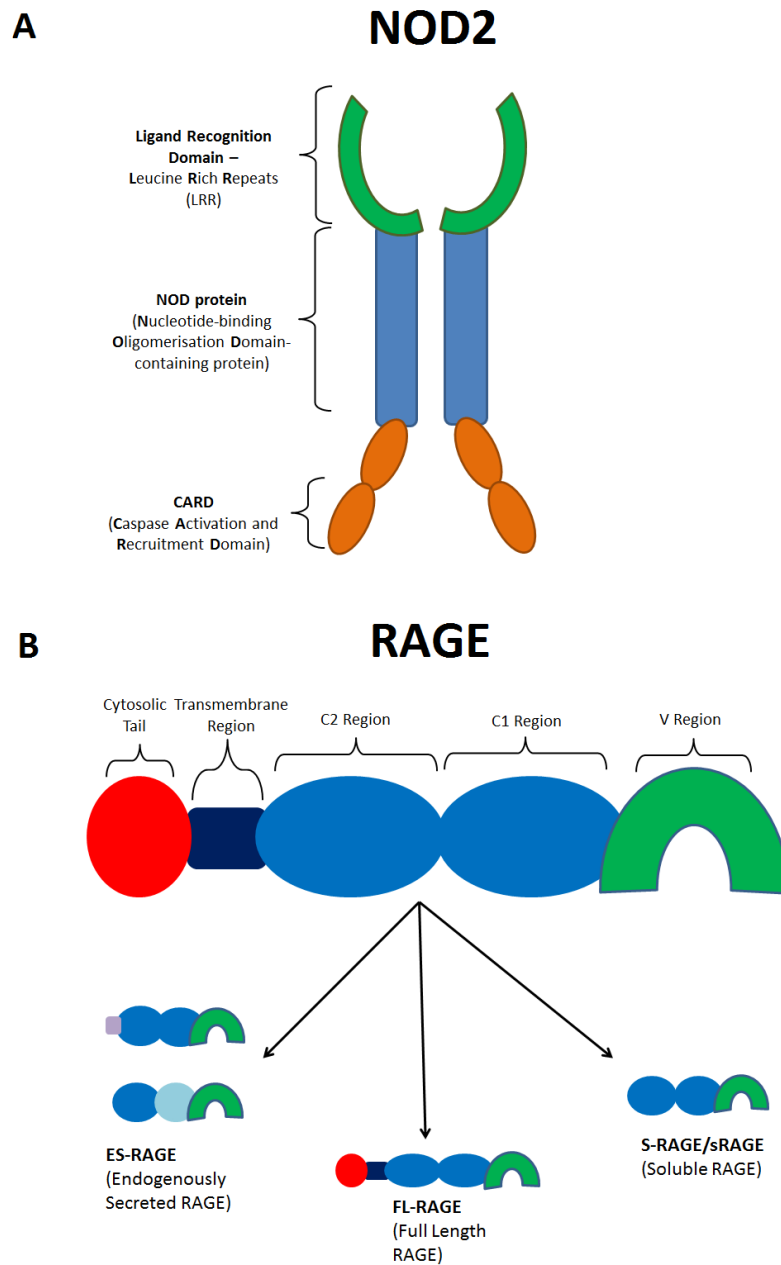
Figure 4.1: Structure of NOD2 and RAGE receptors, along with the isoforms of RAGE.

A: NOD2 basic structure, the LRR region is for binding, whilst the CARD region is involved with signalling. **B:** Receptor for advanced glycation end-products. The V region is for binding to ligands, and is a constant feature in most variants. sRAGE is the cleaved form without transmembrane or cytosolic tail regions. ES-RAGE represents a range of splice variants, often without anchoring regions and can vary in structure more than FL-RAGE or sRAGE. FL-RAGE is anchored to the cell membrane and can signal through inflammatory pathways via the cytosolic tail region. FL-RAGE is the most common variant present in most cells [777].
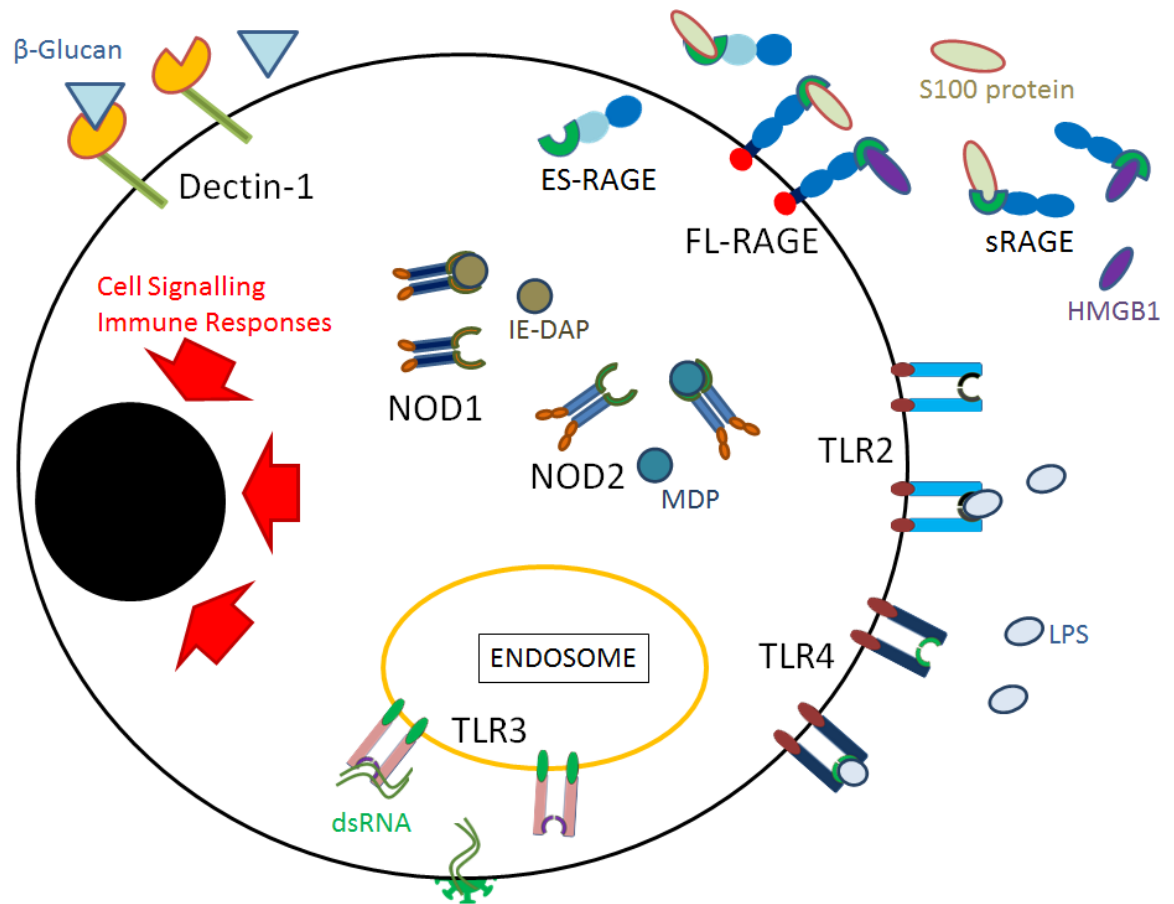
Figure 4.2: Distribution of selected pattern recognition receptors on and inside a cell coupled with their cognate ligands.

Receptors from the Toll-like receptors (TLRs), NOD proteins, Dectin-1 (a C-type Lectin receptor) and RAGE isoforms are represented (labels in black writing). Each can bind different ligands, a number are shown (coloured labels), including LPS, Beat-glucan, IE-DAP, MDP, double stranded RNA (dsRNA), HMBG1 and the S100 proteins. Once activated, they activate immune signalling events (such as MAPK signalling) which leads to gene transcription of proteins necessary for pro-inflammatory immune responses.

Table 4.1: Summary of the innate immune ligands (pathogen associated molecular patterns and damage associated molecular patterns) and cytokines selected for the study.

Ligand or Cytokine	Full Name	Receptor(s)	Summary Description	Refs
IFN-γ	Interferon Gamma	IFNGR - Interferon Gamma Receptor	Overexpressed in sarcoidosis granulomata. Th-1 like cytokine.	[12, 280, 281, 778]
TNF-α	Tumour Necrosis Factor Alpha	TNFRSF - TNF-α receptor superfamily	Pro-inflammatory Th1 like cytokine vital in sarcoidosis pathogenesis	[280, 281, 779]
PHA	Phytohaemagglutinin	TCR – T cell Receptor TLRs (Toll-like receptors) 2, 4, 5 and 6	Plant lectin – agglutiniating and leukocyte activating mitogen.	[697, 699]
MDP	Muramyl Dipeptide	NOD2	Component of peptidoglycan in bacterial cell walls in Gram positive and negative organisms. NOD 2 ligand that is ubiquitously expressed by bacteria.	[758, 780]
S100A12	Calcium-binding protein A12	RAGE - Receptor for advanced glycation end-products	Calcium binding protein, important in various regulatory and metabolic processes. A DAMP released in cell necrosis and stress; RAGE ligand. Present in higher levels in sarcoidosis granulomata.	[773, 781]
S100B	Calcium-binding protein B	RAGE CD166	Usually described as an astrocyte specific calcium binding protein (however, can be present in other cell types, such as macrophages). Released from cells damaged in various brain injury or disease states. Binds to RAGE and CD166.	[781-783]
S100A9	Calcium-binding protein A9	RAGE TLR4	Also known as MRP14, forms a heterodimer with S100A8 to activate TLR4 and RAGE inflammatory pathways. Implicated in TB, cancer and other inflammatory conditions as a damage indicator.	[781, 784, 785]
β-glucan	1,3 Beta-glucan	CLEC7A (Dectin-1) TLR2	Polysaccharide component of Zymosan. Present in various yeast and fungal cell walls, as well as plants and bacteria. Recognised by TLR2.	[758, 786, 787]
Poly I:C	Polyinosinic:polycytidylic acid	TLR3	Structurally similar to double stranded RNA and acts as a stimulant for TLR3. Only viruses have dsRNA.	[758, 788]
HMGB1	High-mobility Group Protein B1	RAGE TLR4 TLR2	High-mobility Group Protein B1. The 'classic' DAMP, a chromatin binding protein. Binds to RAGE and TLR4 to initiate inflammatory responses. Monocytes can be 'induced' to secrete HMGB1 in response to cytokines	[761, 789, 790]
IE-DAP	γ-D-Glu-mDAP	NOD1	Dipeptide present in peptidoglycan in all Gram negative and some Gram positive bacteria. Binds to intracellular NOD1 receptors.	[758, 780]
LPS	Lipopolysaccharide	TLR 4 Also Caspase 4/5/11 and TLR2	Endotoxin present on Gram negative bacterial cell membranes. Initiate a strong immune response through innate receptors such as TLR2 and TLR4.	[758, 791]

4.2 Methods

4.2.1 Sarcoidosis Relevant Cytokines and Innate Immune Receptor Ligands

A panel of potential cytokines and ligands were selected for use within whole blood assays and PBMC stimulations for flow cytometry (Table 4.2). A single final concentration was selected for each stimulant: 5 ng/ml for cytokines, 100 ng/ml for S100 proteins and 10 µg/ml for all other proteins. 3 µg of PHA, a sub-optimal dose, was included in the panel for reference and comparisons. These were concentrations consistent with those used in other reports, sufficient to elicit a response, not necessarily representative of actual *in vivo* concentrations in pathological conditions. All materials were reconstituted as per the manufacturer's instructions. Stock concentrations were created and stored at -20°C, working concentrations were aliquoted and stored at -20°C until required.

Table 4.2: Ligands, cytokines and other stimulant stock concentrations and dilutions.
Ratios represent dilution required from stock concentration, 1:10, 1:100, 1:200 or no dilution.

Stimulant	Stock Concentration	Final Concentration	Dilution in 100µl	Dilution in 1ml
IFN-γ	10µg/ml	5ng/ml	5µl – 1:100	5µl – 1:10
TNF-α	10µg/ml	5ng/ml	5µl – 1:100	5µl – 1:10
PHA	1mg/ml	3µg/ml	3µl – 1:10	3µl – Stock
MDP	1mg/ml	10µg/ml	10µl – 1:10	10µl – Stock
S100A12	100µg/ml	100ng/ml	10µl – 1:100	10µl – 1:10
S100B	100µg/ml	100ng/ml	10µl – 1:100	10µl – 1:10
S100A9	100µg/ml	100ng/ml	10µl – 1:100	10µl – 1:10
Beta-glucan	20mg/ml	10µg/ml	10µl – 1:200	10µl – 1:20
Poly I:C	10mg/ml	10µg/ml	10µl – 1:100	10µl – 1:10
HMGB1	1mg/ml	10µg/ml	10µl – 1:10	10µl – Stock
IE-DAP	10mg/ml	10µg/ml	10µl – 1:100	10µl – 1:10
LPS	1mg/ml	10µg/ml	10µl – 1:10	10µl – Stock

4.2.2 Stimulation of Whole Blood with Innate Immune Ligands and Cytokines

Working concentrations of the panel of ligands and cytokines were made to achieve a final concentration necessary for whole blood assays (Table 4.1). 10.1 µl of each stimulant dilution were added to appropriate labelled 2ml skirted round bottom centrifuge tubes. A control tube containing 10.1 µl of sterile PBS was included in all experiments, hereafter termed 'non-stimulated'. Whole blood was collected from healthy controls and patients with sarcoidosis as previously described (Sections 2.1 and 2.7); for each tube 1ml of blood was added and the samples placed in a 37°C incubator for 16 hours. After incubation, blood was centrifuged and plasma isolated as previously described (Sections 2.12). Samples were stored at -20°C in labelled 1.5ml centrifuge tubes until required.

4.2.3 ELISA Analysis of Blood Plasma for the Cytokines IFN-γ, TNF-α, IL-10, IL-6, IL-8, IL-4 and IL-12(p70)

Plasma obtained from whole blood stimulated with cytokines and ligands was thawed on ice until completely liquid. The basic procedure for the ELISA was followed, as summarised previously (Section 2.13). Samples were run in duplicate, including the recombinant protein standards. Dilutions of the plasma would be required, depending on the stimulant, donor type and the type of cytokine being detected. This was optimised in earlier experiments, though the majority were diluted 1:2 for most stimuli and cytokines being detected. Stimulants such as LPS would require a dilution factor of between 1:100 – 1:5000; IL-6 and IL-8 ELISAs would also require higher dilution factors for even baseline readings (1:10 – 1:100). Data outside of the readable range would be repeated with a different dilution factor. The minimum detectable concentrations for each ELISA were as follows: IFN-γ = 15.6pg/ml, IL-10 = 7.8pg/ml, TNF-α = 15.6pg/ml, IL-6 = 15.6pg/ml, IL-8 = 31.2pg/ml, IL-4 = 7.8pg/ml and IL-12(p70) = 31.2pg/ml.

4.2.4 Extracellular staining and flow cytometry analysis of PBMCs for RAGE

The RAGE antibody was a mouse monoclonal IgG1κ (Section 2.3.4) recognising N-terminal sequences of RAGE, ES-RAGE and ENRAGE isoforms. A matched mouse monoclonal IgG1κ

was used as a control. Both were unconjugated antibodies, thus they required a secondary antibody with a fluorescent FITC tag (Section 2.3.4); both primary antibodies were used at 10 µg/ml and the secondary antibody used at 5 µg/ml. The procedure for extracellular staining of PBMCs with primary and secondary antibodies was followed as previously described (Section 2.9). As a secondary antibody recognising mouse antibodies was used, any single conjugated antibodies used in unison for immunophenotyping (mouse anti-CD3 APC for example) were added after the incubation with secondary antibody to prevent binding of the secondary to these non-RAGE antibodies. Samples were analysed as previously described (Sections 2.9 and 2.11).

4.2.5 Intracellular and staining and flow cytometry analysis of PBMCs for RAGE and NOD2 receptors in response to the ligand stimulation

PBMCs were isolated from whole blood as described previously (Section 2.7) and seeded in a 96 well plate at 2×10^6 cells/ml. These were stimulated with appropriate ligands/cytokines for 6 hours at the concentrations previously described in Table 4.2. A modified form of the intracellular staining procedure (Section 2.10) was performed on cells after the end of the incubation. Owing to the use of mouse anti-human primary antibodies for NOD2 and RAGE with a FITC conjugated anti-mouse secondary antibody, staining for CD3 would have to occur after the secondary antibody step to prevent the secondary antibody binding to the mouse anti-human CD3. Previous attempts to use an anti-CD14 antibody for monocytes showed that this antibody was not suitable to differentiate cells once permeabilised, therefore monocytes were defined based on forward and side scatter characteristics.

In summary, cells were washed with FACs blocking solution post-incubation, then fixed with paraformaldehyde and permeabilised with 90% methanol as previously described (Section 2.10). There was then a 1 hour incubation for blocking with FACs blocking solution, followed by addition of 10 µg/ml primary antibody (anti-NOD2, anti-RAGE or isotype controls) for 1 hour on ice. Cells were then washed with FACs blocking solution, followed by 1 hour incubation with 5 µg/ml of the FITC secondary antibody. After washing, cells were incubated for 1 hour with 20 µg/ml of mouse IgG solution to block

any binding sites present on antibodies currently present on the cells (on primary antibodies). Cells were washed again, followed by staining with the anti-CD3 antibody for 1 hour, followed by washing and preparation as previously outlined (Section 2.9).

Data was interpreted using the standard procedure outlined in Section 2.11**.** Forward and side scatter voltages were adjusted to compensate for fixed cells, which were typically smaller. Voltages were also adjusted as appropriate for fluorescent channels, as background fluorescence was higher in intracellular flow cytometry. Gating for CD3 was completed on 1% of the isotype of lymphocyte gated PBMCs, as it was for NOD2 and RAGE on lymphocytes. Lymphocyte expression was calculated using the geometric mean of the NOD2 or RAGE antibody minus the geometric mean of the isotype control for each treatment. The change in expression was then calculated using these values.

Monocyte isotype control fluorescent signals were very high, despite attempts to reduce this non-specific signal. The geometric mean of populations in RAGE monocyte staining were based on gated positive signals set at 10% of the isotype control for non-stimulated cells, as peaks were skewed in a non-Gaussian distribution or bi-modal in histogram analyses and could not be taken in total or easily assessed for positive and negative populations. Data was then processed as the change in geometric mean from the non-stimulated population to ensure consistency. NOD2 on monocytes established the expression of non-stimulated cells using the geometric mean of the NOD2 antibody minus the elevated isotype control staining. All other monocyte NOD2 data was presented as the change in expression, which was taken using unprocessed geometric mean values for non-stimulated and stimulated samples. As NOD2 histogram peaks were unimodal, this presented the most accurate way of assessing expression. Data from these were n = 3 for NOD2 and RAGE, therefore statistical significance was undertaken using a Student's T-test, though it is acknowledged that with such small numbers of samples, any significance would be considered subject to consideration of this limitation. All statistical data was calculated using Graphpad Prism 7.

4.3 Results

4.3.1 Whole blood cytokine release after stimulation with DAMPs, PAMPs and cytokines

As previously observed for PHA and SEA stimulation of whole blood for 16 hours, IL-12(p70) was not detectable in any sample stimulated with any of the ligands or cytokines. Whether this is the result of the minimum threshold of detectability being too high, at approximately 30 pg/ml, the cytokine not being released into the plasma or being transiently released has not been established. Nor was there any detectable IL-4 in plasma after 16 hours with any stimuli. No data will be presented for these cytokines, as they do not display any positive levels for n = 5 in healthy controls and patients with sarcoidosis.

4.3.1.1 Untreated Whole Blood

Cytokine release from whole blood untreated with stimuli, using PBS, was quantified for healthy controls and patients with sarcoidosis to determine whether there was a baseline concentration present after the 16 hours (Figure 4.3). There was no statistically significant difference in IFN-γ, IL-10, TNF-α or IL-6 concentrations between healthy controls and patients with sarcoidosis. The majority of healthy controls displayed no detectable levels of IFN-γ, only 1 individual had approximately 50 pg/ml present. By contrast, 50% of patients displayed some detectable IFN-γ. IL-10 release also showed that patients with sarcoidosis tended to have higher IL-10 release than controls, with a median concentration of 8.68 pg/ml (IQR 32.00) compared with 0.572 pg/ml (IQR 8.425) respectively. TNF-α release was minimal in most individuals, with 3 out of 7 healthy controls and 2 out of 5 patients presenting with some degree of TNF-α, though the median release was 0 pg/ml. Unlike other cytokines, there was typically IL-6 present in samples for all individuals, though there was quite a bit of variation between individuals in each cohort, with a median release of 136.2 pg/ml (IQR 2353.1) in patients with sarcoidosis and 196.6 pg/ml (IQR 711.01) in healthy controls.

Given the variation in release of untreated samples from individuals, all subsequent work with whole blood stimulations were calculated and are presented as a change in cytokine release from non-stimulated. This would ensure that the actual influence of a stimuli could be assessed, though this could be either positive or negative depending on whether an individual sample from a donor displayed cytokine levels in untreated samples.
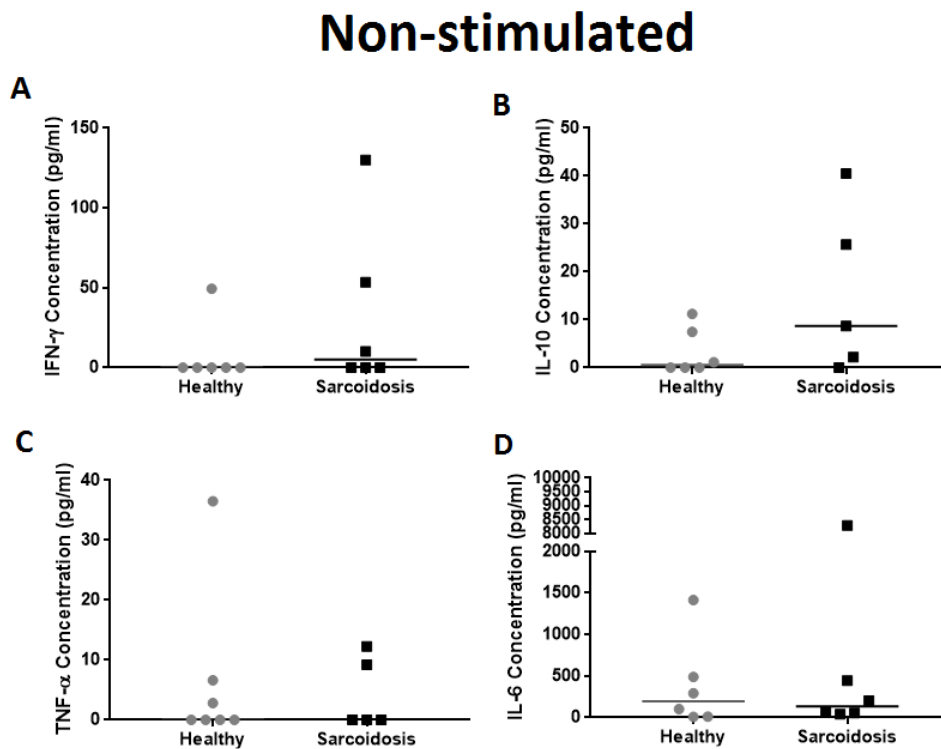
Figure 4.3: Cytokine release from non-stimulated whole blood in patients with sarcoidosis and healthy controls.

Whole blood, treated with only PBS added as a control, after 16 hours incubation was analysed for cytokine release by ELISA. Healthy controls (grey circles) and patients with sarcoidosis (black squares) represented with median (grey or black line respectively). **A:** IFN-γ release (p = 0.303). **B:** IL-10 release (p = 0.191). **C:** TNF-α release (p > 0.999). **D:** IL-6 release (p = 0.937). Statistical significance stated relates to the difference between healthy controls and patients with sarcoidosis, determined using the Mann-Whitney U test; minimum n = 5, maximum n = 7.

4.3.1.2 Th1-like cytokine Stimulation

Sarcoidosis is characterised by the release of increased levels Th-1 cytokines from leukocytes in tissue, including TNF-α and IFN-γ [12, 280, 281]. TNF-α is of particular

importance in sarcoidosis and has been shown to be released more readily in response to PHA from blood leukocytes in the disease (Section 3.3.8). Whilst IFN-γ did not show this same response in whole blood, this cytokine is important in the pathogenesis of sarcoidosis and was not proportionally diminished in whole blood with the reduced T-lymphocyte count (Sections 3.3.3 and 3.3.8). To identify whether the application of these cytokines to a whole blood assay would stimulate a different response between healthy controls and patients with sarcoidosis, recombinant TNF-α or IFN-γ were added to whole blood in the absence of any additional external stimuli. For both cytokines, the plasma TNF-α and IFN-γ concentrations, as well as IL-10 and IL-6 release, were determined after 16 hours of incubation. Addition of TNF-α and IFN-γ and subsequent analysis of plasma for the same cytokines was to identify whether leukocytes were utilising the TNF-α and IFN-γ added to blood and whether this would lead to autologous release of the cytokines.

As shown in Figure 4.4, there was no statistically significant difference in cytokine release in response to the addition of either IFN-γ or TNF-α. Despite adding 5ng/ml of these stimuli externally (equating to 5000 pg/ml), after 16 hours of incubation with whole blood there was no significant change in the detectable levels of TNF-α or IFN-γ in the majority of samples, with some even displaying reduced levels of these secreted cytokines. This would suggest that the added TNF-α and IFN-γ have been used/internalised/degraded, otherwise there would be an increase of approximately 5000 pg/ml in the samples equivalent to the amount added. This does not translate to increased IL-10 levels, as the majority of controls and patients showed a minimal decrease in this cytokine. Although IFN-γ stimulation typically lead to either increased or decreased IL-6 release from whole blood depending on the individual, the addition of TNF-α lead to increased IL-6 release from 4 out of 5 healthy controls, yet half of the population of patients displayed an increased IL-6 release and half showed a reduction in IL-6 (Figure 4.4 C). There was significant variation between patients however, as 1 patient displayed a vast reduction IL-6 release (7645 pg/ml), whereas the other 2 were only marginally reduced (less than -20 pg/ml). It should be noted that the same patient also displayed a significantly reduced IL-6 release to IFN-γ also, suggesting that this was not specific to TNF-α. Overall, the data indicates that responses to these Th1-like cytokines are not enhanced from whole blood leukocytes in patients with sarcoidosis compared with controls.

TNF and IFN
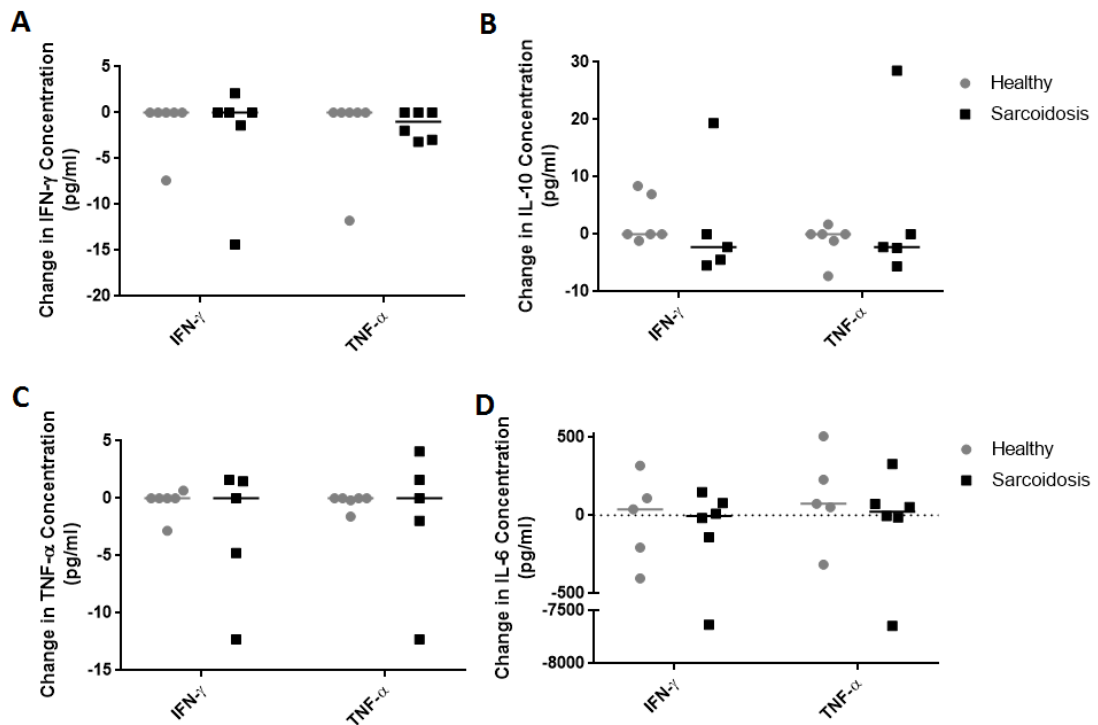
Figure 4.4: Change in cytokine release from whole blood stimulated with IFN-γ or TNF-α in patients with sarcoidosis and healthy controls.

Whole blood was analysed for cytokine release by ELISA after stimulation with 5 ng/ml of IFN-γ or TNF-α (as labelled on the X axis). Incubation was for 16 hours. Healthy controls (grey circles) and patients with sarcoidosis (black squares) represented with median (grey or black line respectively). **A:** Change in IFN-γ release. **B:** Change in IL-10 release. **C:** Change in TNF-α release. **D:** Change in IL-6 release. No statistical significance between controls and patients for any of the cytokines released for both TNF-α and IFN-γ stimulation. Significance determined using the Mann-Whitney U test; minimum n = 5, maximum n = 6.

4.3.1.3 Bacterial PAMPs – LPS, MDP and IE-DAP

Bacterial and mycobacterial aetiologies for sarcoidosis have remained amongst the favourite possibilities for a number of researchers [462, 471, 487, 493], therefore examining PAMPs from bacterial sources was important to establish whether there was greater immune sensitivity to these stimuli from patients with sarcoidosis. For this, lipopolysaccharide (LPS), muramyl dipeptide (MDP) and IE-DAP (γ-D-Glu-mDAP/D-

glutamyl-meso-diaminopimelic acid) were used to activate receptors TLR4, NOD2 and NOD1 respectively.

Figure 4.5: Change in cytokine release from whole blood stimulated with LPS in patients with sarcoidosis and healthy controls.

Whole blood was analysed for cytokine release by ELISA after stimulation with 10 µg/ml of LPS. Incubation was for 16 hours. Healthy controls (grey circles) and patients with sarcoidosis (black squares) represented with median (grey or black line respectively). **A:** Change in IFN-γ release (p = 0.247). **B:** Change in IL-10 release (p > 0.999). **C:** Change in TNF-α release (p = 0.662). **D:** Change in IL-6 release (p = 0.093). Statistical significance stated relates to the difference between healthy controls and patients with sarcoidosis, determined using the Mann-Whitney U test; minimum n = 5, maximum n = 6.

LPS is a potent stimulator of immune responses, inducing a much higher concentration of cytokine release than other ligands and cytokines used in this panel of stimuli (Figure 4.5). Though none of the cytokines analysed displayed a statistically significant difference in release between patients with sarcoidosis and controls, there were trends worth noting. 4 out of 5 patients with sarcoidosis had a change in IFN-γ well below the median release observed in healthy controls when stimulated with LPS; median change in IFN-γ of 2642

pg/ml (IQR 10730) compared with 7278 pg/ml (IQR 5048). There was no significant difference in IL-10 release between the 2 cohorts; median of 2693 pg/ml (IQR 2328) for patients with sarcoidosis compared with 2520 (IQR 883) in controls. Nor was TNF-α significantly increased or decreased to a statistically significant degree in patients, despite a reduced median of release; median of 6413 pg/ml (IQR 8817) for patients with sarcoidosis compared with 8118 (IQR 8249) in healthy controls. However, the median change in IL-6 release of healthy controls (264567 pg/ml, IQR 199349) was higher than in patients (149337 pg/ml, IQR 136404). Taking these together, there is an indication that the immune response to LPS may not be as robust in a proportion of patients when compared with that of healthy controls.

A similar trend was also observed for MDP stimulation of whole blood (Figure 4.6). Unlike LPS, MDP did not induce a significant increase in IFN-γ; patients with sarcoidosis actually displayed a reduced median release of IFN-γ (-4.07 pg/ml, IQR 11.43) from unstimulated samples. This was specific to IFN-γ however, as all other cytokines tested displayed increased levels after stimulation with MDP. Again, whilst not statistically significant, the trends observed were interesting. The change in IL-10 was greater in patients with sarcoidosis after MDP treatment; median 170.9 pg/ml (IQR 219.7) compared with 69.72 (IQR 88.93) in healthy controls. The change in TNF-α was not significantly different between the two cohorts, but the change in IL-6 concentration was below the bottom range of healthy controls (7349pg/ml) in 4 out of 6 patients with sarcoidosis. Healthy controls appeared to be capable of a greater inflammatory response when challenged with MDP in whole blood stimulations.

Much like with MDP, IE-DAP did not induce significant IFN-γ release from whole blood; though unlike MDP, there was also not a significant pro-inflammatory or anti-inflammatory cytokine response observed (**Figure 4.7**). 3 out of 5 patients with sarcoidosis displayed an increase in IL-10 release, compared with 2 out of 6 healthy controls. Aside from 1 healthy control and 1 patient, IE-DAP did not significantly increase TNF-α release from whole blood. When looking at the change in IL-6 release, 4 out of 5 healthy controls saw an increase in cytokine concentrations upon stimulation with IE-DAP, whereas only 3 out of 5 patients had increased IL-6, though the difference was not significant. This suggested that NOD1 and NOD2 responses were very different in profile,

it appeared that MDP stimulation was typically more potent than IE-DAP using comparable concentrations.

MDP

Figure 4.6: Change in cytokine release from whole blood stimulated with MDP in patients with sarcoidosis and healthy controls.

Whole blood was analysed for cytokine release by ELISA after stimulation with 10 µg/ml of MDP. Incubation was for 16 hours. Healthy controls (grey circles) and patients with sarcoidosis (black squares) represented with median (grey or black line respectively). **A:** Change in IFN-γ release (p = 0.333). **B:** Change in IL-10 release (p = 0.247). **C:** Change in TNF-α release (p = 0.792). **D:** Change in IL-6 release (p = 0.180). Statistical significance stated relates to the difference between healthy controls and patients with sarcoidosis, determined using the Mann-Whitney U test; minimum n = 5, maximum n = 6.

IE-DAP
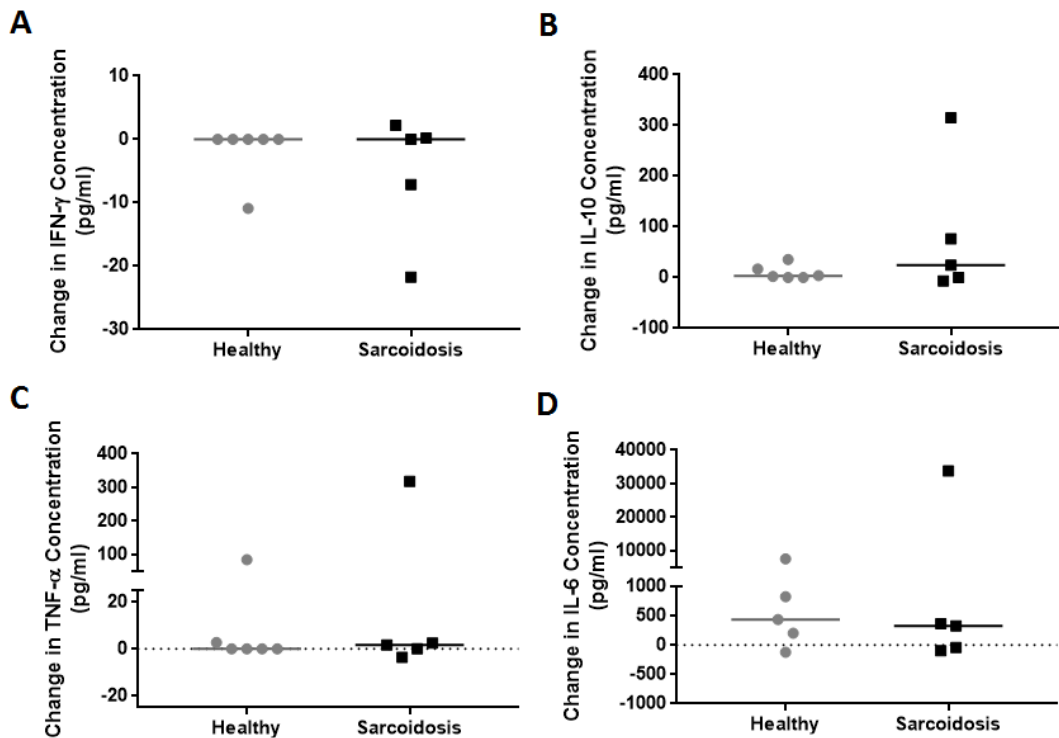
Figure 4.7: Change in cytokine release from whole blood stimulated with IE-DAP in patients with sarcoidosis and healthy controls.

Whole blood was analysed for cytokine release by ELISA after stimulation with 10 µg/ml of IE-DAP. Incubation was for 16 hours. Healthy controls (grey circles) and patients with sarcoidosis (black squares) represented with median (grey or black line respectively). **A:** Change in IFN-γ release (p = 0.879). **B:** Change in IL-10 release (p = 0.656). **C:** Change in TNF-α release (p = 0.924). **D:** Change in IL-6 release (p = 0.841). Statistical significance stated relates to the difference between healthy controls and patients with sarcoidosis, determined using the Mann-Whitney U test; minimum n = 5, maximum n = 6.

4.3.1.4 Other PAMPs – Poly I:C and Beta-glucan

The role of viruses in the development of sarcoidosis has been investigated but is often limited and features contradictory findings [490, 491]. Fungal causes have also been examined [492], again with limited focus in comparison to bacterial and environmental causes. Poly I:C was selected as a model to test TLR3 stimulation (Figure 4.8), whereas Beta-Glucan from yeast (β-glucan) was used as a fungal TLR2 agonist (Figure 4.9).

Poly I:C stimulation did not lead to significant increases in IFN-γ release from whole blood; 1 patient displayed a reduction in the cytokine of approximately 37 pg/ml. Whilst the majority of healthy controls (5 out of 6) also did not show significant increases in IL-10 in response to this stimuli, 3 out of 5 patients with sarcoidosis displayed an increase of over 100 pg/ml of IL-10. With the exception of a couple of outliers, TNF-α release was not significantly increased in whole blood with the application of this stimuli and there was not a statistically significant difference in the change in IL-6 release in response to Poly I:C, despite patients displaying an increased median value of 2889 pg/ml (IQR 10157) compared with 843.7 pg/ml (IQR 4557.5) in healthy controls.

The fungal β-glucan stimulant showed similar results to Poly I:C stimulation in terms of IFN-γ release, with no significant increases present in either cohort, and the change in IL-10 release was higher in 3 out of 5 patients with sarcoidosis compared with controls. Surprisingly, the same three patients with elevated IL-10 responses to Poly I:C were also the same three patients with elevated IL-10 responses to β-glucan (data not shown). The TNF-α response was greater for β-glucan than Poly I:C, though there were not obvious significant differences between release in patients and controls aside from a slightly reduced median value in the former, 5.66 pg/ml (IQR 31.26) compared to 9,49 pg/ml (IQR 49.33) respectively. IL-6 release in response to the stimuli was lower in the majority of patients with sarcoidosis, 4 out of 6 were approximately at the bottom end of the range (3199pg/ml) present in healthy controls. The median value of 3241 pg/ml (IQR 8042) in patients compared with 7969 pg/ml (IQR 9239) in controls highlights this feature. It should be noted, that whilst two thirds of patients also displayed reduced MDP IL-6 release, these were not comprised of the exact same patients. The data for both Poly I:C and β-glucan displays reductions in IL-6 response in a proportion of patients with sarcoidosis, but no significant alterations in TNF-α. The variation in IL-10 response is also worthy of note.
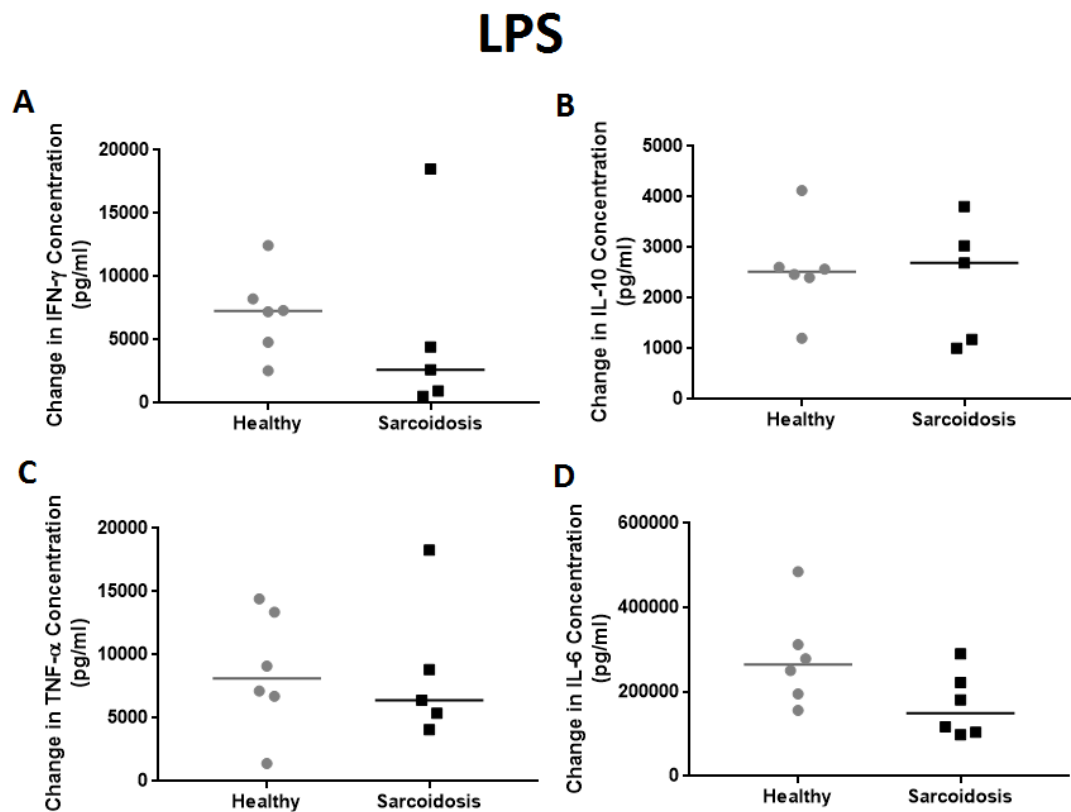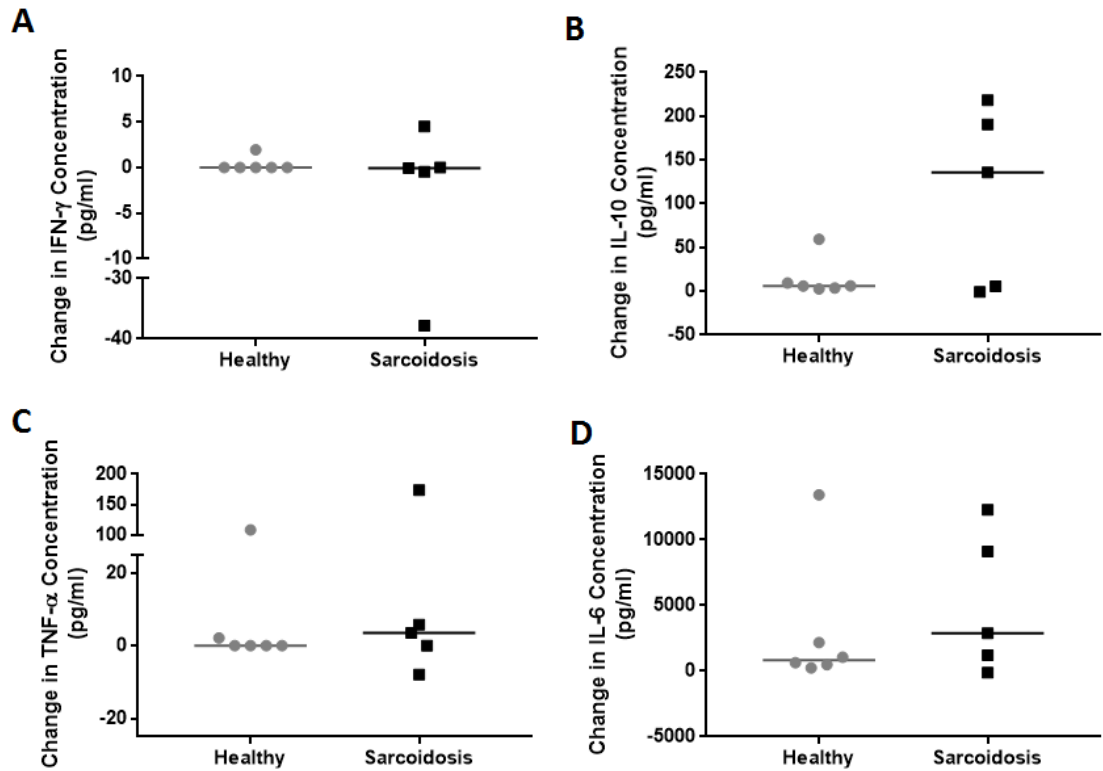
Figure 4.8: Change in cytokine release from whole blood stimulated with Poly I:C in patients with sarcoidosis and healthy controls.

Whole blood was analysed for cytokine release by ELISA after stimulation with 10 µg/ml of Poly I:C. Incubation was for 16 hours. Healthy controls (grey circles) and patients with sarcoidosis (black squares) represented with median (grey or black line respectively). **A:** Change in IFN-γ release (p = 0.253). **B:** Change in IL-10 release (p = 0.429). **C:** Change in TNF-α release (p = 0.654). **D:** Change in IL-6 release (p = 0.537). Statistical significance stated relates to the difference between healthy controls and patients with sarcoidosis, determined using the Mann-Whitney U test; minimum n = 5, maximum n = 6.

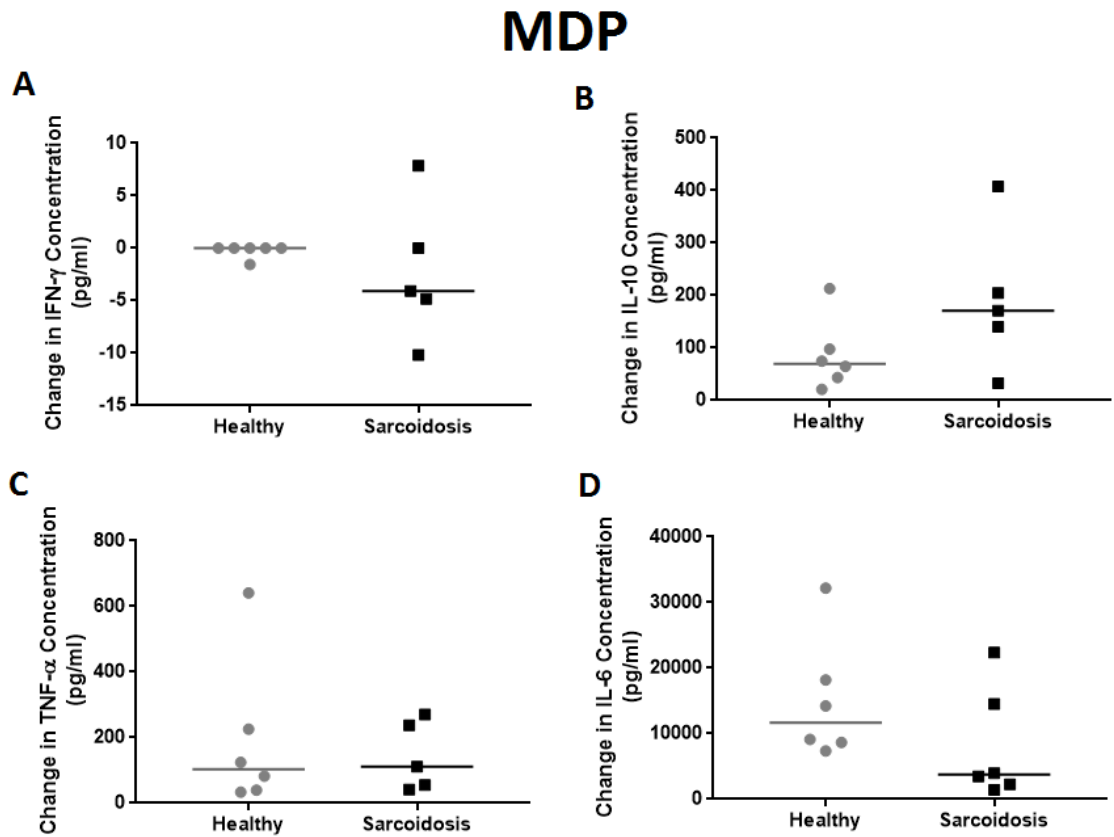# Beta-Glucan
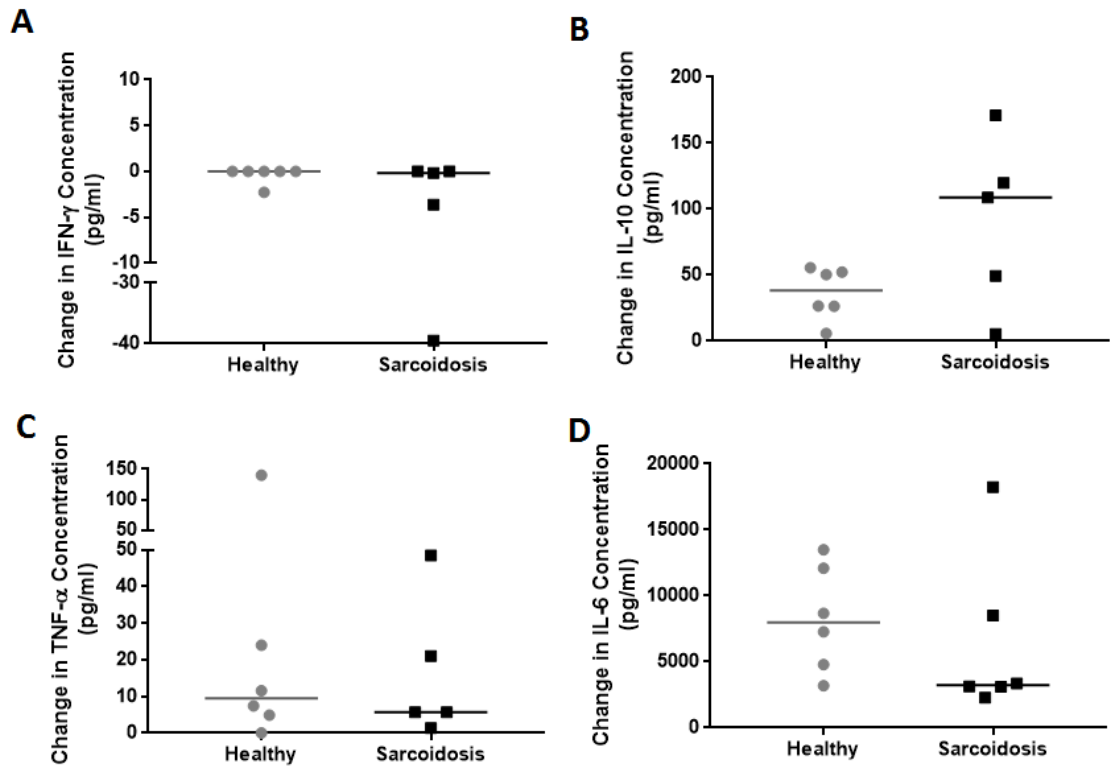
Figure 4.9: Change in cytokine release from whole blood stimulated with Beta Glucan (β-glucan) in patients with sarcoidosis and healthy controls.

Whole blood was analysed for cytokine release by ELISA after stimulation with 10 μg/ml of β-glucan. Incubation was for 16 hours. Healthy controls (grey circles) and patients with sarcoidosis (black squares) represented with median (grey or black line respectively). **A:** Change in IFN-γ release (p = 0.152). **B:** Change in IL-10 release (p = 0.329). **C:** Change in TNF-α release (p = 0.894). **D:** Change in IL-6 release (p = 0.240). Statistical significance stated relates to the difference between healthy controls and patients with sarcoidosis, determined using the Mann-Whitney U test; minimum n = 5, maximum n = 6.

4.3.1.5 DAMPs – The RAGE ligands

The receptor for advanced glycation end-products (RAGE) can bind a range of ligands, including members of the S100 protein family and HMGB1 [768-770]. A selection of RAGE ligands were used in the whole blood assay, including HMGB1 (Figure 4.10), S100B (Figure 4.11), S100A9 (Figure 4.12) and S100A12 (Figure 4.13).

In summary, there was no statistically significant difference in cytokine release between patients with sarcoidosis and healthy controls for any of the tested DAMPs with any of the cytokines analysed. IFN-γ release was not significantly altered for the majority of healthy controls in response to any of the DAMPs. In contrast, patients with sarcoidosis tended to display a small fall in the concentration of IFN-γ released, though the most prominent of these was still less than a 30 pg/ml change observed for HMGB1 (Figure 4.10). Overall, the data suggests that IFN-γ release is not significantly altered in whole blood after the 16 hour stimulation. Neither was TNF-α induced by these DAMPs in the majority of donors (Figures 4.10 – 4.13); there was no significant difference between patients with sarcoidosis and healthy controls when assessing this cytokine. IL-10 displayed some differences, though these tended to be minor in scale. HMGB1 did not induce a large increase in IL-10 in most patients or controls; modest rises were observed in 2 patients (approximately 40 pg/ml and 80 mg/ml) and 2 healthy controls (approximately 20 pg/ml and 60pg/ml). Similar low level increases were observed in patients in response to S100B, S100A9 and S100A12. This should be put in some context, as the changes were all below 100 pg/ml at the highest, whereas MDP was able to induce over that within patients, as was β-glucan stimulation. HMGB1 induced a large change in IL-6 from only 1 patient and 1 control, the majority of individuals displayed limited increases in this cytokine (Figure 4.10). A similar trend was also observed for all the S100 proteins tested (Figures 4.11 – 4.13). Overall, in isolation, these stimuli appeared to have a limited impact on cytokine release from whole blood after 16 hours. Whether this is a feature relating to the time used in these experiments, the system of whole blood employed or whether they require additional stimulants to be used in unison was not determined in these experiments.

HMGB1
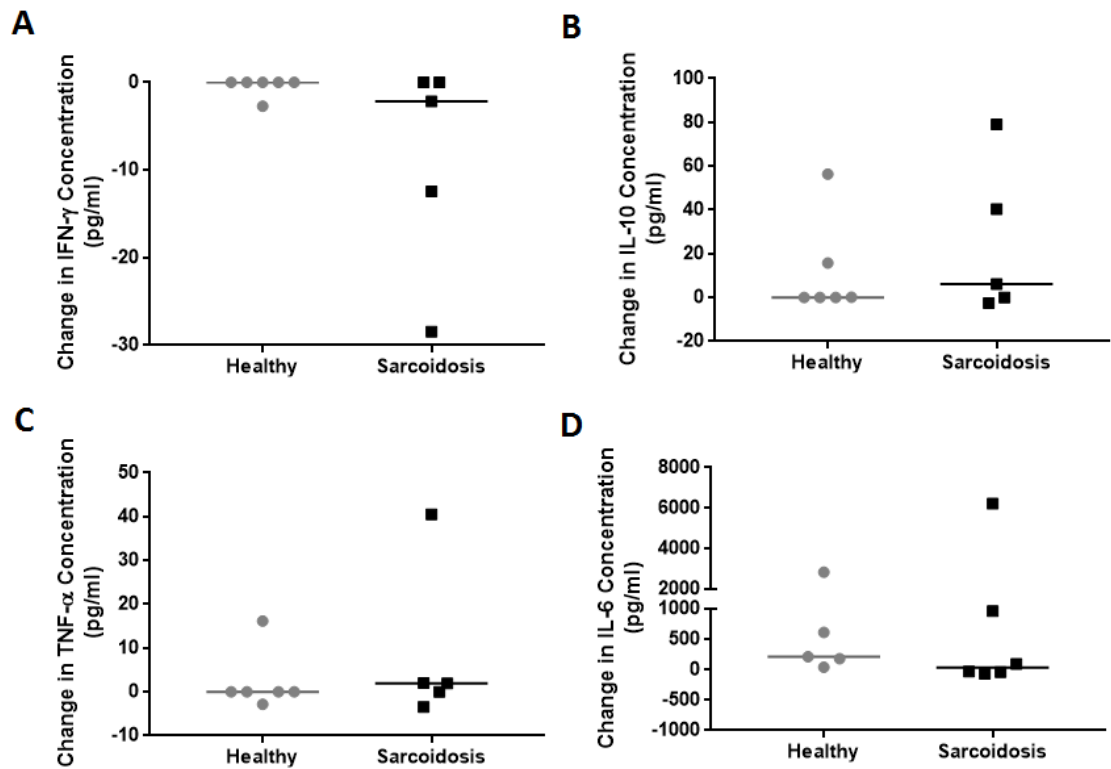
Figure 4.10: Change in cytokine release from whole blood stimulated with HMGB1 in patients with sarcoidosis and healthy controls.

Whole blood was analysed for cytokine release by ELISA after stimulation with 10 µg/ml of HMGB1. Incubation was for 16 hours. Healthy controls (grey circles) and patients with sarcoidosis (black squares) represented with median (grey or black line respectively). **A:** Change in IFN-γ release (p = 0.152). **B:** Change in IL-10 release (p = 0.829). **C:** Change in TNF-α release (p = 0.526). **D:** Change in IL-6 release (p = 0.429). Statistical significance stated relates to the difference between healthy controls and patients with sarcoidosis, determined using the Mann-Whitney U test; minimum n = 5, maximum n = 6.

S100B
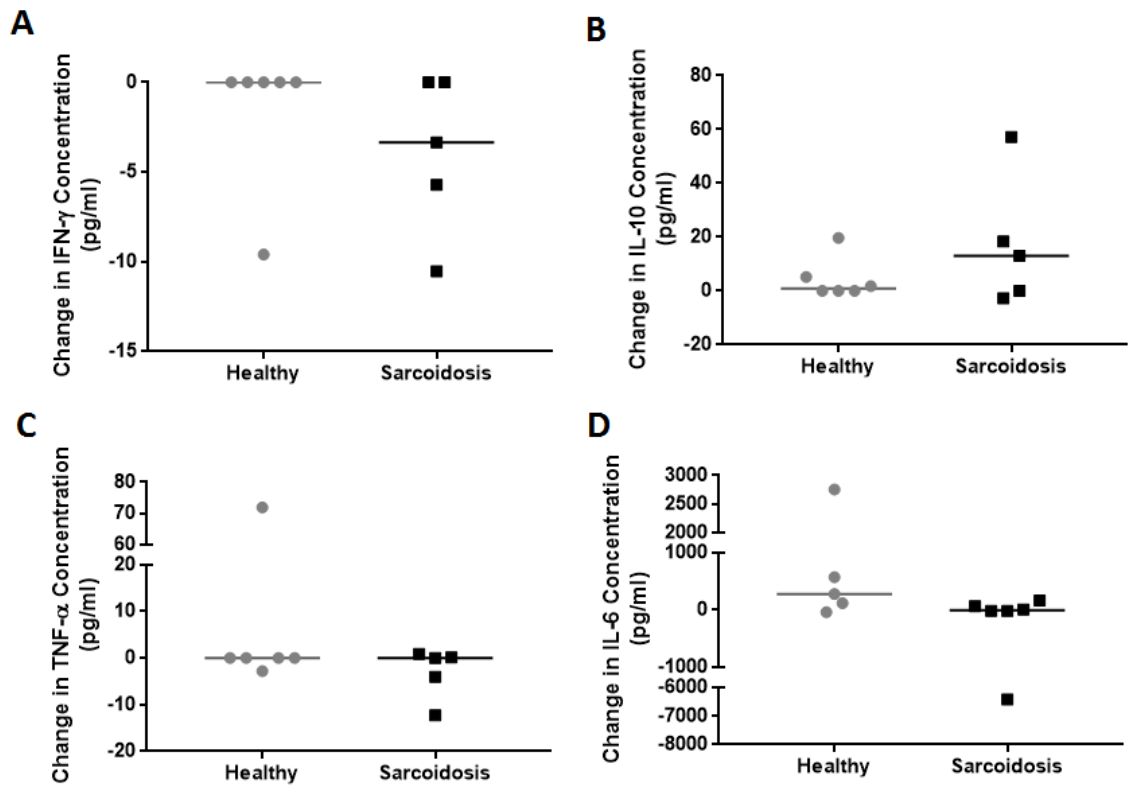
Figure 4.11: Change in cytokine release from whole blood stimulated with S100B in patients with sarcoidosis and healthy controls.

Whole blood was analysed for cytokine release by ELISA after stimulation with 100 ng/ml of S100B. Incubation was for 16 hours. Healthy controls (grey circles) and patients with sarcoidosis (black squares) represented with median (grey or black line respectively). **A:** Change in IFN-γ release (p = 0.273). **B:** Change in IL-10 release (p = 0.684). **C:** Change in TNF-α release (p = 0.771). **D:** Change in IL-6 release (p = 0.126). Statistical significance stated relates to the difference between healthy controls and patients with sarcoidosis, determined using the Mann-Whitney U test; minimum n = 5, maximum n = 6.

S100A9
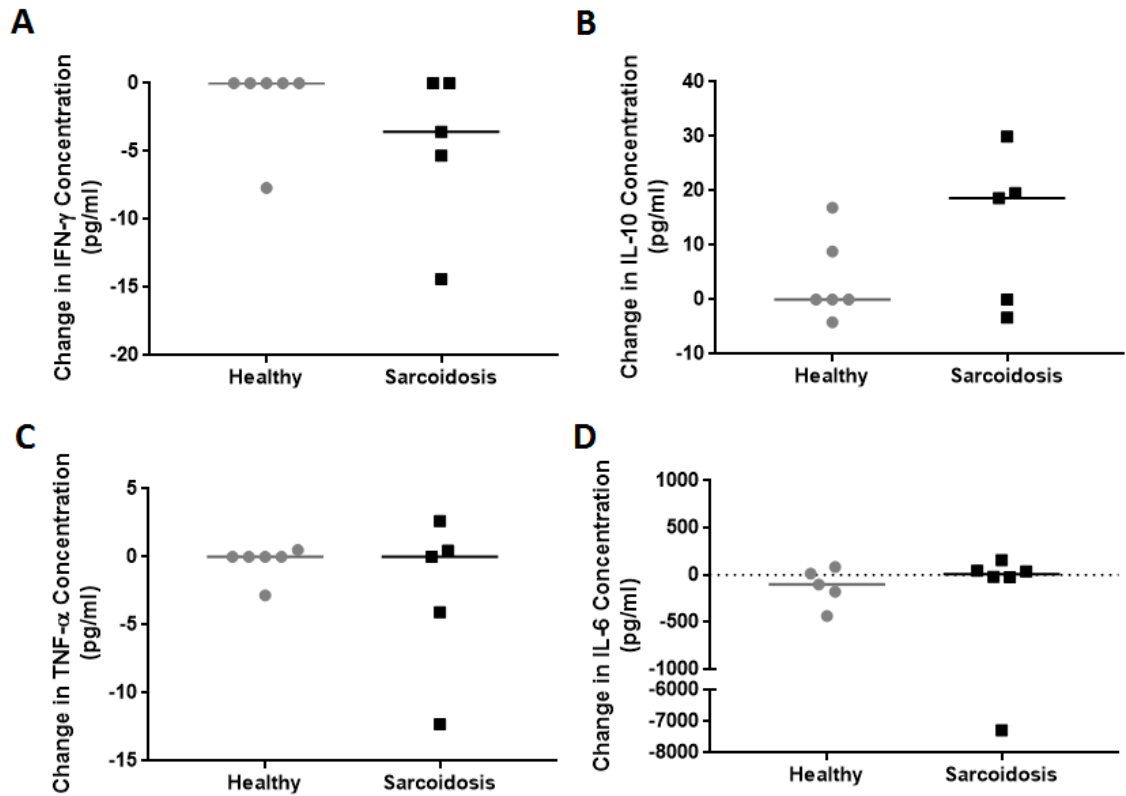
Figure 4.12: Change in cytokine release from whole blood stimulated with S100A9 in patients with sarcoidosis and healthy controls.

Whole blood was analysed for cytokine release by ELISA after stimulation with 100 ng/ml of S100A9. Incubation was for 16 hours. Healthy controls (grey circles) and patients with sarcoidosis (black squares) represented with median (grey or black line respectively). **A:** Change in IFN-γ release (p = 0.273). **B:** Change in IL-10 release (p = 0.260). **C:** Change in TNF-α release (p = 0.900). **D:** Change in IL-6 release (p = 0.537). Statistical significance stated relates to the difference between healthy controls and patients with sarcoidosis, determined using the Mann-Whitney U test; minimum n = 5, maximum n = 6.

S100A12
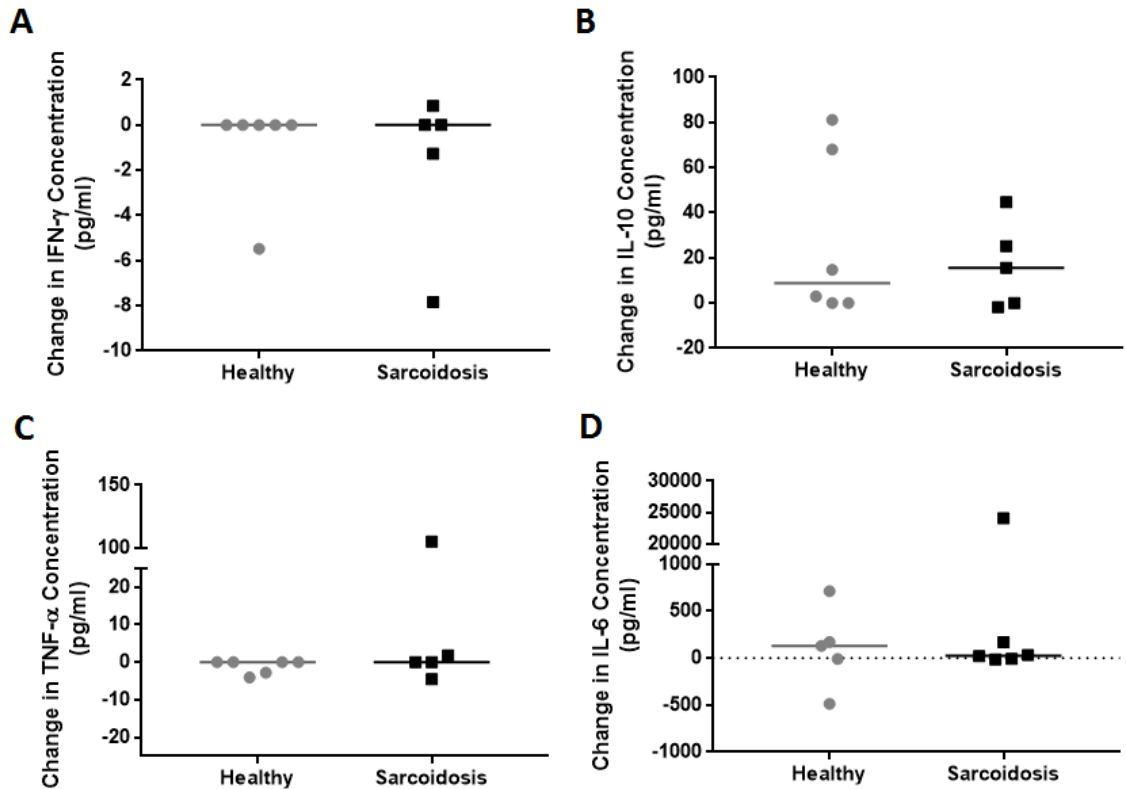
Figure 4.13: Change in cytokine release from whole blood stimulated with S100A12 in patients with sarcoidosis and healthy controls.

Whole blood was analysed for cytokine release by ELISA after stimulation with 100 ng/ml of S100A12. Incubation was for 16 hours. Healthy controls (grey circles) and patients with sarcoidosis (black squares) represented with median (grey or black line respectively). **A:** Change in IFN-γ release (p = 0.879). **B:** Change in IL-10 release (p = 0.794). **C:** Change in TNF-α release (p = 0.238). **D:** Change in IL-6 release (p = 0.931). Statistical significance stated relates to the difference between healthy controls and patients with sarcoidosis, determined using the Mann-Whitney U test; minimum n = 5, maximum n = 6.

4.3.2 Cell Surface and intracellular expression of RAGE in PBMCs

The RAGE ligands had not induced a significant response from whole blood assays, though this was a multi-celled system with a number of confounding elements. To identify whether RAGE was differentially expressed on leukocyte cell surfaces in sarcoidosis, the receptor was stained on PBMCs and assessed using flow cytometry. The antibody selected for detection of RAGE was capable of detecting all forms of the receptor, FL-RAGE, ES-RAGE and sRAGE, therefore it was not specific for the full length functional form (FL-RAGE).
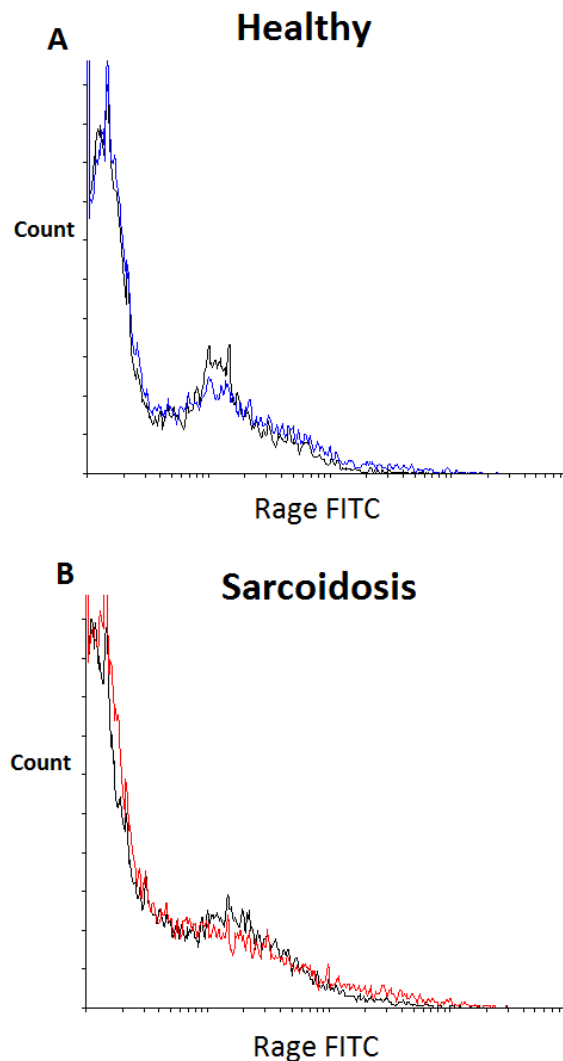
Figure 4.14: Representative histograms of extracellular staining of PBMCs for RAGE.

The RAGE receptor was stained using a primary unconjugated mouse anti-human antibody, followed by an anti-mouse FITC secondary antibody. Whole PBMCs represented for 10,000 events. **A:** Healthy control PBMCs stained with an isotype control antibody (black line, geomean 2.98) and the RAGE antibody (blue line, geomean 3.21). **B:** Sarcoidosis patient PBMCs stained with an isotype control antibody (black line, geomean 3.16) and the RAGE antibody (red line, geomean 3.53).
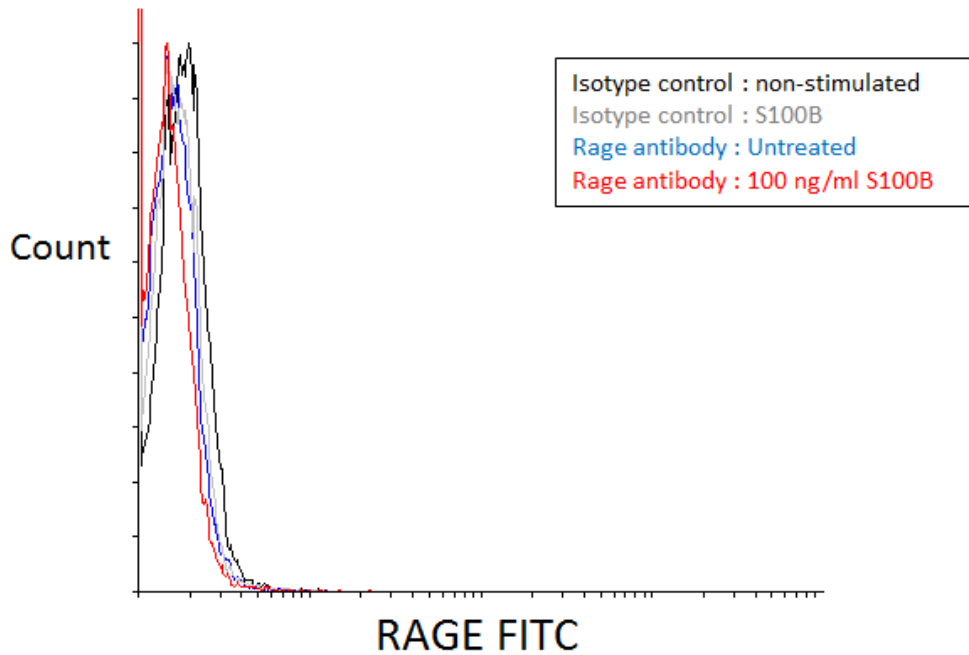
Figure 4.15: Representative histograms of extracellular staining of lymphocyte gated PBMCs for RAGE.

The RAGE receptor was stained using a primary unconjugated mouse anti-human antibody, followed by an anti-mouse FITC secondary antibody. Whole PBMCs represented for 10,000 events. Cells were either untreated (PBS) or treated with 100 ng/ml of S100B protein for 6 hours. Black line is the isotype control antibody for untreated cells (geomean 1.68); grey line is the isotype control for cells treated with S100B (geomean 1.53); Blue line is the Rage antibody on untreated cells (geomean 1.51); red line is the Rage antibody on S100B treated cells (geomean 1.35).

Despite multiple attempts to detect the receptor on PBMC cell surfaces of both healthy controls and patients with sarcoidosis, there was no significant expression present on either T-lymphocytes or monocytes; summarised with representative histograms in Figure 4.14. A range of different RAGE ligands (S100 proteins and HMGB1) and TNF-α were used in an attempt to stimulate increased RAGE expression on cell surfaces, though no RAGE was detectable (Figure 4.15). The decision was made to move to intracellular flow cytometry to detect changes in RAGE expression in response to stimuli.

All the ligands and cytokines used in the whole blood assay were also used on PBMCs from patients with sarcoidosis and healthy controls (Table 4.2). The main areas of interest were on the RAGE ligands HMGB1, S100B, S100A9 and S100A12, as well as the cytokines TNF-α and IFN-γ. The impact of these stimulants on RAGE expression was quantified for both expression and the percentage of RAGE$^+$ cells in T-lymphocytes. PBMCs were treated for 6 hours, an earlier time point than the 16 hours used in whole blood assays, selected to attempt to see the early effects of stimulation that might be consistent with innate immune responses.

The majority of peripheral lymphocytes expressed intracellular RAGE (Figure 4.16), the difference between CD3$^-$ and CD3$^+$ was not significant; the two populations tended to mirror each other in both expression of the receptor and changes induced upon stimuli. Therefore, CD3$^+$ was focussed on instead of looking at CD3$^-$, as the latter would require differentiation to establish B-lymphocyte versus NK cell RAGE expression with additional markers not used in these experiments. The distribution could be Gaussian and unimodal for expression (Figure 4.16 B and D), or non-Gaussian or bimodal (Figure 4.16 A and C). Whilst expression level was considered the principle area of focus, the percentage of RAGE$^+$ T cells was also calculated to accommodate for shifts in cell populations after stimulation, where the percentage of positive cells could increase. Monocyte isotype staining was high in both patients with sarcoidosis and healthy controls (Figure 4.17), which required additional steps in processing data (outlined in Section 4.2.5). The observed peaks were assumed to indicate the presence of RAGE, as the isotype controls were significantly more fluorescent than those on lymphocytes, though confidence in this assessment is not 100%.

Figure 4.18 displays the intracellular expression of RAGE on T-lymphocytes and monocytes. A total of 3 healthy controls and 3 patients with sarcoidosis were tested for RAGE expression experiments. The basal level of intracellular RAGE varied between donors, for example, 2 patients with sarcoidosis had low T-lymphocyte RAGE expression (GMFI below 25), whereas one patient had high expression (GMFI of 138). There was no clear significant difference between patients and controls in terms of overall expression as a result. The percentage of RAGE$^+$ T-lymphocytes was similar within patients with

sarcoidosis (range 43.26 – 62.7%), whereas healthy controls displayed a significant variation in percentage positive cells (range 6.12 – 69.88%); Appendix A4.1.
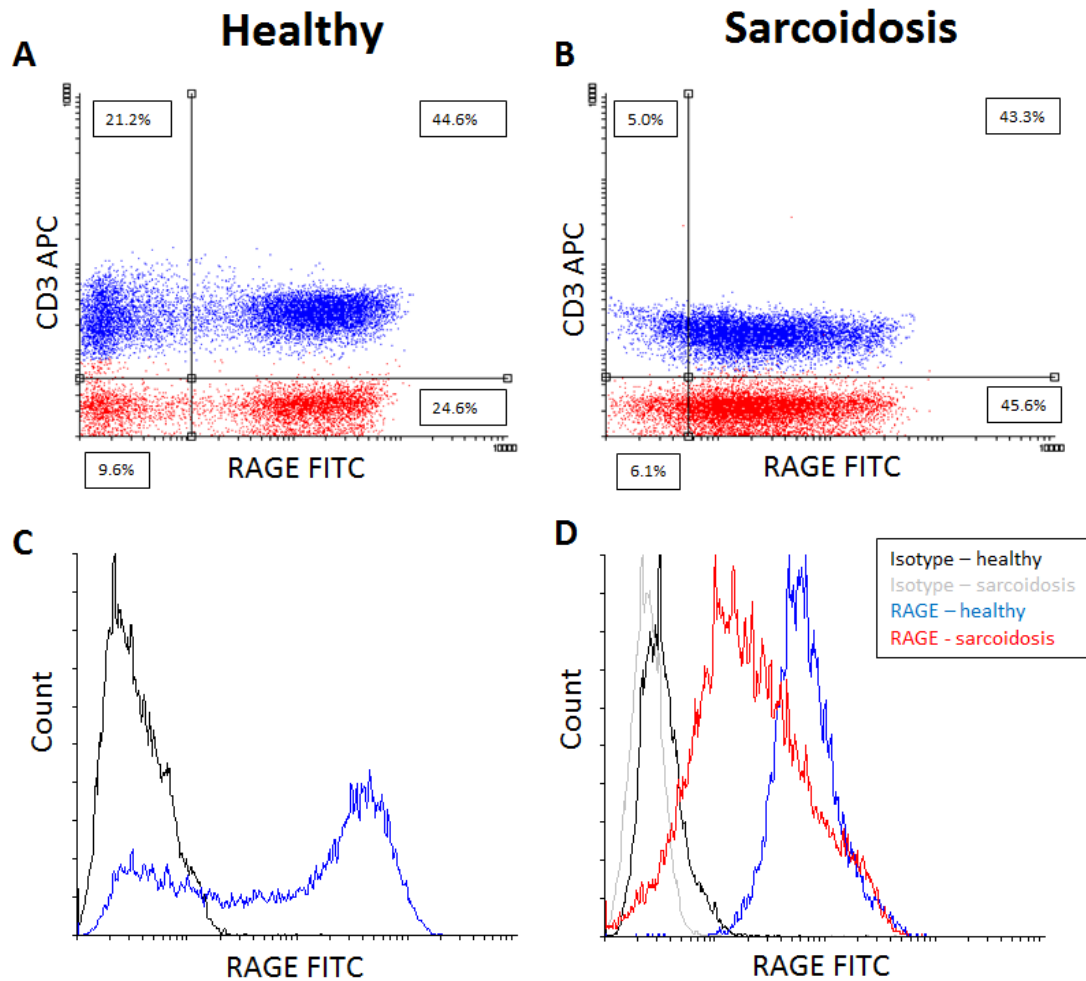
Figure 4.16: Representative dot plots and histograms of intracellular staining of lymphocyte gated PBMCs for RAGE.

The RAGE receptor was stained using a primary unconjugated mouse anti-human antibody, followed by an anti-mouse FITC secondary antibody. **A** and **B:** Representative dot plots of lymphocytes stained for CD3 (APC, Y axis) and RAGE (FITC, X axis) for a healthy control (**A**) and a patient with sarcoidosis (**B**). Percentages are displayed in boxes for each quadrant. Blue populations are CD3$^+$ and red populations CD3$^-$ lymphocytes established by gating using histograms. **C:** Representative histogram of a healthy donor showing RAGE staining (blue line) against the isotype control (black line) for CD3$^+$ gated T-lymphocytes with a positive and negative population. Isotype geomean is 3.41; RAGE antibody staining has a geomean value of 217.1 for positive cells, 71.22% of cells are positive. **D:** Representative histogram of RAGE staining in CD3$^+$ gated T-lymphocytes for a patient with sarcoidosis and healthy control. Black line is the isotype control antibody for healthy cells (geomean 3.34); grey line is the isotype control for sarcoidosis (geomean 2.41); Blue line is the RAGE antibody on untreated healthy cells (geomean 66.97); red line is the RAGE antibody on untreated sarcoidosis cells (geomean 22.11).
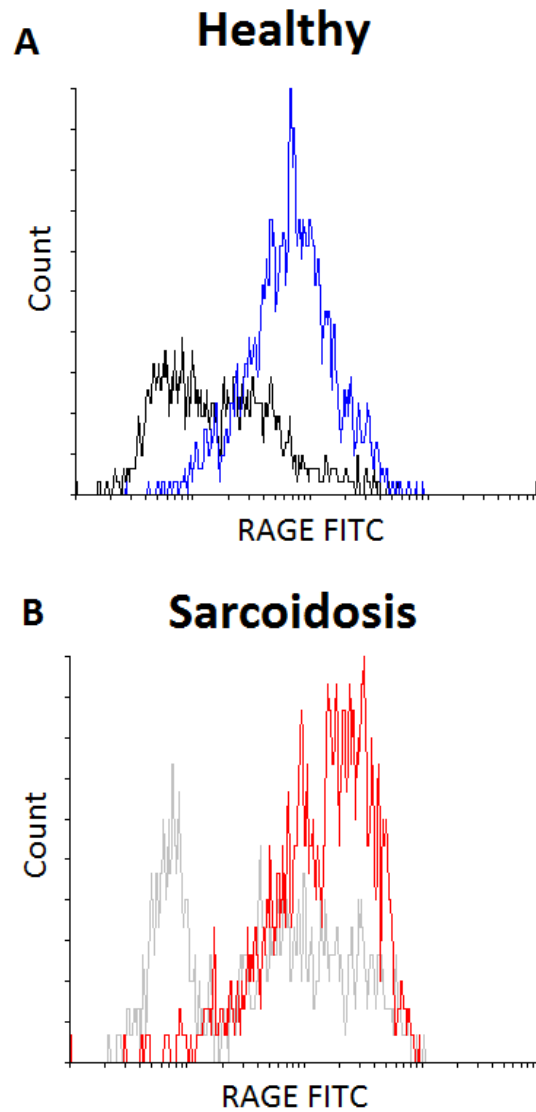
Figure 4.17: Representative histograms of intracellular staining of monocyte gated PBMCs for RAGE in a patient with sarcoidosis and healthy control.

The RAGE receptor was stained using a primary unconjugated mouse anti-human antibody, followed by an anti-mouse FITC secondary antibody. **A:** Representative histogram of monocytes from a healthy control, with the isotype control antibody (black line; geomean 17.66) and RAGE antibody (blue line; 67.87). **B**: Representative histogram of monocytes from a patient with sarcoidosis, with the isotype control antibody (grey line; geomean 44.44) and RAGE antibody (red line; 133.03).
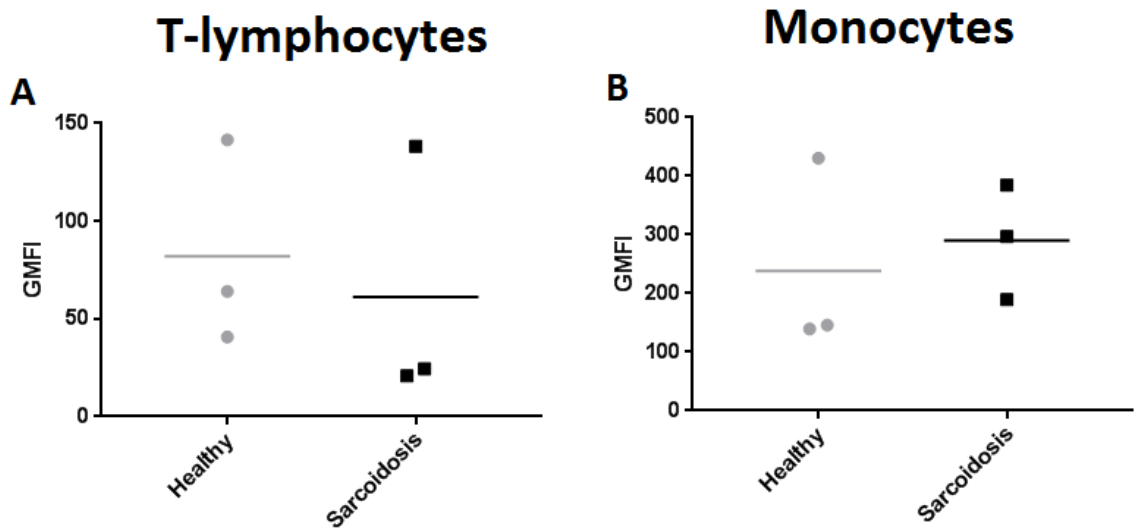
Figure 4.18: Basal expression of intracellular RAGE in T-lymphocytes and monocytes of patients with sarcoidosis and healthy controls.

Expression of RAGE determined by flow cytometry using geometric mean fluorescence intensity (GMFI). Expression for healthy controls (grey circles, n = 3) and patients with sarcoidosis (black squares, n = 3) displayed with mean (grey and black horizontal lines respectively). **A:** RAGE expression in T-lymphocytes. Mean values = 82.11 and 61.13 for controls and patients respectively. **B**: RAGE expression in monocytes. Mean values = 238.6 and 290.2 for controls and patients respectively. No statistical significance displayed (p > 0.05) using the Student's T-test.

In order to identify whether a stimuli was having an impact on RAGE expression, the change in RAGE expression and change in the percentage of RAGE$^+$ cells from baseline were calculated. RAGE ligands were used to stimulate PBMCs, including HMGB1, S100B, S100A9 and S100A12 (Figure 4.19). In healthy controls, 2 out of 3 individuals saw a reduction in T-lymphocyte RAGE expression when cells were treated with all of the ligands used; the remaining control showed a limited increase in expression (GMFI < 10), though the percentage of RAGE positive cells increased in 2 patients for S100B, S100A9 and S100A12 stimulation. The inverse was observed for patients with sarcoidosis, where expression of RAGE on T-lymphocytes increased in response to HMGB1, S100B and S100A9. The 2 patients on the whole also did not see a significant reduction or increase in the percentage of RAGE$^+$ T cells either (< 10 % above or below 0). The remaining patient

displayed a significant reduction in both RAGE expression (GMFI > -50) and the percentage of positive T cells (> -20 %) for the S100 proteins, but less of a change for HMGB1. There was no significant difference in RAGE expression observed between patients with sarcoidosis and healthy controls in monocytes (Figure 4.20), though overall the ligands did not lead to an increase in RAGE expression in monocytes in the whole population. The data suggests that intracellular RAGE may not naturally increase in healthy controls, whereas 2/3 patients with sarcoidosis showed increased T-lymphocyte RAGE in response to RAGE ligands.

Using cytokines known to play a role in the pathogenesis of sarcoidosis, intracellular RAGE expression was detected for the same patients (Figure 4.21). There was not a significant difference in RAGE expression when cells were stimulated with IFN-γ for either T-lymphocytes or monocytes. By contrast, whereas healthy controls responded to TNF-α stimulation by increasing T-lymphocyte and monocyte RAGE expression in all individuals, patients with sarcoidosis either displayed no difference in expression or a reduction in RAGE in both cell types. The number of individuals used does not allow for accurate determination of statistical significance, though this difference highlights a possible difference in response to this cytokine between patients with sarcoidosis and healthy controls.

Other PAMPs were used in the stimulation of PBMCs, including LPS, MDP, IE-DAP, Poly I:C, Beta-glucan and the lectin PHA; RAGE expression was characterised for both T-lymphocytes and monocytes and percentage of positive cells were calculated for T cells (Appendix A4.2 and A4.3). There appeared to be no significant difference in monocyte RAGE expression between healthy controls and patients with sarcoidosis for any of the ligands tested. The trend in both populations was for LPS stimulation to reduce RAGE expression, whereas PHA activation demonstrably increased expression of the receptor (Appendix A4.3). A similar trend was also observed in T-lymphocytes, where expression of RAGE increased with PHA stimulation, though LPS did not display a reduction in receptor expression in 2 out of 3 patients; RAGE did reduce in all healthy controls upon LPS stimulation (Appendix A4.2). This needs to be contrasted with the percentage of RAGE^{+} cells, where LPS increased the percentage of positive T cells, yet reduced percentages were observed in 2 out of 3 controls and patients upon PHA stimulation. All other stimuli

displayed no significant differences in expression or the percentage of positive cells between controls and patients, though individual differences were displayed for stimuli in particular donors (Appendix A4.2).

Although not conclusive, given the limited numbers of patients and controls, there were trends suggesting that RAGE ligands were not capable of eliciting an increase in intracellular RAGE for all individuals, and that patients with sarcoidosis may also not be responding to TNF-α-stimulated increases in intracellular RAGE production.
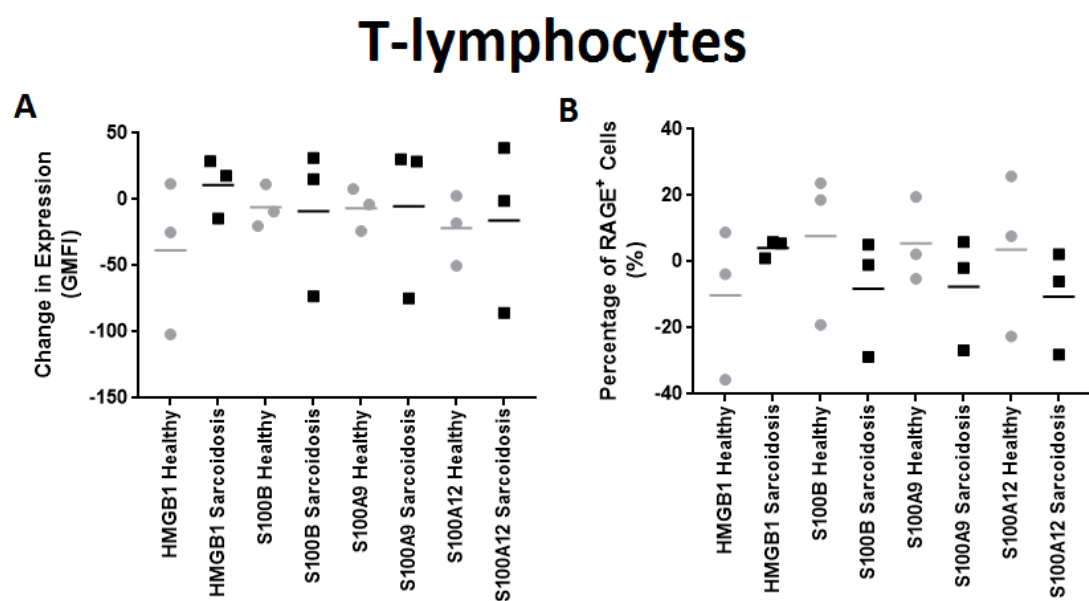
Figure 4.19: Change in expression of intracellular RAGE and percentage of RAGE+ T-lymphocytes in response to RAGE ligands HMGB1, S100B, S100A9 and S100A12.

Expression of RAGE and percentage positive RAGE expression as determined by flow cytometry using geometric mean fluorescence intensity (GMFI) and percentage (%). Expression or percentage for healthy controls (grey circles, n = 3) and patients with sarcoidosis (black squares, n = 3) displayed with mean (grey and black horizontal lines respectively). PBMCs were incubated for 6 hours with either HMGB1 (10 µg/ml), S100B (100 ng/ml), S100A9 (100 ng/ml) or S100A12 (100 ng/ml). **A:** RAGE expression in T-lymphocytes. Mean values from left to right = GMFI of -38.39, 10.72, -5.95, -8.86, -6.57, -5.32, -21.76, -15.96. **B**: Percentage of RAGE+ T-lymphocytes. Mean values from left to right = -10.2, 4.16, 7.74, -8.19, 5.53, -7.59, 3.64, -10.6 %. No statistical significance displayed (p > 0.05) using the Student's T-test.
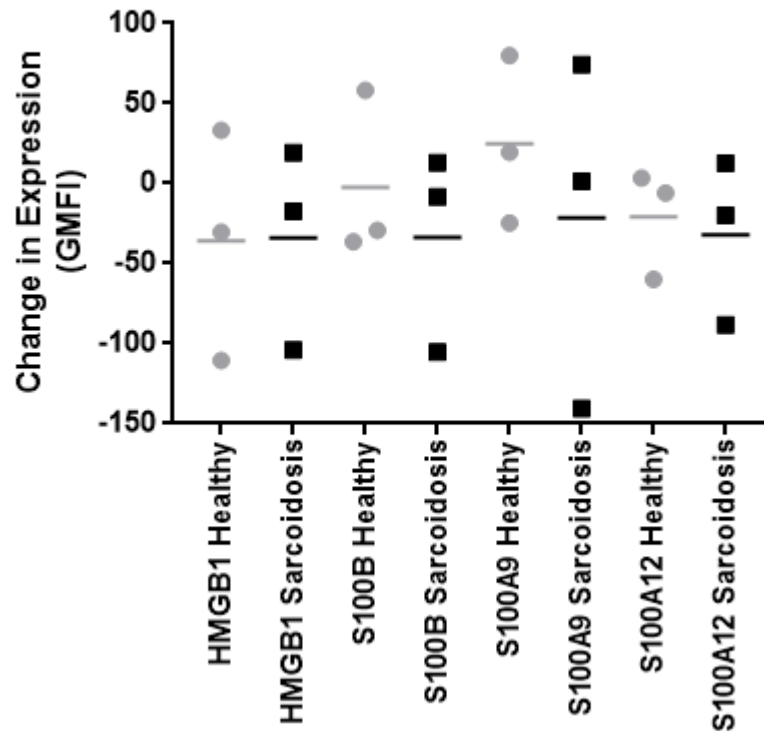
Figure 4.20: Change in the expression of intracellular RAGE in monocytes in response to RAGE ligands HMGB1, S100B, S100A9 and S100A12.

Expression of RAGE as determined by flow cytometry using geometric mean fluorescence intensity (GMFI). Expression for healthy controls (grey circles, n = 3) and patients with sarcoidosis (black squares, n = 3) displayed with mean (grey and black horizontal lines respectively). PBMCs were incubated for 6 hours with either HMGB1 (10 µg/ml), S100B (100 ng/ml), S100A9 (100 ng/ml) or S100A12 (100 ng/ml). Mean values from left to right = GMFI of -35.79, -34.01, -2.473, -33.59, 24.93, -21.57, -20.76, -31.9. No statistical significance displayed (p > 0.05) using the Student's T-test.
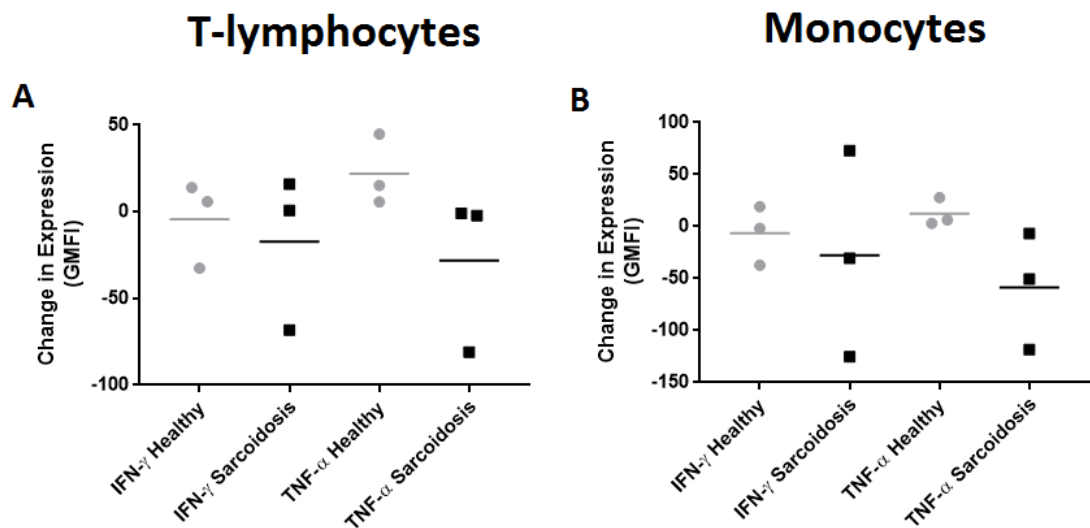
Figure 4.21: Change in the expression of intracellular RAGE in T-lymphocytes and monocytes in response to IFN-γ and TNF-α.

Expression of RAGE determined by flow cytometry using geometric mean fluorescence intensity (GMFI). Expression for healthy controls (grey circles, n = 3) and patients with sarcoidosis (black squares, n = 3) displayed with mean (grey and black horizontal lines respectively). PBMCs were incubated for 6 hours with either 5 ng/ml of TNF-α or 5ng/ml of IFN-γ. **A:** RAGE expression in T-lymphocytes. Mean values for IFN-γ stimulations = GMFI of -4.26 and -17.21 for controls and patients respectively. Mean values for TNF-α stimulations = GMFI of 21.9 and -28.05 for controls and patients respectively. **B**: RAGE expression in monocytes. Mean values for IFN-γ stimulations = GMFI of -6.61 and -27.65 for controls and patients respectively. Mean values for TNF-α stimulations = GMFI of 12.41 and -58.56 for controls and patients respectively. No statistical significance displayed ($p > 0.05$) using the Student's T-test.

4.3.3 NOD2 expression in PBMCs

NOD2 expression was of interest as the receptor is considered a risk factor in Crohn's Disease [429, 430] and mRNA expression in BAL cells from patients with sarcoidosis have shown increased expression of the receptor [792]. As the receptor is intracellular, a similar intracellular flow cytometry protocol was used as had been performed for RAGE. 3 patients with sarcoidosis and 3 healthy controls were characterised for their NOD2 expression on T-lymphocytes and monocytes in response to the NOD2 ligand MDP, NOD1

ligand IE-DAP, TLR4 ligand LPS and the cytokines TNF-α and IFN-γ, as these all related to bacterial immune responses.
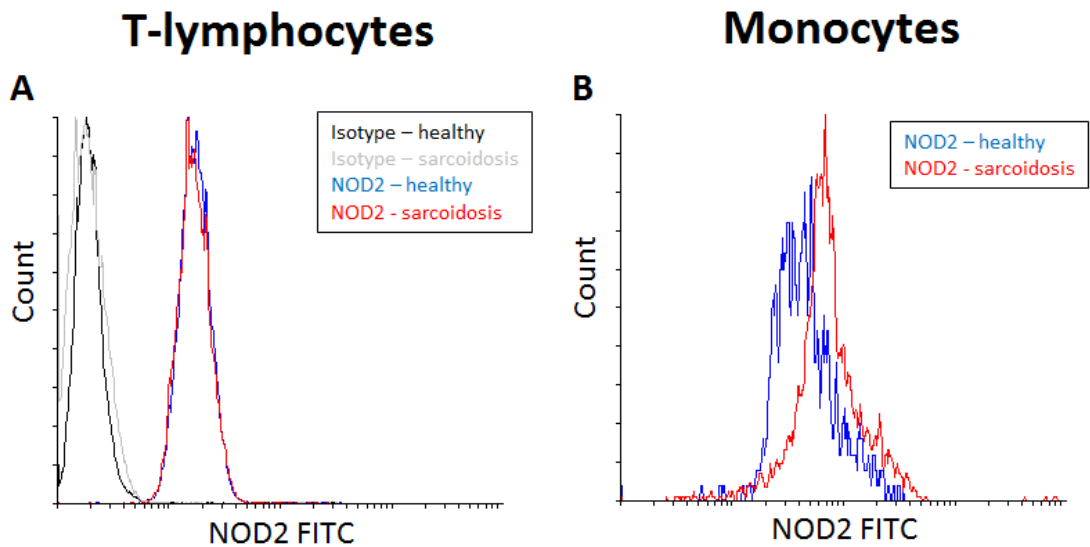
Figure 4.22: Representative histograms of intracellular staining of T-lymphocytes and monocytes for NOD2.

The NOD2 receptor was stained using a primary unconjugated mouse anti-human antibody, followed by an anti-mouse FITC secondary antibody. **A**: Representative histogram of NOD2 staining in CD3^{+} gated T-lymphocytes for a patient with sarcoidosis and healthy control. Black line is the isotype control antibody for healthy cells (geomean 1.95); grey line is the isotype control for sarcoidosis (geomean 1.87); Blue line is the NOD2 antibody on untreated healthy cells (geomean 17.22); red line is the NOD2 antibody on untreated sarcoidosis cells (geomean 16.9). **B**: Representative histogram of NOD2 staining in monocytes for a patient with sarcoidosis and healthy control. Blue line is the NOD2 antibody on untreated healthy cells (geomean 44.9); red line is the NOD2 antibody on untreated sarcoidosis cells (geomean 72.4). Isotype controls omitted for clarity in comparison; isotype for healthy control = GMFI of 15.61, isotype for sarcoidosis patient = GMFI of 30.91.
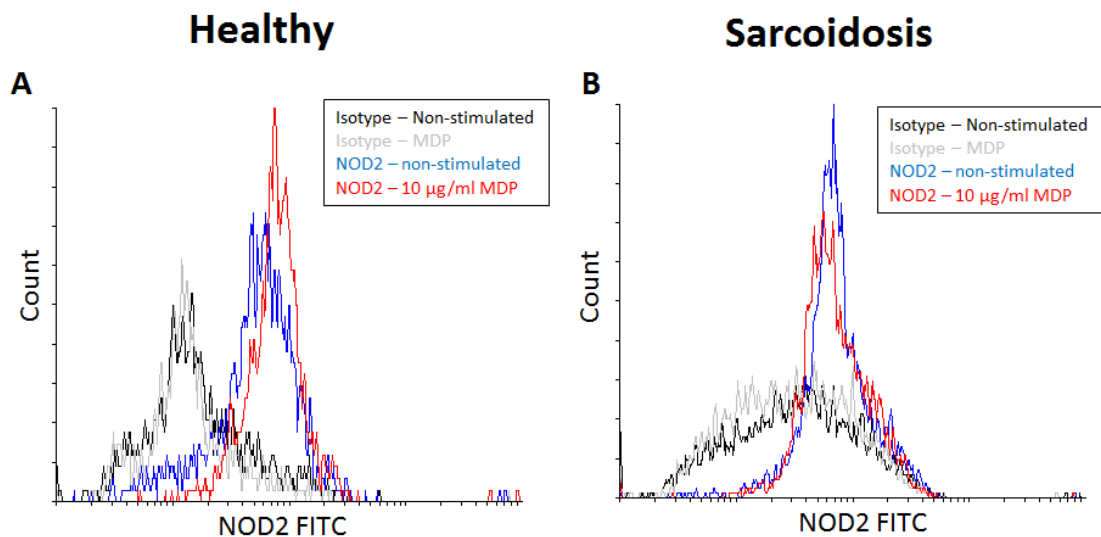
Figure 4.23: Representative histograms of NOD2 intracellular staining of monocytes stimulated with MDP in patients with sarcoidosis and healthy controls.

The NOD2 receptor was stained using a primary unconjugated mouse anti-human antibody, followed by an anti-mouse FITC secondary antibody. PBMCs were incubated with 10 µg/ml of MDP for 6 hours. **A**: Representative histogram of NOD2 staining in monocytes for a healthy control. Black line is the isotype control antibody for non-stimulated (geomean 16.79); grey line is the isotype control for MDP stimulation (geomean 14.53); Blue line is the NOD2 antibody on untreated cells (geomean 50.28); red line is the NOD2 antibody on MDP treated cells (geomean 75.75). **B**: Representative histogram of NOD2 staining in monocytes for a patient with sarcoidosis. Black line is the isotype control antibody for non-stimulated (geomean 30.00); grey line is the isotype control for MDP stimulation (geomean 29.09); Blue line is the NOD2 antibody on untreated cells (geomean 73.44); red line is the NOD2 antibody on MDP treated cells (geomean 70.05).

As shown in Figure 4.22, T-lymphocytes clearly expressed NOD2. The monocytes showed similar high isotype control fluorescence, as had been observed with RAGE staining (Figures 4.22 and 4.23). Although the raw, uncorrected NOD2 fluorescence was higher in patients with sarcoidosis than healthy controls, the isotype control antibody was also higher for patients (Appendix A4.5). This is displayed in sample histograms in Figure 4.22, featuring stimulation with MDP or unstimulated cells, where patient monocytes had a higher background staining. The significance of this has not been determined, and may confound the results obtained from the monocyte data of NOD2. The corrected basal

expression of NOD2 is displayed in Figure 4.24, showing no statistically significant increase in monocyte NOD2 expression though the mean was higher in patients. This was taken as the value, though the caveat is that unprocessed NOD2 was higher in patients (Appendix A4.5); a statistically significant difference compared with controls ($p = 0.017$, Student's T-test), which may be the actual value of NOD2 expression. These factors were not significant confounding factors for subsequent data for the stimulated cells, which are represented as a change in expression ignoring the isotype control, which was consistently similar between treatments.
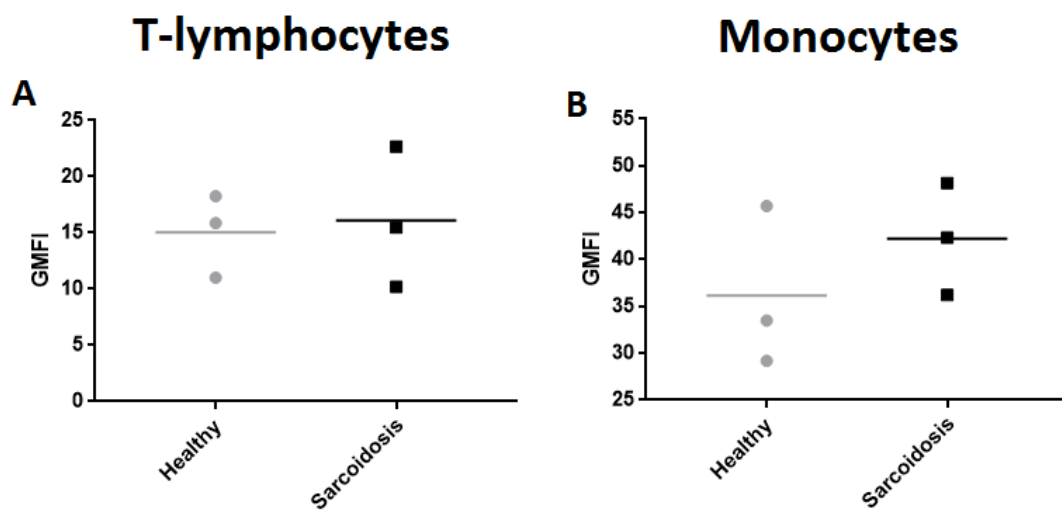
Figure 4.24: Basal expression of intracellular NOD2 in T-lymphocytes and monocytes in patients with sarcoidosis and healthy controls.

Expression of NOD2 as determined by flow cytometry using geometric mean fluorescence intensity (GMFI). Expression for healthy controls (grey circles, n = 3) and patients with sarcoidosis (black squares, n = 3) displayed with mean (grey and black horizontal lines respectively). **A:** NOD2 expression in T-lymphocytes. Mean values = 15.05 and 16.11 for controls and patients respectively. **B**: NOD2 expression in monocytes. Mean values = 36.13 and 42.23 for controls and patients respectively. No statistical significance displayed ($p > 0.05$) using the Student's T-test.

There was a statistically significant difference in the change in NOD2 expression on both T-lymphocytes and monocytes between healthy controls and patients with sarcoidosis after stimulation with MDP (Figures 4.25 A and B, $p < 0.05$, Student's T-test). Whereas patients with sarcoidosis had a reduction in NOD2 expression in response to the stimuli, NOD2 expression increased in healthy controls. In monocytes, the same was also

observed for IE-DAP stimulation, where the PAMP led to a reduction in patients' NOD2 expression but healthy control NOD2 increased ($p < 0.05$, Student's T-test). There was no significant difference in IFN-γ or LPS responses between patients and controls, though both T-lymphocyte and monocyte NOD2 expression increased after TNF-α treatment in healthy controls, but again was reduced in patients with sarcoidosis (Figures 4.25 C and D, $p < 0.05$, Student's T-test). Again, with low numbers of samples, this may indicate a possible difference in the NOD2 response to MDP, IE-DAP and TNF-α; though a more thorough examination would have to be performed with more patients and controls to confirm these preliminary findings.
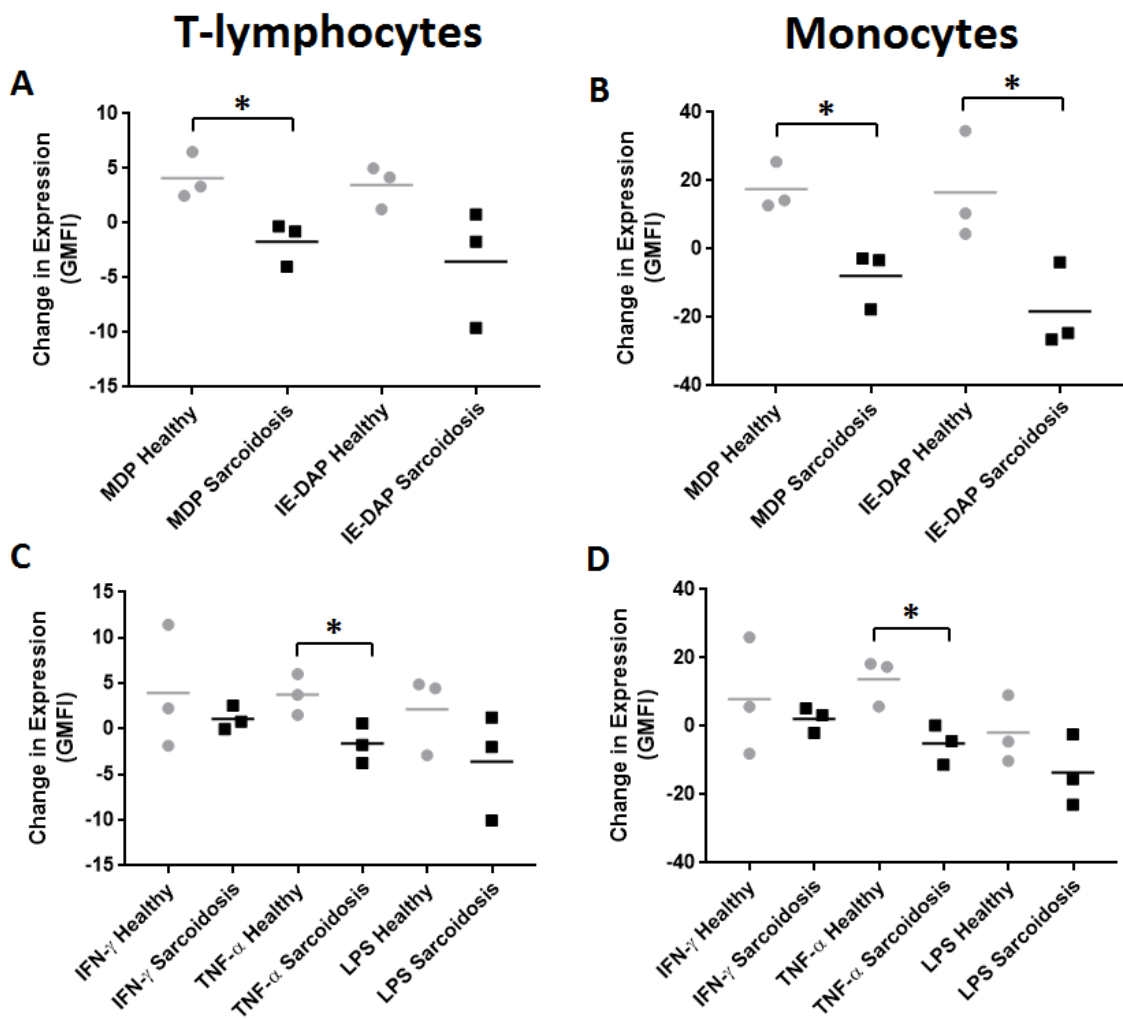
Figure 4.25: Change in the expression of intracellular NOD2 in T-lymphocytes and monocytes in response to IFN-γ, TNF-α, LPS, IE-DAP or MDP in healthy controls and patients with sarcoidosis. Expression of NOD2 determined by flow cytometry using geometric mean fluorescence intensity (GMFI). Expression for healthy controls (grey circles, n = 3) and patients with sarcoidosis (black squares, n = 3) displayed with mean (grey and black horizontal lines respectively). PBMCs were incubated for 6 hours with either 5 ng/ml of TNF-α, 5ng/ml of IFN-γ, 10 μg/ml of MDP, 10 μg/ml of IE-DAP or 10 μg/ml of LPS. **A:** NOD2 expression in T-lymphocytes. Mean values for MDP stimulations = GMFI of 4.11 and -1.69 for controls and patients respectively. Mean values for IE-DAP stimulations = GMFI of 3.48 and -3.50 for controls and patients respectively. **B:** NOD2 expression in monocytes. Mean values for MDP stimulations = GMFI of 17.48 and -7.88 for controls and patients respectively. Mean values for IE-DAP stimulations = GMFI of 16.50 and -18.31 for controls and patients respectively. **C:** NOD2 expression in T-lymphocytes for TNF-α, IFN-γ and LPS stimulation. Mean values from left to right = GMFI of 3.96, 1.11, 3.76, -1.62, 2.15 and -3.60. **D:** NOD2 expression in monocytes for TNF-α, IFN-γ and LPS stimulation. Mean values from left to right = GMFI of 7.89, 2.15, 13.77, -5.13, -1.85, -13.60. Significance determined using a Student's T test. * = p < 0.05.

4.4 Discussion

The role of certain innate immune receptors has been investigated previously in sarcoidosis by other groups, typically focussing on the Toll-like receptors and even NOD receptors [266, 268, 269, 436, 437]. There has been limited work reported on RAGE and sarcoidosis [773], none of which has looked at the expression of the receptor on/in peripheral blood mononuclear cells. This study sought to characterise the immune responses of whole blood leukocytes in patients with sarcoidosis after stimulation with a variety of PAMPs and DAMPs. This was to attempt to identify whether the increased pro-inflammatory release from PHA induced activation in patients was associated with a particular pattern recognition receptor. Innate immune ligands representing a range of different possible aetiological sources in the disease and capable of binding to a variety of pattern recognition receptors (Table 4.1) were used to stimulate whole blood. After 16 hours, it was found that there was no clear statistically significant differences in cytokine release between patients with sarcoidosis and healthy controls in response to any of the stimuli used in this assay. Expression of the RAGE receptor was not significantly detected on the surface of PBMCs, whereas intracellular RAGE isoforms were observed inside the cell. There was no significant difference in RAGE expression in T-cells and monocytes between patients and controls. There was also no statistically significant difference in the change in RAGE expression between patients with sarcoidosis and healthy controls when PBMCs were treated with RAGE ligands or a variety of other stimuli. NOD2 expression in both T-lymphocytes and monocytes was not significantly different once interpreted using a standardised methodological approach. The response of monocytes and T-lymphocytes in PBMCs treated with TNF-α and MDP was to increase NOD2 expression in healthy controls. By contrast, TNF-α and MDP led to significant reduction in NOD2 expression in patients with sarcoidosis. IE-DAP stimulation also showed similar findings in monocytes. Despite the previous findings that PHA stimulated whole blood leads to increased IL-6 and TNF-α release from patients with sarcoidosis, and that PHA has been reported to interact with TLRs 2, 4, 5 and 6 [699], more selective activation of these receptors using PAMPs and DAMPs did not lead to increased cytokine release from patients. This indicates that a different mechanism may be responsible for the elevated PHA responses in sarcoidosis, rather than a hyperactive innate immune inflammatory receptor signal.

4.4.1 Bacterial and other Pathogen Innate Immune Responses

Infectious causes remain a possibility for the aetiology of sarcoidosis, particularly considering that mKatG from *Mycobacterium tuberculosis* has been associated with adaptive immune responses in the disease [462, 465], as have *Propionibacterium acnes* antigen [468, 481, 482]. Despite the numerous reports in support of a pathogenic cause for sarcoidosis, the aetiology remains unknown and evidence relating to this is far from conclusive (see Section 1.6). This present study focussed on innate immune ligands rather than antigen-associated adaptive immune responses. The 3 bacterial PAMPs selected each had a different receptor binding profile, meant to cover different PRRs and therefore provide a more rounded view than focussing only on 1 ligand/receptor pathway.

Despite the fact that it has been demonstrated that TLR4, along with TLRs 2, 5 and 6, can be activated by PHA [699], the results showing that LPS induced lower median IL-6, TNF-α and IFN-γ release from whole blood in patients with sarcoidosis compared with healthy controls suggests that TLR4 activation alone cannot account for the differences observed with PHA. It is worth noting that, whilst the median release of these cytokines in patients with sarcoidosis was lower, the difference was not statistically significant, therefore patients did not appear to display a significant anergy to this stimuli. LPS was demonstrably a potent TNF-α and IL-6 inducer. The non-stimulated levels of TNF-α and IL-6 in the blood for most individuals (patients and controls) was below 20 pg/ml and below 500 pg/ml respectively for most individuals, whereas 10 μg/ml of LPS was able to induce a median increase in TNF-α and IL-6 concentration of 8118 pg/ml and 264567 pg/ml. Just to put that in perspective, the PHA dose curves showed that, even at the highest concentration of 100 μg/ml, the lectin induced a mean TNF-α and IL-6 release of only 2832 pg/ml and 92594 pg/ml in healthy controls (Figure 3.24, Section 3.3). It is clear that both cohorts can respond to LPS with a reasonable response. E.*coli* LPS was used due to the ready availability of this reagent. It is acknowledged that LPS from a bacteria resident in the digestive system may not be as physiologically relevant as a lung resident bacteria, thus LPS from resident or pathological Gram-negative bacterial species from the lung would be the ideal source. Given that the aetiology of the disease is unknown and the leading bacterial contender is a Gram-positive bacteria, P. *acnes*, the use of LPS in this context is considered acceptable for a limited exploratory study of this kind. It is notable

that the difference in TNF-α release between patients and controls was relatively small in terms of the median value, whereas IL-6 and IFN-γ showed greater differences in median release between the two groups. This is consistent with the findings of another study using PBMCs to measure TNF-α release in response to LPS, where there was no significant difference in release between healthy controls and patients with the disease [340]. As a T-cell lymphocytopenia has been observed in patients (Section 3.3), the results could be representing an impact of the reduced T-cell number in whole blood for LPS release, as T-cells can release both IFN-γ and IL-6 in the PHA whole blood assays, yet TNF-α seems to be predominantly monocyte restricted. LPS is not an obligate TLR4 agonist; TLR2 and CD14 have been reported to interact with LPS [793] and a recent report has highlighted that it can bind directly to caspases [791], therefore it is difficult to form conclusions about the individual contribution of the receptor without a specific TLR4 activation process. LPS stimulation of alveolar macrophages has shown to lead to elevated IL-6 and TNF-α release in patients with sarcoidosis [341]. The current study has not performed work on purified cells types, though it would be useful in future studies to look at LPS response in sarcoidosis on separated monocytes, T-lymphocytes or other leukocytes to test whether the current results are a product of multiple cell interactions.

Whole blood stimulation with MDP and IE-DAP, NOD2 and NOD1 ligands respectively [780], displayed a similar pattern to those observed for LPS, adding support for the concept that the T-cell lymphocytopenia may be impacting overall cytokine responses in whole blood for these specific ligands. The response to MDP was more pronounced than that of IE-DAP, though the significance of this was not interrogated further.

In support of the theory that the reduction in T-lymphocyte number in sarcoidosis might be having a general impact on these assays, Beta-glucan, which is both a Dectin-1 and TLR2 agonist from fungal cell walls [758, 786, 787], also displayed reduced median IL-6 release from whole blood. TLR3 stimulation through Poly I:C, a synthetic compound that 'mimics' the structure of double stranded RNA [788], showed limited IFN-γ and TNF-α release, with non-significant but elevated median IL-6 release from patients. TLR3 is difficult to assess however, as it is unlike other TLRs, TLR3 uses a MYD88 independent adapter protein [764], whereas other TLRs can use MYD88 dependent activation of downstream pathways [764, 794]. The implications are that different gene transcription

events can occur upon stimulation with these different types of receptor pathways, therefore some differences are likely to be observed between the receptor types. Either way, it appears that most PAMPs may be negatively affected by the reduced T-lymphocyte number, though a necessary assessment in more detail would be required to assess this further.

4.4.2 NOD2 Expression on T-lymphocytes and Monocytes

One of the main findings of this work relates to the expression of the NOD2 receptor itself. Intracellular flow cytometry was used to determine expression in T-lymphocytes and monocytes; the receptor was expressed in both cell types. The work was preliminary and only performed in 3 patients and 3 healthy controls, owing to limitations discussed in more detail later (see Chapter 6). Despite this, the initial results highlighted significant differences in patient response to MDP and TNF-α in terms of NOD2 expression; both stimuli led to a reduction in NOD2 in patients and an increase in the receptor in controls. The full significance of this cannot be determined at this present time, as further repeats would need to be performed and a more thorough investigation using different experimental strategies is required. However, MDP did not lead to increased whole blood pro-inflammatory release in patients. There was a trend suggesting that patients may release less IL-6 upon MDP stimulation, though a lack of statistical significance limits what can be concluded from this. Nor was TNF-α addition to whole blood enough to stimulate a reduced or increased cytokine release in either cohort. Healthy controls demonstrably increase NOD2 expression after stimulation with both stimuli, which is consistent with another report which described TNF-α induced increase in the receptor expression [795]. To help explain the finding that patients respond in the opposite manner to healthy controls depends on determining the basal expression of the receptor, which is an issue for this current study. As mentioned during the results section, the raw data without correcting for the isotype control showed patients displayed an increased basal NOD2 expression in monocytes. However, the isotype control staining was very high in all intracellular experiments, particularly in monocytes. This is not unusual; another group stained eosinophils for NOD2 and their intracellular control also showed a relatively high background binding that somewhat overlaps the 'positive' staining [796]. The higher

background staining in this report might be related to the use of a primary and secondary conjugated antibody, rather than a single antibody with a conjugated fluorescent tag. In the experience of the author, use of a secondary antibody after a primary in intracellular flow cytometry does potentially lead to increased non-specific binding and higher fluorescence. This may not have been an issue, aside from the much higher isotype control staining on sarcoidosis patient monocytes compared with healthy controls. The author has no specific explanation for this observed difference; any suggestions would merely be speculation at present without further investigations. This does have the effect of reducing the values obtained for patient NOD2 baseline expression, therefore the conclusion becomes that NOD2 expression is not significantly increased in monocytes of patients with sarcoidosis. Whether or not this is a true reflection of the expression in monocytes is unknown, however, the T-lymphocytes which did not suffer from this high staining issue showed no significant difference between the expression of NOD2 in the cohorts. The mirroring of NOD2 reduction in response to TNF-α and MDP in both T-cells and monocytes does suggest that the reduction observed is not specific to one cell type. An initial hypothesis could be that the MDP and TNF-α could be interacting with other immune components altered in sarcoidosis that are skewing immune response away from the NOD2 pathway. Alternatively, there could be a deficiency or alteration in NOD2 in these patients leading to a reduction in the receptor upon stimulation with these two activators. Despite the limited data available for sarcoidosis and NOD2, some reports have suggested NOD2 expression is increased in sarcoidosis [792], though the results presented in the current study cannot support or refute this entirely. Another study by Wikén *et al* [268] suggested that a combination of MDP and TLR2 stimulation enhanced cytokine responses from PBMCs in sarcoidosis, though interestingly the same report also highlighted that MDP alone (and LPS also) did not lead to elevated TNF-α and IL-6 release from patients with sarcoidosis compared with controls, supporting the findings presented for the current research in whole blood. The discussions about association of NOD2 with Crohn's Disease have been mentioned previously, though the summary that Crohn's is associated with loss of function mutations in the gene, whereas the early onset sarcoidosis and Blau syndrome are considered to have gain of function mutations is worthy of note [268]. The findings presented here may relate more closely with Crohn's Disease rather than EOS or Blau syndrome, in that reduced sensitivity to MDP and TNF-α and a limited response to MDP stimulation would indicate that a loss of function is more

likely. A study on susceptibility of patients with particular NOD2 polymorphisms suggest that the cohort may not have a universal risk factor for NOD2, but certain polymorphisms may relate to the severity of pulmonary symptoms in patients [435]. More work would be necessary related to identifying if the patients in this study's cohort had polymorphisms in NOD2 in order to make this determination.

4.4.2 The Receptor for Advanced Glycation End-products – RAGE

RAGE is responsible for signalling in response to damage/necrosis and cell stress and is a multi-ligand receptor capable of binding to a variety of DAMPs [797-799]. Dubaniewicz [512] has proposed that 'danger signals' acting through PRRs may have a role to play in sarcoidosis; this part of the study sought to investigate whether patients would display altered expression of RAGE on peripheral leukocytes and different responses to DAMPs in the whole blood. Much like with NOD2, the findings are limited by low numbers of donors used for this portion of the study, yet it provides a preliminary look at this receptor and DAMPs in the context of the disease.

There was no statistically significant differences in RAGE ligand induced cytokine release from the whole blood of patients with sarcoidosis compared with healthy controls. In fact, the stimulation with these DAMPs tended to have a limited effect on the release of cytokines in general; any small changes were usually manifest with reductions in released cytokines rather than increases (with the exception of IL-10). There are a number of caveats attached to these findings. Firstly, the whole blood assays were all performed for 16 hours, consistent with the previous whole blood assays, though it is acknowledged that the kinetics of any stimuli used (PAMP or DAMP) may have completely different optimal release. Whilst the PHA results had been optimised for this, there was not sufficient time and resources to perform a full kinetic profile for all the stimuli and dose responses, which might also be insufficient/sub-optimal. This was considered an acceptable compromise, as this data was being used to attempt to find possible receptors that could be responsible for the results observed with PHA at 16 hours. Further work is necessary to establish whether DAMPs such as these require a longer or shorter incubation. It is also possible that the DAMPs require other co-stimulatory

signals/cytokines/proteins for activity in a system such as this. This need for a synergistic activation has been demonstrated for HMGB1, which releases low levels of cytokines by itself but can enhance the activity of other immune stimulants, such as ATP (adenosine triphosphate) and LPS [800, 801]. A different study has shown that HMBG1 stimulated TNF-α release from human umbilical vein endothelial cells [802], so this may depend on the system used and the cell types being tested. It would suggest that, for future work, DAMPs should be used in combination with existing stimuli, such as TNF-α or LPS, as well as being used individually.

Despite the full length receptor being described as cell surface receptor, attempts in this study to detect RAGE on monocyte and T-lymphocytes proved unsuccessful. Although the full length RAGE (FL-RAGE) is purported to be a cell surface receptor, demonstration of this in flow cytometry by other authors is difficult to find. One group stated that the receptor was present on cell surfaces on monocytes as detected by flow cytometry, though only the mean fluorescence intensity (MFI) value is stated with no visual representation of the staining using histograms or dot plots to ascertain the level beyond the MFI number [803]. THP-1 cells, a model for monocyte/macrophages, also displayed low levels of surface expression using this method until an overexpression system was employed [804]. Other groups often describe expression using different techniques, including mRNA analysis, western blotting and IHC tissue staining [768, 805, 806], though care needs to be taken in interpreting mRNA RAGE expression and assuming this translates to FL-RAGE surface expression, as it has been shown that there is a significantly lower level of protein expression compared with mRNA levels [807].

The decision to stain intracellular RAGE expression had two primary issues. The first was the same as the issues that arose with intracellular NOD2 staining of monocytes; the background staining in these cells using the antibody set up (which was the same for RAGE as NOD2) was very high. Despite this, a method was employed to attempt to correct for this issue, though patients with sarcoidosis still displayed a demonstrably increased background staining compared with healthy control monocytes. If a similar system were to be employed in the future, a thorough interrogation of the method would need to be made. The other main issue related to the specificity of the antibody, which was capable of recognising a sequence common to FL-RAGE, but also soluble RAGE

(sRAGE) and endogenous secretory RAGE (ES-RAGE). The soluble form is cleaved from FL-RAGE by the action of metalloproteases (sheddases) such as ADAM10 (a disintegrin and metalloproteinase domain-containing protein10) and MMP9 (matrix metallopeptidase 9) [804, 808], whereas ES-RAGE are a collection of secreted splice variants of RAGE [777]. ES-RAGE and sRAGE lack the transmembrane domain and cytosolic tail required for signalling (see Figure 4.1), but can both bind to RAGE ligands, therefore they are potentially a regulatory feature involved in RAGE signalling [777, 797, 809]. The antibody would not be capable of differentiating between these isoforms, so the assumption that RAGE relates to the active inflammatory FL-RAGE cannot be made. However, it has been demonstrated that FL-RAGE is the main transcribed form of RAGE [777], therefore this limitation has to be taken with this consideration in mind.

The lack of a statistically significant difference in RAGE expression between healthy controls and patients with sarcoidosis, both at baseline and in response to either RAGE ligands or other stimuli suggests that the receptor is not radically altered in the disease. There were trends suggesting some possible areas that could be explored in the future, should there be renewed interest in looking at RAGE in more detail in sarcoidosis. The main finding that may be worth exploring with increased numbers of patients and controls would be the response of RAGE in patients to TNF-α treatment. Much like the situation seen with NOD2, sarcoidosis patients saw a reduction in RAGE or no significant increase in both T-cells and monocytes, whereas TNF-α led to increased RAGE protein expression in healthy controls. The response from controls was broadly consistent with the findings from another study, where RAGE mRNA and protein expression were both increased after TNF-α stimulation in skin cells [810]. Another report also confirms that TNF-α can induce RAGE expression [811], which makes the result from sarcoidosis patients unusual. Though, based on the NOD2 results, this could represent a more systemic issue that merits further investigation.

Having characterised the RAGE protein in sarcoidosis, there remains questions to be answered about the disease and further work that can be completed to expand on this limited preliminary study of the receptor and its DAMP ligands. One feature not analysed was the soluble form of the protein, sRAGE, which can be detected in plasma or media from cells in a soluble form [812]. What is particularly interesting is that soluble RAGE

from the blood of patients with sarcoidosis has been investigated in a single report, though this study showed that more severe sarcoidosis was associated with lower circulating levels of sRAGE [813]. Given that RAGE has been implicated in a number of inflammatory diseases [814], it would be of interest to determine whether the soluble form of this receptor is a potential marker of disease activity or of particular prognostic value in sarcoidosis. There is insufficient data based on this preliminary study to identify whether this would be a reasonable direction for future work in sarcoidosis, though it is worth considering depending on time and the availability of resources.

4.4.3 Conclusions

The aim of this part of the project was to attempt to identify possible innate immune receptor and cognate ligands that were altered in patients with sarcoidosis that might account for the differences previously obtained for enhanced PHA sensitivity. As PHA has been associated with TLRs, a wide range of potential PAMPs and DAMPs were used in the same whole blood assay system and tested for responses. The overall conclusions were that there was no significant differences in cytokine release as a result of any PAMP or DAMP stimulation. The response of whole blood to LPS stimulation was also not significantly different between healthy controls and patients with sarcoidosis. This is important, as the current paradigm has suggested that patients display a generalised peripheral anergy. This data would suggest otherwise and merits further investigation of the immune responses to LPS stimulation in particular isolated leukocyte types, particularly monocytes and the intermediate subset specifically. RAGE expression was also not significantly altered in the disease, though NOD2 expression appears to be distinctly altered in sarcoidosis monocytes and T-cells upon stimulation with TNF-α and MDP. This data indicates that, under the conditions tested, the innate immune receptors selected are unlikely to account for the elevated PHA responses by themselves, as a single stimuli did not lead to significantly increased IL-6 or TNF-α release from whole blood. It is possible that, despite the stated association of PHA with TLRs 2, 4, 5 and 6 [699], it might be that in the whole blood system, or as a result of higher PHA concentrations, other immune receptors are responsible for the elevated responses observed previously which were not stimulated by any of the ligands selected, for example, none of the ligands are

believed to activate TLR6 directly. Also, given the large range of inflammatory TLRs, NLRs, RLRs, CLRs and other possible receptors that can be expressed on leukocytes [511], it is possible that an unidentified receptor interaction may be responsible for the increased activity in sarcoidosis and be stimulated by PHA.

The ligands/cytokines selected for this study were limited to a single concentration based on previous work by other authors. As the PHA dose responses showed that the difference observed in patients with sarcoidosis was concentration dependent, IL-6 and TNF-α release were statistically significant in patients at 30-100 µg/ml of PHA; it is possible that other stimuli may only display enhanced effects at higher concentrations. This is something that could be investigated in any subsequent studies in the future, should this work be expanded upon later. For this, each stimuli would need to be validated in a similar way to PHA, with multiple patients and controls and various concentrations of each stimuli. For the sake of practicality, only using a limited number of stimuli in testing with a narrower field of inquiry would be necessary for such a study. The results of the previous PHA dose responses had led to this investigation of pattern recognition receptors in patients with sarcoidosis. The preliminary findings were not comprehensive enough to exclude all possibilities, yet there are other hypotheses that could be tested that might relate to the enhanced pro-inflammatory release in patients with sarcoidosis. Given that blood in healthy controls tended to 'peak' in terms of the cytokine release at 10 µg/ml of PHA stimulation before plateauing, whereas blood from patients with sarcoidosis showed continual increases in cytokine release well above this concentration of PHA (Figure 3.24, Chapter 3), it is possible that regulatory control may have a role to play in this mechanism. A lack or reduction in specific immune regulatory signalling could account for this. Therefore the next step was be to investigate immune regulation of inflammation in sarcoidosis.

Chapter 5
Regulatory Immune Receptors in Sarcoidosis Peripheral Blood Leukocytes

5.1 Introduction

Sarcoidosis is a disease characterised by increased inflammatory responses in tissue, though the question remains as to the cause of this elevated immune response. Whether it is the result of a persistent pathogenic challenge, an aberrant inflammatory signalling pathway or dysregulated control of immune response has yet to be determined [68].

Regulation of immunity is vital to permit the immune system to respond to pathogens while remaining tolerant to self and preventing excessive inflammation that may inflict "friendly fire" damage to host tissue [815]. The immune system must react to a variety of challenges, though there is often a fine line between a proportionate response and an overactive or underactive one. A number of diseases represent situations where there is an imbalance in this response; cancer often occurs in/encourages a climate where immune response is circumvented/evaded [816], whereas an autoimmune disease such as systemic lupus erythematosus (SLE) is characterised by an overactive immune response causing damage to the body itself [817]. In addition to pathways necessary for inflammation, there are also regulatory immune pathways necessary to control the system. Given the elevated local tissue inflammation that occurs in sarcoidosis in the apparent absence of a persistent, invasive and pathogenic microorganism [1], it is possible that abnormalities of regulatory pathways that normally act to dampen immune responses could explain some of the immunological features of sarcoidosis.

A number of mechanisms exist that can regulate inflammation, including pre and post-translational, as well as transcriptional control [818, 819]. In terms of cell signalling, a number of receptors exist that can regulate immune responses. Collectively termed 'Regulatory Immune Receptors', these can respond to either a specific ligand/protein/cytokine or a number of different stimuli to control the inflammatory response. There are receptors and their cognate ligands/cytokines that achieve this in a variety of ways. An example of some of these proteins and their expression on monocytes and T-lymphocytes from human peripheral blood are summarised in Figure 5.1.
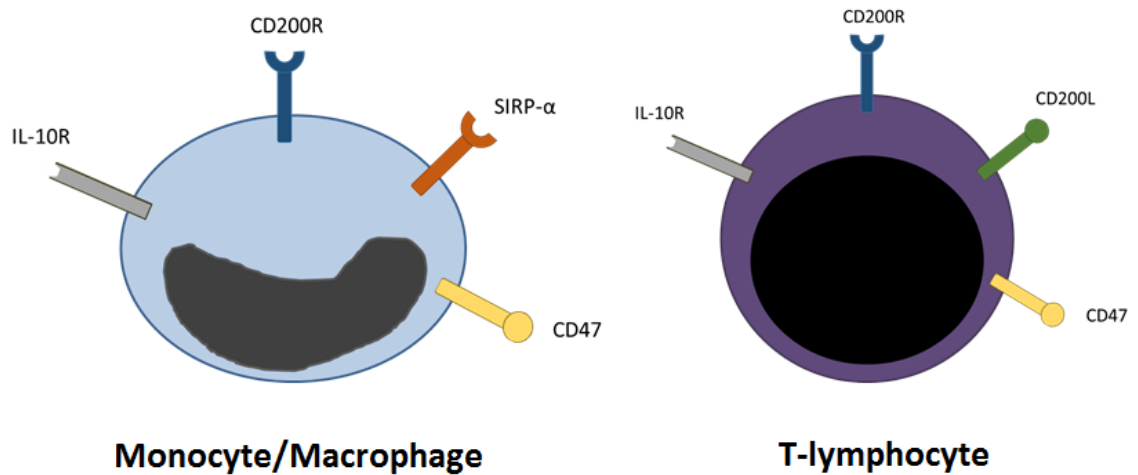
Figure 5.1: Distribution of immune regulatory receptors and ligands for these receptors on monocyte/macrophage cells and T-lymphocytes.

The CD200 receptor (CD200R) is expressed on both cell types, whereas its cognate ligand CD200L (also called CD200) is absent on monocytes/macrophages. The IL-10 receptor (IL-10R) and CD47 (the ligand for SIRP-α) are present on both cell types, whereas SIRP-α is exclusively on monocytes/macrophages.

Interleukin 10 (IL-10) is the archetypal regulatory cytokine involved in control of inflammatory Th1 immune activity. IL-10 is produced primarily by monocytes and regulatory T-lymphocytes and acts through its receptor IL-10R expressed by T cells, monocytes, and macrophages [820]. IL-10 has been reported to be suppressed by NOD2 in Crohn's disease [821], a granulomatous disease affecting the gastrointestinal tract. It has been reported that sarcoidosis blood monocytes produced smaller amounts of IL-10 than controls and were less able to suppress T cell proliferation [822].

Monocytes and macrophages express signal-regulatory protein-alpha (SIRP-α), another regulatory immune receptor which binds to ubiquitously expressed CD47 and acts as an anti-phagocytic signal [823]. Another receptor-ligand regulatory pairing is the CD200 receptor (CD200R) and CD200 ligand (CD200L or CD200). The CD200R/CD200L axis has been shown to be vital in maintaining immune homeostasis in the lungs of mice [334, 824]. CD200R is expressed most strongly by monocytes and macrophages [825]. Many cell types express its cognate ligand CD200L, which engages CD200R leading to reduced MAPK

(Mitogen-Activated Protein Kinase) signalling through recruitment of RASGAP (Ras GTPase activating protein) by DOK2 (Docking protein 2) [824]. Reduced CD200R signalling has been implicated in the pathology of joint inflammation [826], neurodegeneration [827], and cancer [828].
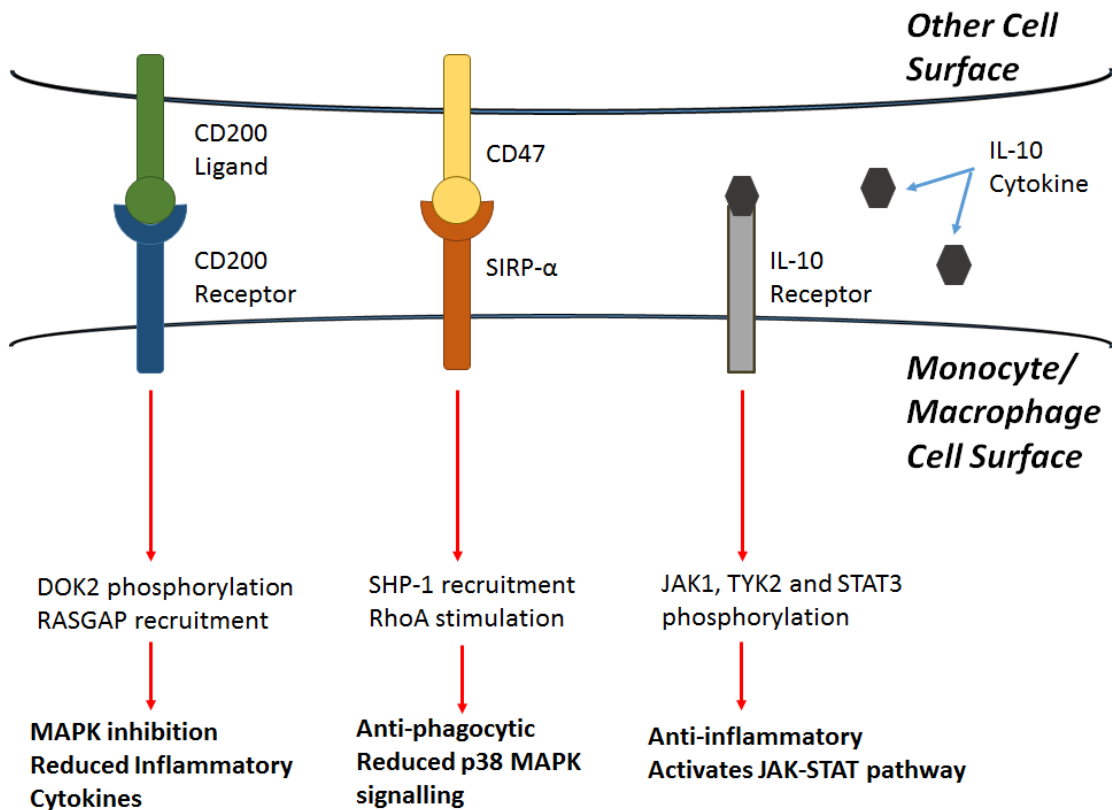
Figure 5.2: Summary of the principle activity for a selection of immune regulatory receptors present on monocyte/macrophage cells.

A summary of these regulatory receptors and ligands, along with the principle mode of action, is presented in Figure 5.2. The IL-10 receptor has not been studied in sarcoidosis previously (to the best of the author's knowledge), though the IL-10 cytokine has been studied in the disease [822, 829]. There has been no research on SIRP-α in the context of sarcoidosis PBMCs and currently no study on CD200R/CD200L in sarcoidosis. The primary aim of this work was to characterise the expression of the regulatory receptors IL-10R, SIRP-α and CD200R, as well as the receptor/ligand CD47 and CD200L, on peripheral blood

leukocytes in patients with sarcoidosis to identify whether there were any differences between patients and healthy controls. Secondary to this, if there was any significant difference observed involving a receptor or ligand, this would be investigated further to identify whether this had a role in peripheral blood immune responses. There would be a particular focus on pro-inflammatory cytokine release from whole blood in response to PHA, as this had previously been shown to be elevated in patients with sarcoidosis, with a view to identifying whether any differences in regulatory receptor interactions could explain the previous observations.

5.2 Methods

5.2.1 Cohort

A subset of individuals within the main healthy control and sarcoidosis cohort were used for work relating to the regulatory receptors. A maximum n = 17 was used for both cohorts, depending on the experiment. These belonged to the latter donors, as the study was undertaken after the initial work had started (Chapters 3 and 4). Lymph node and lung biopsy samples were obtained following appropriate consent procedures (Section 2.1) from sarcoidosis patients within this cohort of n = 17. There was overlap between some individuals in the n = 17 subset of the cohorts and previous work in immunophenotyping and whole blood assays, whereby individual donors would undergo a combination of experiments allowing for correlations between findings.

5.2.2 Cell Surface Staining of Peripheral Blood Mononuclear Cells for Regulatory Receptors and their Cognate Ligands

PBMCs were isolated from whole blood and prepared as previously described (Sections 2.1 and 2.7). A standard live cell extracellular flow cytometry staining protocol was used (Section 2.9). Preliminary tests identified cell types expressing the receptor and ligand, informing future test combinations. Antibodies are listed previously (Section 2.3.5, Table 2.4) and were titrated as previously described (Section 2.8). Based on these titrations, 10

µg/ml was selected as the final concentration for these antibodies. For CD200R, CD3 and CD14 were used to identify T-lymphocyte and monocyte populations, whereas CD3 and CD19 were used for CD200L. SIRP-α/β was dual expressed with CD14, whereas both CD14 and CD3 were used in combinations with CD47. IL-10R was also combined with either CD3 or CD14. Data was collected and interpreted as previously described (Sections 2.9 and 2.11), though representative samples were increased from 20000 events to 50000 events to allow for greater discrimination in individuals with lower monocyte numbers.
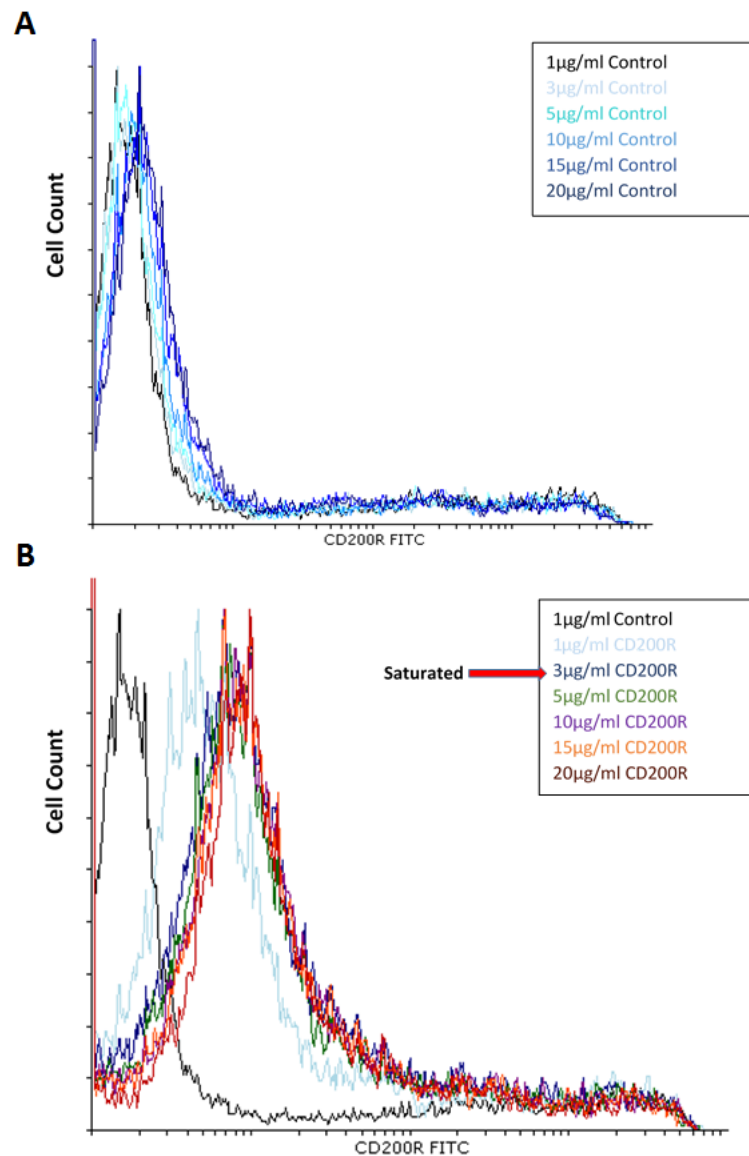
Figure 5.3: Titration of CD200R blocking antibody and a control antibody on lymphocytes.

PBMCs were stained for extracellular CD200R using concentrations between 1-20 µg/ml of anti-CD200R blocking antibody. The above representative histograms display the titration of **A:** the control antibody and **B:** the anti-CD200R at varying concentrations on lymphocyte gated cells. CD200R saturates at 3 µg/ml on lymphocytes.

5.2.3 Blockade of CD200R and CD200L in Whole Blood Cultures and PBMC Cultures

Heparinised whole blood was collected from healthy volunteers and processed within 1 hour of collection, as previously described (Section 2.1). PBMCs were isolated and prepared as previously described (Section 2.7). Antibodies used for functional studies are listed in Section 2.3.6. Anti-CD200R was a polyclonal antibody and anti-CD200L was a monoclonal antibody; both were used with matched isotype control antibodies. Both antibodies were titrated on PBMCs and assessed by flow cytometry as previously described (Section 2.8). As these were unconjugated, a secondary polyclonal FITC anti-mouse raised in goat and FITC anti-goat raised in donkey were used (at 5 µg/ml) for the CD200L and CD200R antibodies respectively. An example of the titration of the CD200R blocking antibody is displayed in Figure 5.3, showing that for this antibody, 3 µg/ml was sufficient to saturate lymphocytes. To accommodate whole blood and other possible confounding variables, 10 µg/ml of the antibodies were used in the blocking experiments.

Depending on the experiment, PHA at 100 µg/ml was used to stimulate the cells in culture, always with an additional set of tubes containing PBS as a control. Each was used in triplicate, with each tube in a set containing either a PBS control, control antibody or the CD200R/CD200L blocking antibody. These antibodies (or PBS control) were added to tubes prior to the addition of whole blood or PBMC cultures. The whole blood or PBMC cultures were added to 2ml round bottom skirted microfuge tubes, as had been used in whole blood assay experiments (Section 2.12). For PBMC cultures, cells were adjusted to 2×10^6 cells/ml in RPMI media, and 0.5 ml of this was added to each tube. Whole blood was used unaltered, 0.5 ml of which was added to each tube.

Cultures were incubated with the blocking antibodies and controls for 1 hour at 37°C in an incubator prior to the addition of the PHA (or PBS controls). After this initial antibody incubation, the PHA was added to cultures for an end concentration of 100 µg/ml. Cultures were incubated at 37 °C and 5% CO_2 in an incubator for 16 hours. The plasma or RPMI supernatant was then extracted using the method previously described (Section 2.12) and stored at -20°C until required.

Plasma or supernatant was run using the same ELISA method previously applied for whole blood assays and PBMC cultures (Section 2.13). Dilution of the plasma or PBMC

supernatant was dependent on the type of stimulant used and the particular cytokine being analysed (Section 2.13 and Section 3.2.7). Supernatant or plasma was analysed for IFN-γ, TNF-α, IL-6 and IL-8 (for CD200L blocking experiments) and the data analysed as previously described (Section 2.12 and Section 2.16), with dilution of the starting culture accounted for in final calculations.

5.2.4 Activation of CD200R using Soluble CD200L Recombinant Protein in Whole Blood Cultures

Heparinised whole blood was collected from healthy volunteers and processed within 1 hour of collection, as previously described (Section 2.1). PBMCs were isolated and prepared as previously described (Section 2.7). Recombinant human CD200 Fc Chimera (CD200L) and a control recombinant human IgG1 Fc (Section 2.5) were used. After an initial trial, a concentration of 1 µg/ml was selected for these proteins. 100 µg/ml of PHA would be used to stimulate cells from either PBMC or whole blood, with 1 µg/ml of anisomycin used as a purported positive control in MAPK activation and a negative PBS control. Preliminary testing indicated that isolated PBMC cultures were not as effective in this assay, thus the focus was on whole blood assays. Each stimulant was used in triplicate, with each tube in a set containing either a PBS control, the CD200L protein or the control protein. The whole blood (1ml) was added to 2ml round bottom skirted microfuge tubes, as had been used in whole blood assay experiments (Section 2.12). As the agonistic effects of CD200L were to be observed, blood was stimulated for 1 hour with PHA, anisomycin or PBS controls at 37°C and 5% CO_2 in an incubator, followed by the addition of the CD200L, control protein or PBS control depending on the tube. This was then incubated for a total of 16 hours (15 hours post PHA/anisomycin stimulation) at 37°C and 5% CO_2. The plasma was then extracted using the method previously described (Section 2.12) and stored at -20°C until required.

Plasma was run using the same ELISA method previously applied for whole blood assays and PBMC cultures (Section 2.13). Dilution of the plasma was dependent on the type of stimulant used and the particular cytokine being analysed (Section 2.13 and Section 3.2.7). Plasma was analysed for TNF-α and IL-6 concentrations and the data analysed as

previously described (Section 2.12 and Section 2.16), with dilution of the starting culture accounted for in final calculations.

5.2.5 Immunohistochemistry (IHC) – CD200R and CD200L in Sarcoidosis Tissue

Tissue acquired by informed consent and local ethical approval was obtained in formalin fixed paraffin embedded (FFPE) samples and prepared at 5µm on slides as previously described (Section 2.14). Slides from lung and lymph node biopsies were stained using haematoxylin and eosin as previously described (Section 2.14). The procedure for immunohistochemistry (IHC) was followed as described in Section 2.15. A range of optimising steps with antibodies listed in 2.3.7 were required to identify suitable antibodies for staining CD200L and CD200R. The final CD200L staining utilised the polyclonal goat antibody (R&D, AF2724) and normal goat IgG control (R&D, AB-108-C). The anti-CD200L antibody was reconstituted at 200 µg/ml, the goat IgG was adjusted to match this concentration from stock of 1mg/ml. Antibody concentrations were optimised using dilutions between 1:50 – 1:200; final dilutions of 1:200 were selected based on this preliminary work, leading to a final concentration of 1 µg/ml used in the IHC staining. The CD200R staining used a mouse monoclonal anti-human CD200R IgG1 antibody (AbD Serotec, MCA 2282T) with matched mouse monoclonal IgG1 antibody (Biolegend, 400101). These antibodies were both used at a dilution of 1:50 to obtain a working concentration of 10 µg/ml. For all experiments, a control slide was included that did not have a primary antibody, only the secondary antibody applied. These were all negative for the experiments undertaken.

5.2.6 Correlation between Regulatory Immune Receptor Expression and other Factors

Data acquired from SIRP-α/CD47, IL-10R and CD200R/L expression was correlated with a range of clinical, immunophenotyping and PHA whole blood assay data obtained from the experimental work undertaken as part of Chapter 3. This was treated in the same manner as previously described (Section 3.2.9), though the reduced size of the cohort and more

limited overlapping of experiments meant the total number within each correlation could be smaller than those used previously.

5.3 Results

5.3.1 The Expression of IL-10R on T-lymphocytes and Monocytes

IL-10 cytokine release in whole blood had previously been shown to not be significantly altered in sarcoidosis blood when compared with healthy controls in response to either PHA or SEA (Section 3.3.8 and 3.3.13). In spite of this, there was a robust TNF-α and IL-6 response to PHA in patient samples (Section 3.3.8). Expression of the cognate IL-10 receptor (IL-10R) was therefore characterised on peripheral blood mononuclear cells to determine whether sarcoidosis patients displayed a reduced expression of this receptor on either T-lymphocytes or monocytes that might account for the elevated PHA induced pro-inflammatory cytokine release.

IL-10R was present on both lymphocytes and monocytes and tended to be more heavily expressed in the latter cell type (Figure 5.4). T-lymphocyte mean IL-10R expression was greater in patients with sarcoidosis (GMFI 3.38 ± SEM 0.448) than in healthy controls (GMFI 2.71 ± SEM 0.236), though this was not statistically significant (p = 0.189, Student's T test). The same was true of monocytes, where patients has an increased mean IL-10R expression relative to controls, GMFI 8.47 ± SEM 1.049 compared with GMFI 7.38 ± SEM 0.682 respectively, though this was also not statistically significant between the cohorts (p = 0.385, Student's T test).

The expression of the receptor on monocytes was inversely correlated with the release of IL-6 from PHA stimulated whole blood in patients with sarcoidosis (p = 0.033, R^2 = 0.344), but increased T-lymphocyte IL-10R expression was not associated with reduced IL-6 release (Figure 5.5). TNF-α release was not associated with the level of IL-10R expression on either cell type. There was not enough data available to be able to identify whether these associations existed in healthy controls. There was also no direct correlation between IL-10 release in response to PHA and IL-10R expression in either monocytes or T

cells in pooled donors, though the numbers for this were very limited (data not shown). There was no statistically significant correlation between leukocyte cell number and the expression of IL-10R in either heathy or sarcoidosis patients either.
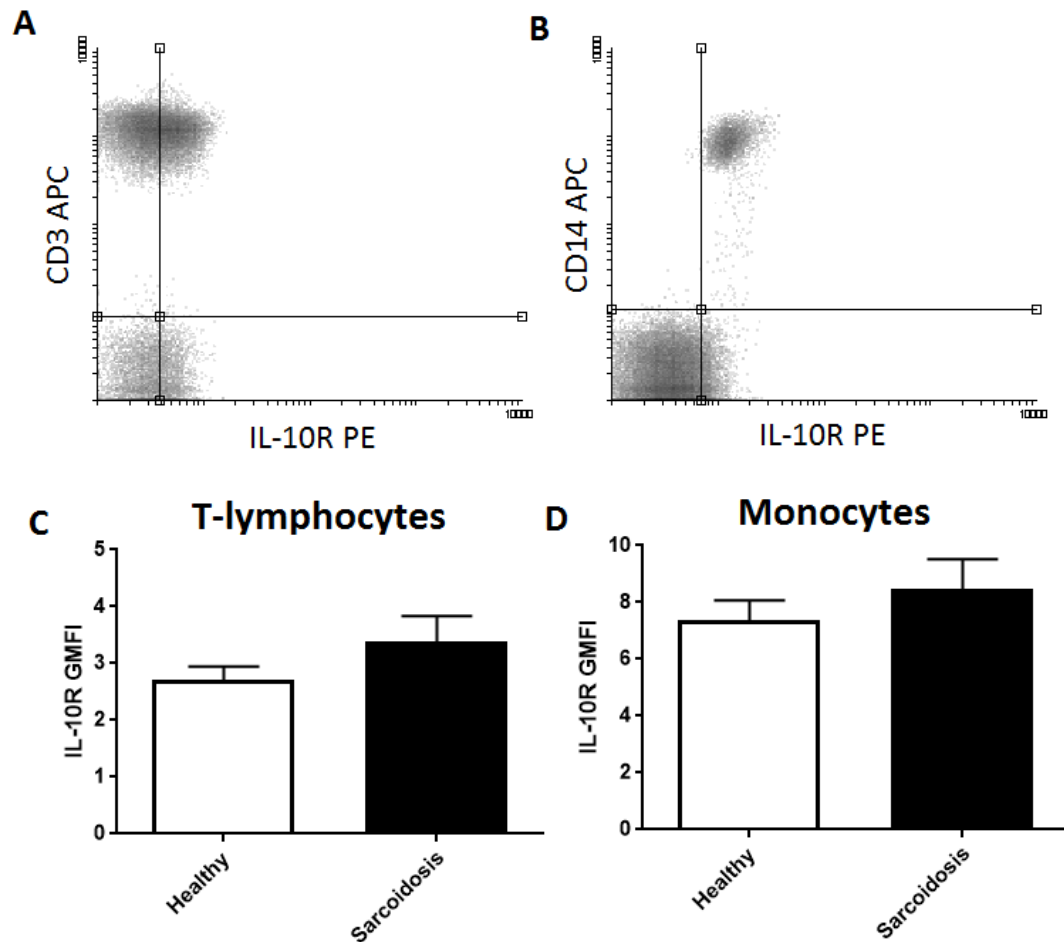
Figure 5.4: IL-10 receptor (IL-10R) expression on T-lymphocytes and monocytes in patients with sarcoidosis and healthy controls.

A: Representative density plot of IL-10R expression on lymphocyte gated healthy PBMCs. CD3 was used to differentiate T-lymphocytes from other lymphocyte subpopulations. Quadrant set to 1% of isotype control on lymphocytes. **B:** Representative density plot of IL-10R expression on healthy PBMCs. CD14 was used to identify monocytes from other PBMC subpopulations. Quadrant set to 1% of isotype control on whole PBMC population. **C:** IL-10R expression on T-lymphocytes in patients with sarcoidosis (black, n = 9) compared with healthy controls (white, n = 10). No statistical significance (p = 0.189), as determined using a Student's T test. **D:** IL-10R expression on monocytes in patients with sarcoidosis (black, n = 9) compared with healthy controls (white, n = 10). No statistical significance (p = 0.385), as determined using a Student's T test. Data in **C** and **D** displayed as mean ± SEM.
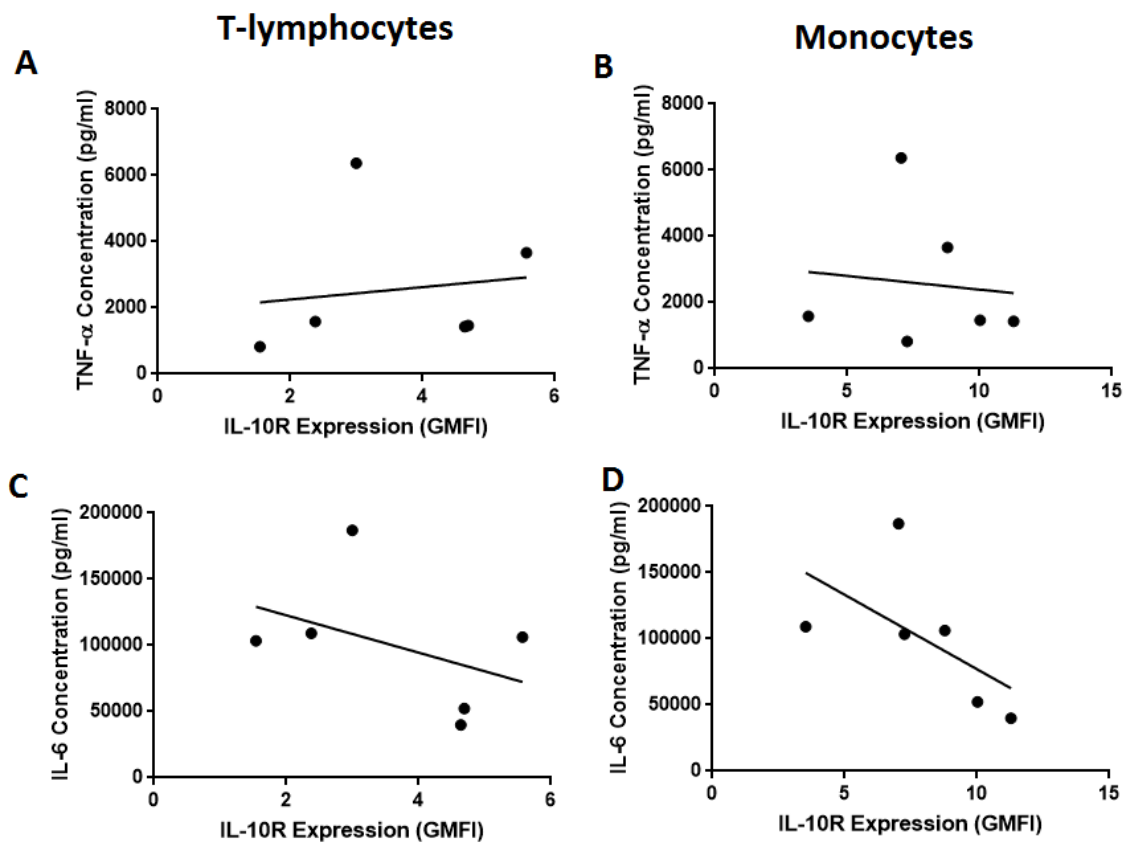
Figure 5.5: IL-10R expression correlated with PHA induced cytokine release from whole blood assays in patients with sarcoidosis.

T-lymphocyte IL-10R expression (**A** and **C**) and monocyte IL-10R expression (**B** and **D**) were correlated with TNF-α release (**A** and **B**) and IL-6 release (**C** and **D**) from whole blood in response to 100 µg/ml stimulation with PHA. There was no statistically significant correlation in **A** (p = 0.497, R^2 = 0.019), **B** (p = 0.419, R^2 = 0.011) or **C** (p = 0.658, R^2 = 0.179). There was a correlation between IL-6 release and IL-10R expression on monocytes (**D**), ***** p = 0.033, R^2 = 0.344. Black line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.

In patients with sarcoidosis, reduced IL-10R expression on T-lymphocytes was associated with higher serum ACE levels (Figure 5.6, p = 0.034, R^2 = 0.411), though there was no correlation between monocyte IL-10R expression and serum ACE (p = 0.613, R^2 < 0.001). Although there was a trend in monocyte IL-10R to correlate inversely with levels of CRP and PV (Figure 5.6), neither this nor the T-lymphocyte IL-10R were statistically significant. Other clinical features were not statistically significant when correlated with IL-10R expression (Appendix A5.1), the low numbers (n=9) means that it is difficult to state with

confidence whether an association exists when 2 or more groups are used to stratify this data.
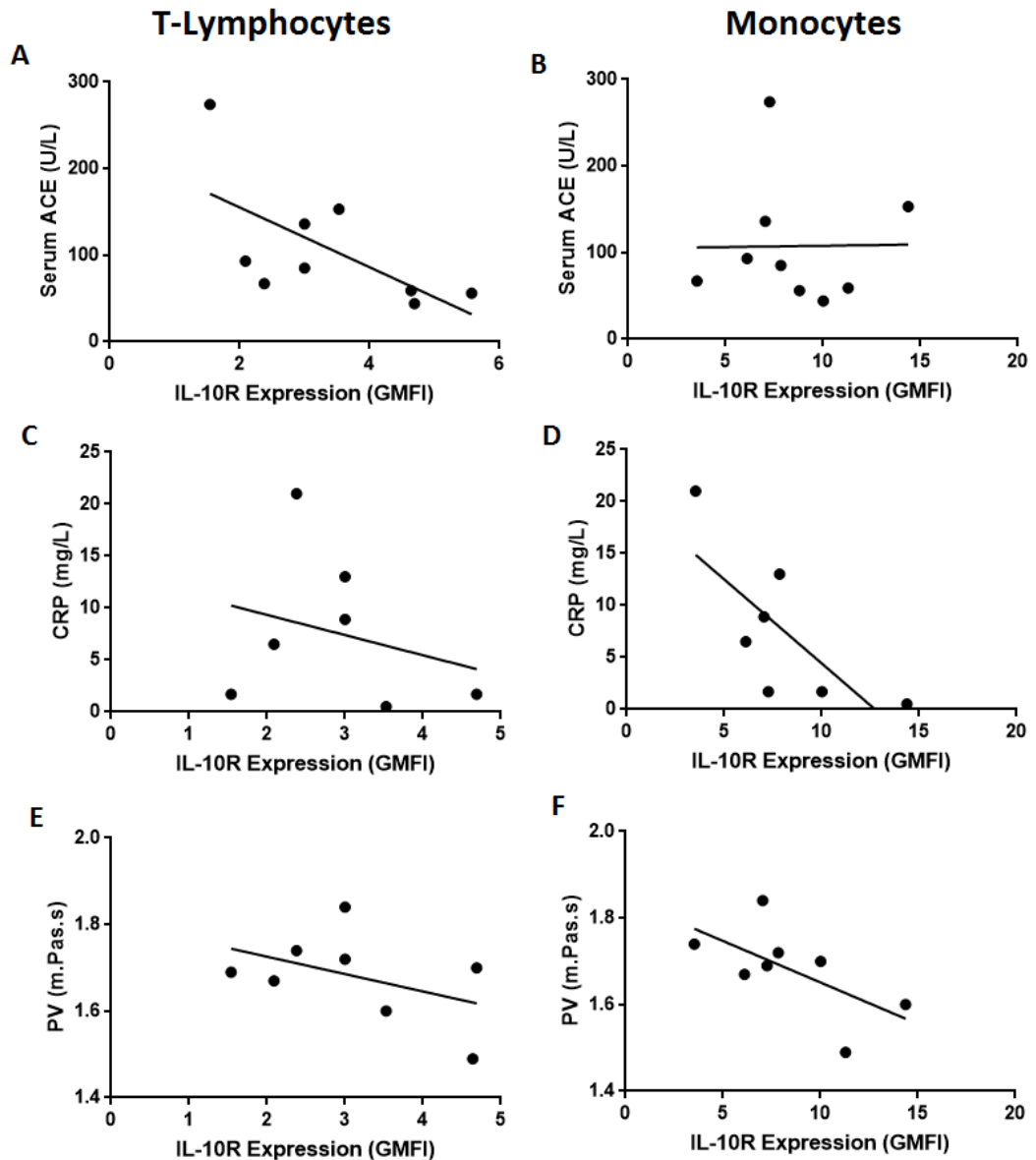
Figure 5.6: IL-10R expression correlated with serum ACE, C-Reactive Protein (CRP) and Plasma Viscosity (PV) in patients with sarcoidosis.

T-lymphocyte IL-10R expression (**A**, **C** and **E**) and monocyte IL-10R expression (**B, D** and **F**) were correlated with serum ACE (**A** and **B**), CRP (**C** and **D**) and PV (**E** and **F**). There was a statistically significant inverse correlation between serum ACE and T-lymphocyte IL-10R expression (**A:** * p = 0.034, R^2= 0.411). There was no statistically significant correlation in **B** (p = 0.613, R^2 < 0.001), **C** (p = 0.559, R^2 = 0.073), **D** (p = 0.079, R^2 = 0.549), **E** (p = 0.609, R^2 = 0.198) or **F** (p = 0.115, R^2 = 0.393)**.** Black line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.

5.3.2 SIRP-α Expression on Monocytes and CD47 Ligand Expression on Peripheral Blood Leukocytes

Unlike IL-10R, SIRP-α is a receptor with a ligand that is bound to the extracellular surface of other cells. The cognate ligand CD47 is itself a receptor that is widely expressed, though SIRP-α has been described as being myeloid specific in expression. To identify whether monocyte SIRP-α expression was increased or decreased in sarcoidosis, PBMCs were stained for this receptor in patients and healthy controls using flow cytometry. Unlike IL-10R, this receptor was restricted to expression on monocytes (Figure 5.7), confirming other reports that this receptor is myeloid specific [830]. All CD14$^+$ cells were SIRP-α positive. Patients with sarcoidosis expressed a remarkably similar level of SIRP-α to healthy controls (Figure 5.7), mean GMFI of 745.3 (± SEM 110.7) compared with 812.2 (± SEM 116.2) respectively. The data indicates that the SIRP-α expression on monocytes is not significantly altered in sarcoidosis.

Despite the similar expression levels of SIRP-α displayed by sarcoidosis patients, the possibility exists that the CD47 ligand that engages with this regulatory receptor could be altered. The ligand was heavily expressed on lymphocyte cell types and monocytes (Figure 5.8). Both CD3$^+$ and CD3$^-$ lymphocytes expressed this ligand, as well as CD14$^+$ cells within PBMCs. Neither T-lymphocytes nor monocytes in sarcoidosis displayed a statistically significant difference in CD47 expression compared with healthy controls (Figure 5.8), though the median in patient monocytes was higher than in controls (GMFI of 410.9 compared with 272.3 respectively). Overall, this suggests that the SIRP-α/CD47 regulatory axis is not significantly altered in sarcoidosis blood leukocytes.
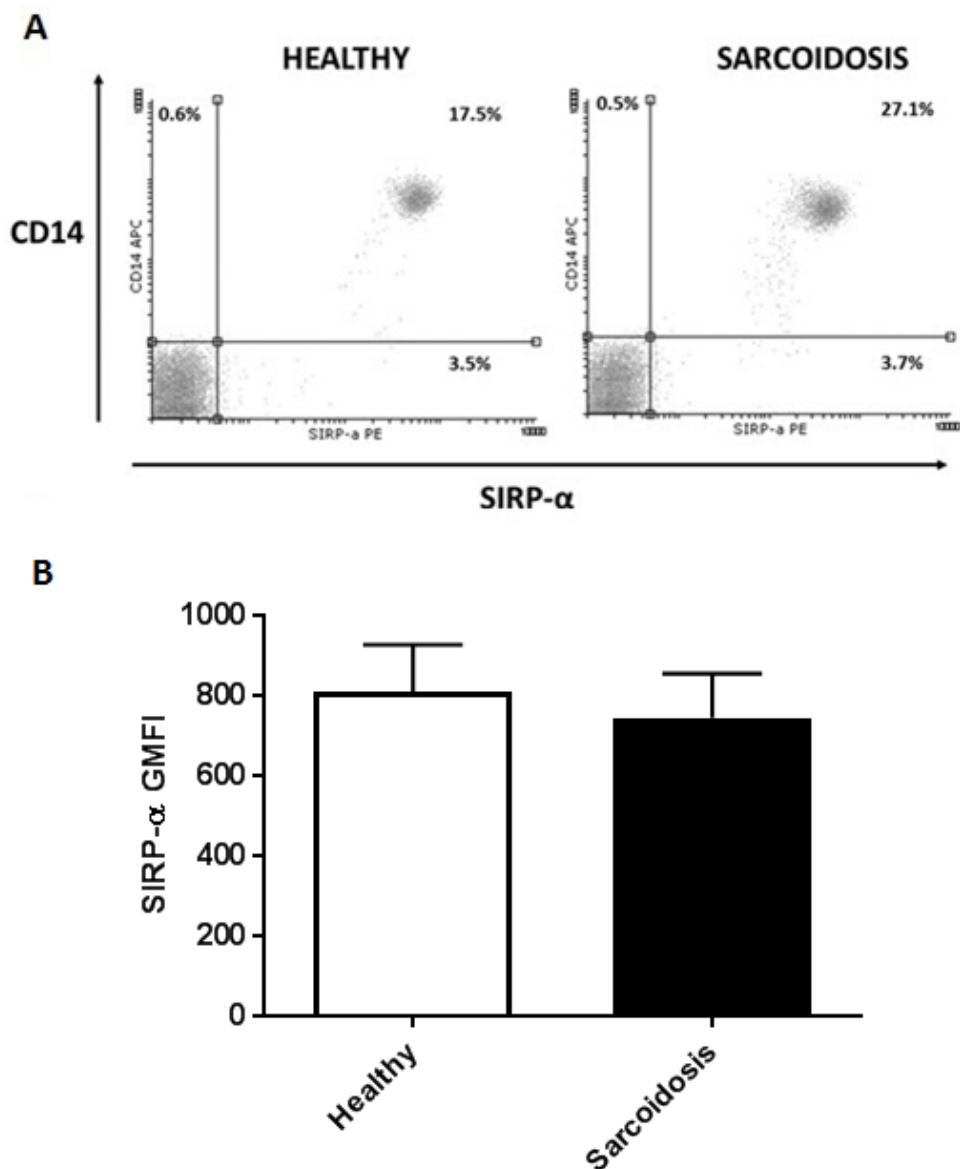
Figure 5.7: SIRP-α expression on monocytes in patients with sarcoidosis and healthy controls.

A: Representative density plots obtained from extracellular staining of PBMCs in a healthy control and a sarcoidosis patient. SIRP-α^{+} cells were exclusively CD14^{+} (monocytes); the proportionally higher percentage of monocytes within the PBMC population is representative of the reduced T-lymphocyte population. **B:** SIRP-α receptor expression (GMFI) on monocytes in healthy controls (white bar, n = 10) and patients with sarcoidosis (black bar, n=9). There was no statistically significant difference in expression (p = 0.684), as determined by Student's T test. Data presented as mean ± SEM.
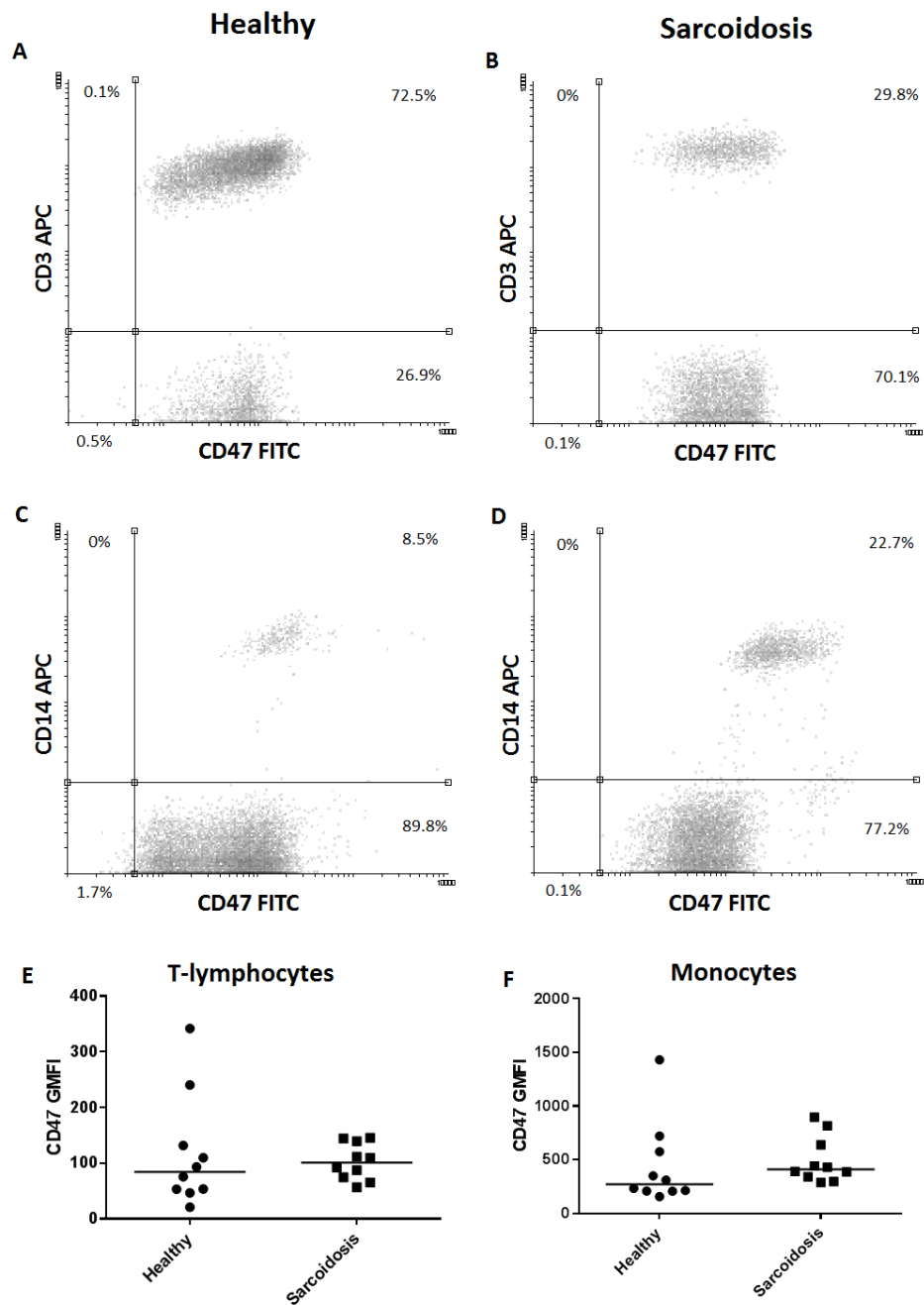
Figure 5.8: CD47 expression on PBMCs in patients with sarcoidosis and healthy controls.

A and **B:** Representative density plots of gated lymphocytes stained with anti-CD3 and anti-CD47 from healthy controls and patients with sarcoidosis respectively. Percentages represent proportion of lymphocytes. **C** and **D:** Representative density plots of PBMCs stained with anti-CD14 and anti-CD47 from healthy controls and patients with sarcoidosis respectively. Percentages represent proportion of total PBMCs. **E** and **F:** CD47 expression in T-lymphocytes (**E**) and monocytes (**F**) in controls and patients with sarcoidosis. Data presented with median (black line) of the GMFI (geometric mean fluorescence intensity). No statistical difference determined using Mann-Whitney U tests, p = 0.481 and p = 0.143 for **E** and **F** respectively.

5.3.4 CD200 Receptor Expression on Peripheral Blood Mononuclear Cells

The CD200R/CD200L axis acts to dampen inflammatory responses by reducing MAPK signalling in leukocytes [824]. SIRP-α expression was similar in monocytes between patients with sarcoidosis and healthy controls. IL-10R was, if anything, likely to be more heavily expressed in patients compared to controls. The expression of CD200R and CD200L was characterised to identify which mononuclear leukocytes expressed these proteins and see if there was any difference in the level of expression that might account for the elevated pro-inflammatory responses observed in sarcoidosis patients observed previously (Section 3.3).

5.3.4.1 CD200R Expression on Monocytes

CD200R was expressed on both the majority of T-lymphocytes and monocytes, but was absent on CD3⁻ lymphocytes (Figure 5.9). On monocytes, the median CD200R expression was reduced in patients with sarcoidosis (GMFI 6.62) compared with healthy controls (GMFI 14.16); both cohorts displaying non-Gaussian distribution (Figure 5.10 B). This was a statistically significant reduction in CD200R expression in patients relative to controls (p = 0.046, Kolmogorov–Smirnov test), with sarcoidosis patients clustering as either high or low expressers. Using a boundary defining low expression below the standard deviation of the healthy population (geometric mean <7.28 fluorescence units), 52.9% of sarcoidosis patients were classified as CD200R^{low} compared with 11.7% of healthy donors (p < 0.0001), the remaining individuals labelled as CD200R^{high}. CD200R on monocytes was inversely correlated with higher IL-6 and TNF-α release from PHA induced whole blood assays in patients with sarcoidosis (Figures 5.10 C and E), narrowly missing statistical significance (p = 0.071 and 0.089 respectively). When healthy controls and patients were pooled, IL-6 release was greater in CD200R^{low} individuals compared with CD200R^{high}, a mean 134239 pg/ml ± SEM 22062 compared with 85010 pg/ml ± SEM 7929 respectively (p = 0.018, Student's T test). Mean TNF-α release was also higher in CD200R^{low} individuals compared with CD200R^{high}, 4570 pg/ml ± SEM 1336 compared with 3026 pg/ml ± SEM 473 respectively, though this was not statistically significant (p = 0.191, Student's T test). This data suggested that a proportion of patients with sarcoidosis had reduced level

CD200R expression on their monocytes which might influence the release of IL-6 and possibly TNF-α release in PHA induced activation of peripheral blood leukocytes.
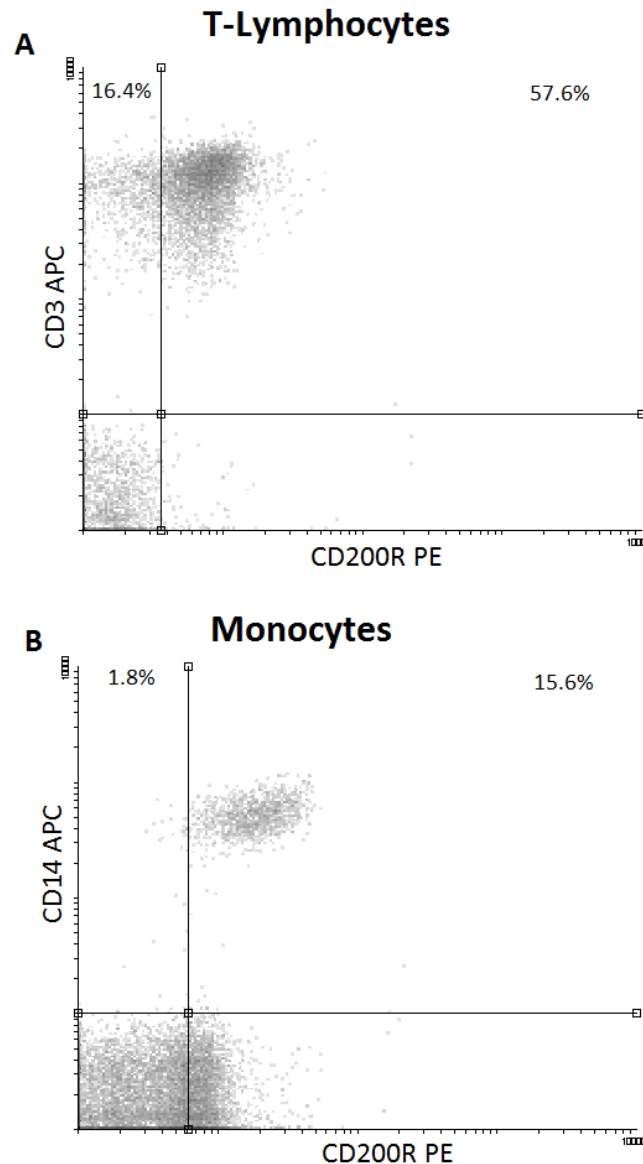
Figure 5.9: Representative density plots of CD200R expression on PBMCs from a healthy control, as detected by flow cytometry.

Figure 5.9 – Representative density plots of CD200R expression on PBMCs from a healthy control, as detected by flow cytometry. **A:** CD200R expression on lymphocyte gated cells, CD3+ T-lymphocytes (upper quadrants) are mostly CD200R+ (upper right quadrant). Percentages displayed represent proportion of lymphocytes; quadrant set to control antibody on gated lymphocyte cells. **B:** CD200R expression on monocytes in PBMCs. The vast majority of monocytes (CD14+) are CD200R+. Percentages displayed represent proportion of PBMCs; quadrant set to control antibody on mixed PBMCs.
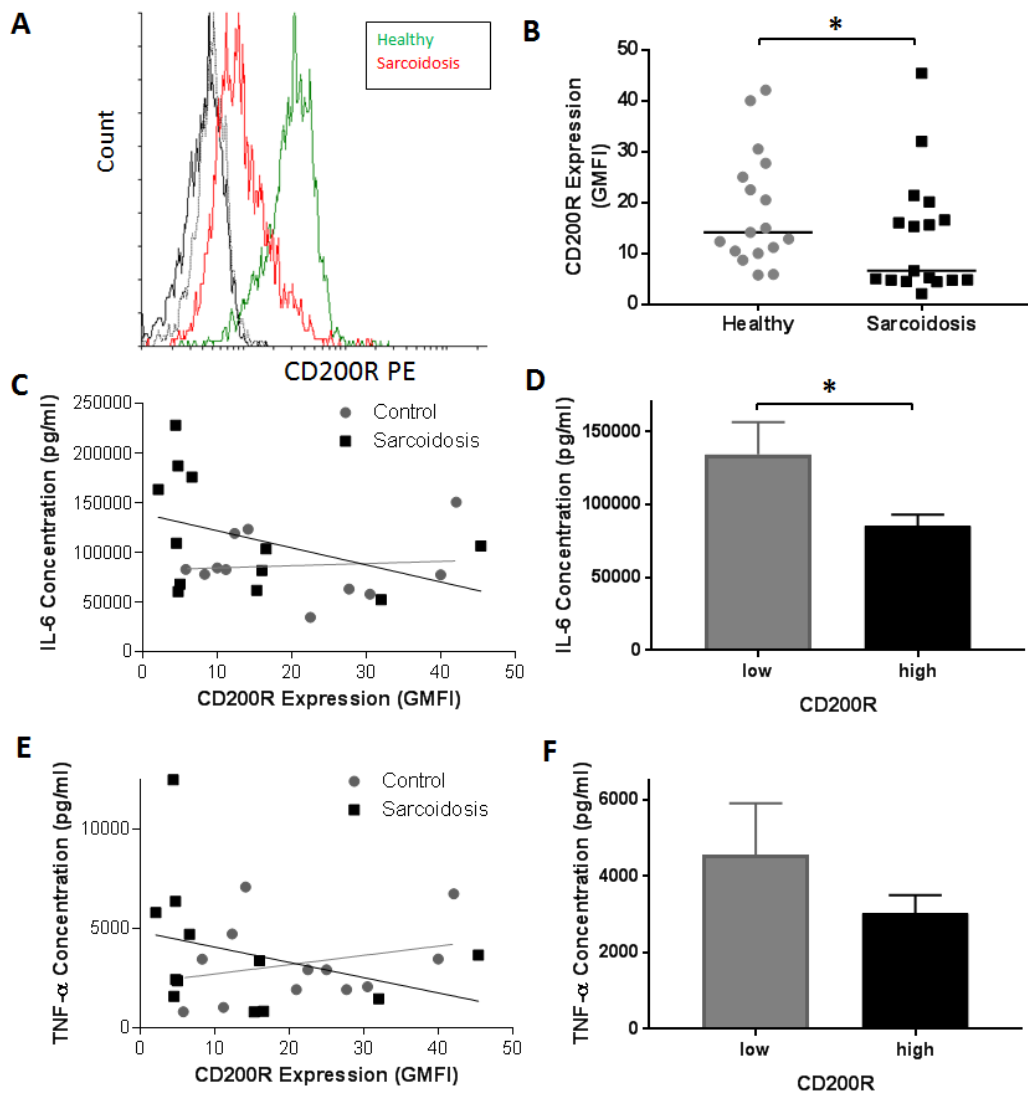
Figure 5.10: CD200R expression on monocytes in patients with sarcoidosis and healthy controls and the correlation between receptor expression and pro-inflammatory cytokine release.

A: Representative flow cytometry histograms showing CD200R expression on monocytes from a patient with sarcoidosis and a healthy control subject. Grey dotted and black dashed lines, isotype control antibodies in healthy control and sarcoidosis respectively; green solid line, CD200R healthy; red solid line, CD200R sarcoidosis. **B:** CD200R expression on monocytes in sarcoidosis and control subjects. Median is indicated by horizontal lines; * p = 0.046 determined using the Kolmogorov–Smirnov test; n = 17 healthy controls, n = 17 sarcoidosis patients. **C:** Relationship between monocyte CD200R expression and whole blood IL-6 release in response to stimulation with 100μg PHA. Black line, sarcoidosis linear regression (n = 13, p = 0.071, R^2 = 0.154); grey line, control linear regression (n = 10, p = 0.673, R^2 = 0.007). **D:** PHA stimulated IL-6 release in CD200R^{low} (n = 8) and CD200R^{high} (n = 15) subjects; * p = 0.018 using Student's T test. **E:** Relationship between monocyte CD200R expression and whole blood TNF-α release in response to stimulation with 100μg PHA. Black line, sarcoidosis linear regression (n = 12, p = 0.089, R^2 = 0.096); grey line, control linear regression (n = 12, p = 0.230, R^2 = 0.076). **F:** PHA stimulated TNF-α release in CD200R^{low} and CD200R^{high} subjects; p = 0.19 using Student's T test.

5.3.4.2 CD200R Expression on T-lymphocytes

CD200R expression on T lymphocytes in sarcoidosis also polarised as predominantly either 'CD200R low' or 'CD200R high' in a bimodal distribution, compared with a unimodal distribution in healthy controls (Figure 5.11). Overall, the median CD200R expression was not significantly different between patients and controls (p = 0.240, Kolmogorov–Smirnov test). This expression of T-lymphocyte CD200R did correlate positively with monocyte CD200R in patients with sarcoidosis (p = 0.026, R^2 = 0.262), though some individuals would express various combinations of either monocyte or lymphocyte CD200R; for example, some would have low CD200R monocyte expression but high T-lymphocyte CD200R expression (Figure 5.11 B).

Using a similar strategy to categorise CD200R expression into groups, T-lymphocyte CD200R was categorised as either CD200R low, medium or high based upon the standard deviation of the healthy population. To distinguish this from monocyte CD200R descriptions, T-lymphocyte CD200R was labelled CD200R$^+$, CD200R^{++} and CD200R^{+++} for low, medium and high expression respectively. These boundaries were defined based upon 1 standard deviation of the healthy population, so that-lymphocyte CD200R$^+$ = GMFI of below 4.55, CD200R^{+++} = above GMFI of 8.8 and CD200R^{++} = between 4.55 and 8.8. Using this, healthy controls were predominantly CD200R^{++} (53%), with equal numbers of CD200R$^+$ and CD200R^{+++} (23.5%), in keeping with a normal distribution. Patients with sarcoidosis were predominantly CD200R^{+++}, 53%, with CD200R$^+$ comprising 35% of the cohort. The dual expression profile with T-lymphocyte and monocyte CD200R for each cohort is shown in **Figure 5.12**, and shows that healthy controls did not have CD200R$^{+/low}$ or CD200R$^{+++/low}$ phenotypes displayed. The only monocyte low CD200R in healthy controls was associated with T-lymphocyte CD200R^{++}, unsurprising given that the majority of the population (53%) belonged to this group. Patients with sarcoidosis tended to either be CD200R$^{+/low}$ or CD200R$^{+++/high}$, though CD200R$^{+++/low}$ was also present in 3 patients. In general, patients with sarcoidosis polarised to the extremes in terms of their CD200R expression, compared to healthy controls who predominantly expressed high CD200R on monocytes.
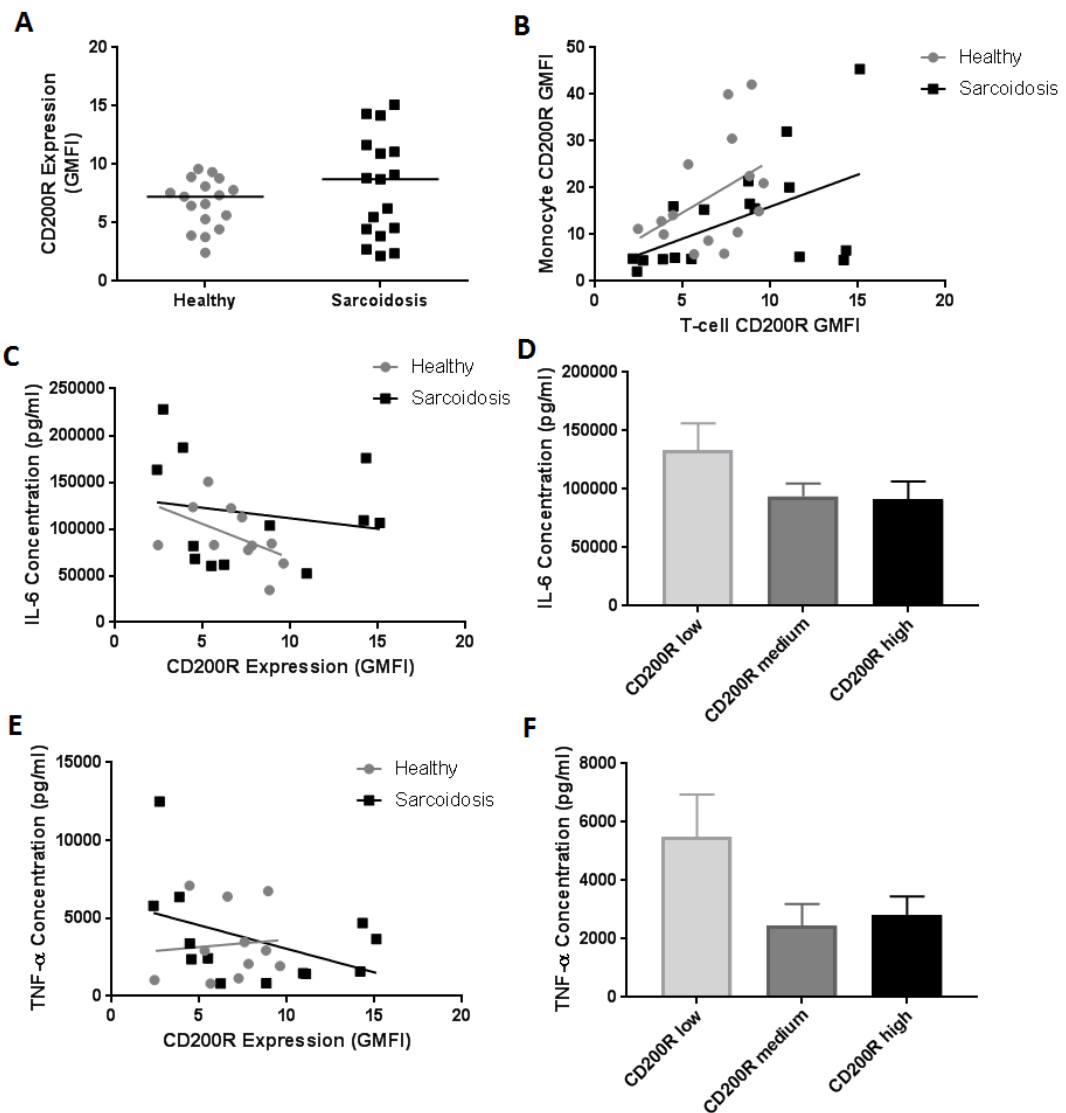
Figure 5.11: CD200R expression on T-lymphocytes in patients with sarcoidosis and healthy controls and the correlation between receptor expression and pro-inflammatory cytokine release.

A: CD200R expression on T-lymphocytes in sarcoidosis and control subjects. Median is indicated by black horizontal lines; p = 0.240 determined using the Kolmogorov–Smirnov test; n = 17 healthy controls, n = 17 sarcoidosis patients. **B:** Association between CD200R expression on monocytes and T-lymphocytes in sarcoidosis and control subjects. Black line, sarcoidosis linear regression (n = 17, * p = 0.026, R^2 = 0.262); grey line, control linear regression (n = 15, p = 0.109, R^2 = 0.186). **C:** Relationship between T-lymphocyte CD200R expression and whole blood IL-6 release in response to stimulation with 100 µg PHA. Black line, sarcoidosis linear regression (n = 12, p = 0.417, R^2 = 0.034); grey line, control linear regression (n = 11, p = 0.052, R^2 = 0.240). **D:** PHA stimulated IL-6 release from pooled healthy controls and sarcoidosis patients in CD200R^{low} (n = 7), CD200R^{medium} (n = 8) and CD200R^{high} (n = 8) subjects; p > 0.05 using One-way ANOVA. **E:** Relationship between T-lymphocyte CD200R expression and whole blood TNF-α release in response to stimulation with 100 µg PHA. Black line, sarcoidosis linear regression (n = 13, p = 0.141, R^2 = 0.190); grey line, control linear regression (n = 11, p = 0.673, R^2 = 0.008). **F:** PHA stimulated TNF-α release from pooled healthy controls and sarcoidosis patients in CD200R^{low} (n = 7), CD200R^{medium} (n = 7) and CD200R^{high} (n = 9) subjects; p > 0.05 using One-way ANOVA.
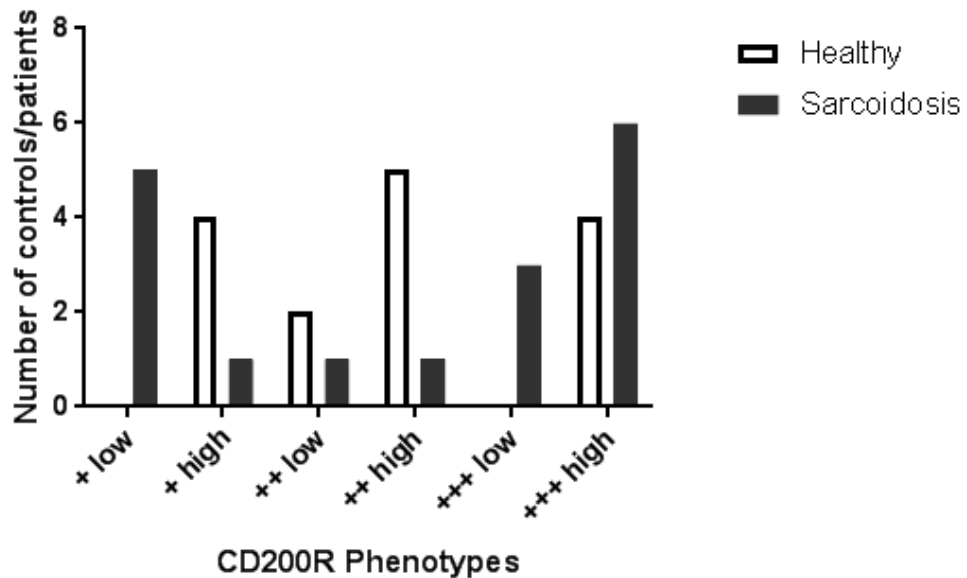
Figure 5.12: The number of healthy controls and patients with sarcoidosis displaying specific phenotypes of CD200R expression on their T-lymphocytes and monocytes.

Patients with sarcoidosis (n = 17, black bars) and healthy controls (n = 15, white bars) are displayed in each category of CD200R expression. Monocytes were classified as 'low' based on GMFI values below 1 standard deviation of the mean GMFI of healthy control CD200R expression (below 7.28), 'high' were values above this value. T-lymphocyte CD200R expression was separated into 3 profiles labelled '+', '++' or '+++' representing low, medium and high expression respectively. This was based on GMFI values for expression in healthy controls, + = GMFI values below 1 SD of the mean (below 4.55), ++ = GMFI values within 1 SD of the mean (within 4.55 – 8.8) and +++ = GMFI values above 1 SD of the mean (above 8.8). Patients with sarcoidosis were classified using these boundaries from healthy controls.

IL-6 release from PHA stimulated whole blood was correlated with healthy control T-lymphocyte CD200R expression (p = 0.052, R^2 = 0.240) more than sarcoidosis patients (p = 0.417, R^2 = 0.034). There was little correlation between T-lymphocyte CD200R expression and TNF-α release in either patients or controls (Figures 5.11 C and E). T-lymphocyte CD200R medium and high did not display any significant difference in IL-6 and TNF-α release from PHA induced whole blood assays when patients and controls were pooled together. There was a tendency for CD200R+ (low T-lymphocyte expression) individuals to release more IL-6 or TNF-α, though this was not statistically significant (p > 0.05 for both,

One-way ANOVA). Whilst the numbers in the cohort were limited, a preliminary breakdown of IL-6 and TNF-α release from pooled individuals who were stratified based upon whether they were CD200R$^{+/low}$, CD200R$^{+/high}$, CD200R$^{++\text{ or }+++/low}$ or CD200R$^{++/\text{ or}}$ $^{+++/high}$ suggested that the greatest release in these cytokines comes within individuals who are phenotypically low in both T-lymphocyte and monocyte CD200R expression (Figure 5.13). Separating only the T-lymphocyte high or low expressing sarcoidosis patients does show a trend towards higher pro-inflammatory cytokine release (Figures 5.13 E and F), though this was not statistically significant given the low numbers of patients involved. CD200R in T-lymphocytes clearly has an impact on TNF-α and IL-6 release in PHA stimulated whole blood, though whether this is the predominant influence compared with monocytes is not known, though there are a greater number of monocyte CD200R^{low} individuals than T-lymphocyte CD200R^{+} in sarcoidosis patients.

5.3.4.3 Correlation between CD200R expression and the Leukocyte Populations

The expression of CD200R was correlated against leukocyte populations to identify whether the number of T-lymphocytes or monocytes may influence the expression. In patients with sarcoidosis, neither the number of T-lymphocytes nor the number of monocytes appeared to correlate with the expression of CD200R on either cell type (Figure 5.14). In healthy controls, the expression of monocyte CD200R positively correlated with T-lymphocyte number (Figure 5.14 B, $p = 0.027$, $R^2 = 0.512$), though no other associations were statistically significant. The importance of the number of leukocytes in CD200R expression appeared limited. The percentage of classical, non-classical and intermediate monocyte subsets within sarcoidosis patients did not correlate with either monocyte or T-lymphocyte CD200R expression (Appendix A5.2), though the expression of the receptor on each individual monocyte subset had not been analysed. This would be an area for future study to identify whether the receptor is differentially expressed within the monocyte population.
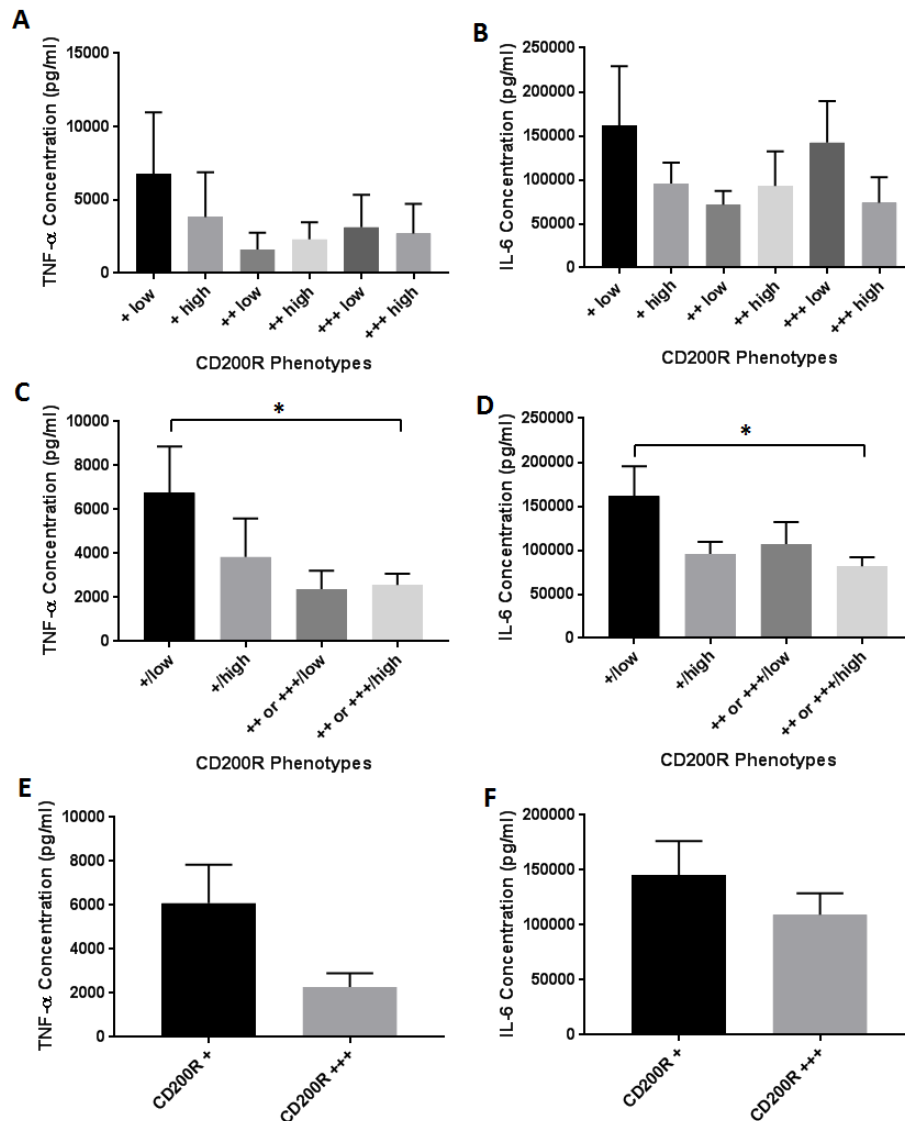
Figure 5.13: – Associations between different CD200R expression profiles and the release of pro-inflammatory cytokines from PHA stimulated whole blood.

CD200R phenotypes are represented as '+', '++' or '+++' for low, medium and high expression of the receptor on T-lymphocytes. Monocyte CD200R was either 'low' or 'high', individuals were classified ranging from CD200R$^{+/low}$ to CD200R$^{+++/high}$. TNF-α release from whole blood assays stimulated with 100 μg/ml of PHA is represented in **A**, **C** and **E,** IL-6 is shown in **B**, **D** and **F**. All data displayed as mean ± SEM. **A** and **B:** TNF-α and IL-6 release from pooled healthy controls and sarcoidosis patients with different CD200R expression profiles. Each phenotype is represented separately. For all data, p > 0.05 using One-way ANOVA, n = 21 for IL-6 release and n = 22 for TNF-α release. **C** and **D:** TNF-α and IL-6 release from pooled healthy controls and sarcoidosis patients comparing CD200R^{+} phenotypes against combined CD200R^{++} and CD200R^{+++} phenotypes; T-lymphocyte medium and high expression subsets were combined. * p = 0.042 and p = 0.029 for TNF-α and IL-6 respectively, determined using One-way ANOVA. **E** and **F:** TNF-α and IL-6 release from patients with sarcoidosis comparing CD200R^{+} and CD200R^{+++} without monocyte expression considered. Neither was statistically significant, p = 0.052 and p = 0.547 for TNF-α and IL-6 respectively, determined using Mann-Whitney U test. TNF-α, n = 5 and n = 6 for CD200R^{+} and CD200R^{+++} respectively. IL-6, n = 5 and n = 5 for CD200R^{+} and CD200R^{+++} respectively.
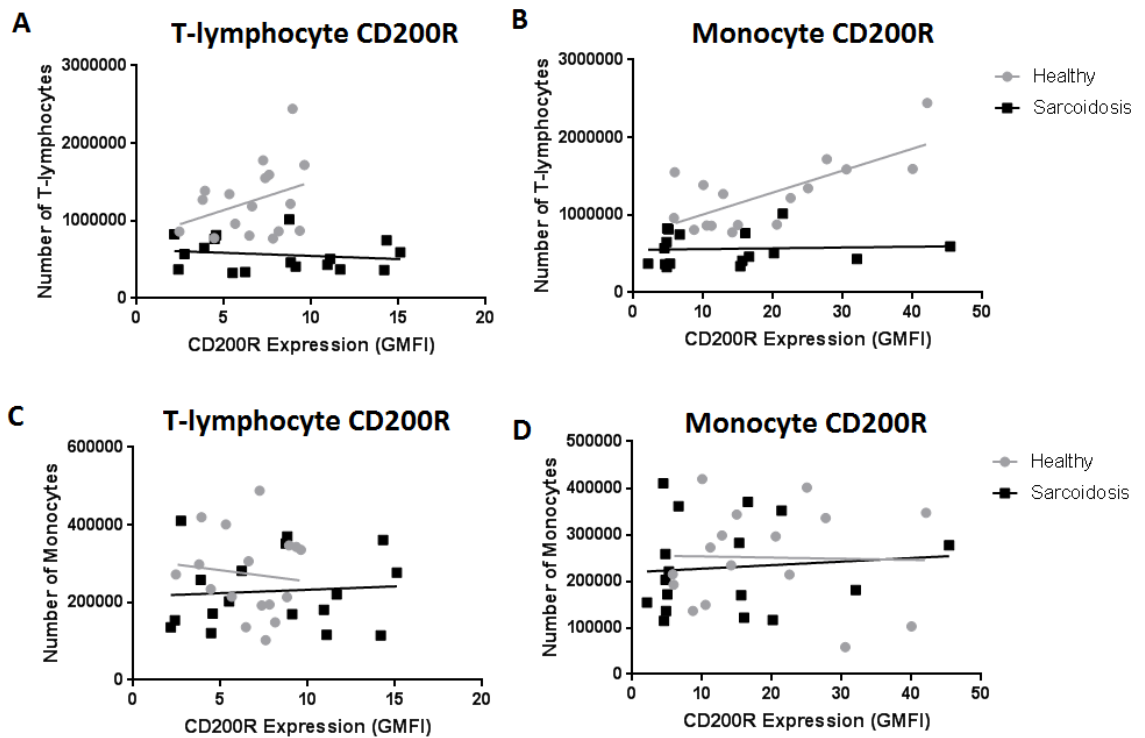
Figure 5.14: Correlation between T-lymphocyte or monocyte CD200R expression and the number of T-lymphocytes or monocytes per ml of blood from patients with sarcoidosis and healthy controls.

Healthy controls are represented by grey circles, patients with sarcoidosis by black squares. Black line represents the sarcoidosis linear regression, grey line represents the control linear regression. **A:** Correlation between T-lymphocyte CD200R expression and the number of T-lymphocytes. No significant correlation in sarcoidosis patients (n = 17, p = 0.372, R^2 = 0.029) or healthy controls (n = 17, p = 0.294, R^2 = 0.114). **B:** Correlation between monocyte CD200R expression and the number of T-lymphocytes. No significant correlation in sarcoidosis patients (n = 17, p = 0.352, R^2 = 0.003). There was a statistically significant association present in healthy controls (n = 16, * p = 0.027, R^2 = 0.512). **C:** Correlation between T-lymphocyte CD200R expression and the number of monocytes. No significant correlation in sarcoidosis patients (n = 17, p = 0.787, R^2 = 0.006) or healthy controls (n = 17, p = 0.787, R^2 = 0.013). **D:** Correlation between monocyte CD200R expression and the number of monocytes. No significant correlation in sarcoidosis patients (n = 17, p = 0.625, R^2 = 0.008) or healthy controls (n = 16, p = 0.697, R^2 < 0.001).

5.3.4.4 The Association between Age and Gender with CD200R expression

There was a skew in age and gender in the sarcoidosis cohort, with a statistically significant difference in the former between patients and controls; patients had a higher mean age and were predominantly male. It was necessary to correlate the age and gender with the expression of the CD200R receptor to identify whether the differences observed in patients were due to these factors. There was no statistically significant association between age and CD200R expression on either T-lymphocytes (p = 0.945, R^2 = 0.009) or monocytes (p = 0.361, R^2 = 0.060) in healthy controls, suggesting that age is not a factor for CD200R expression under normal conditions (Figures 5.15 A and B). However, CD200R positively correlated with age in sarcoidosis patients, on both T-lymphocytes (p = 0.008, R^2 = 0.433) and monocytes (p = 0.042, R^2 = 0.226). As patients tended to be older than healthy controls, the fact that CD200R expression increases with increased age in patients only suggests that the observed reduction in CD200R on patients is a feature specific to the disease.

The impact of gender on CD200R expression is more complex (Figures 5.15 C - F). Female patients tended to express higher T-lymphocyte CD200R than their healthy counterparts (p = 0.043), though there was no significant difference between male patients and male healthy controls (p = 0.930). Whilst male patients, who comprised the majority of the cohort, made up the majority of the CD200R^{low} monocyte expression in the patient group, healthy control males had a higher median than female controls. There was a significant difference in CD200R monocyte expression between male patients with sarcoidosis and male controls (p = 0.035), suggesting that the features of gender may be specific to the disease, rather than being a component of the wider population as a whole.
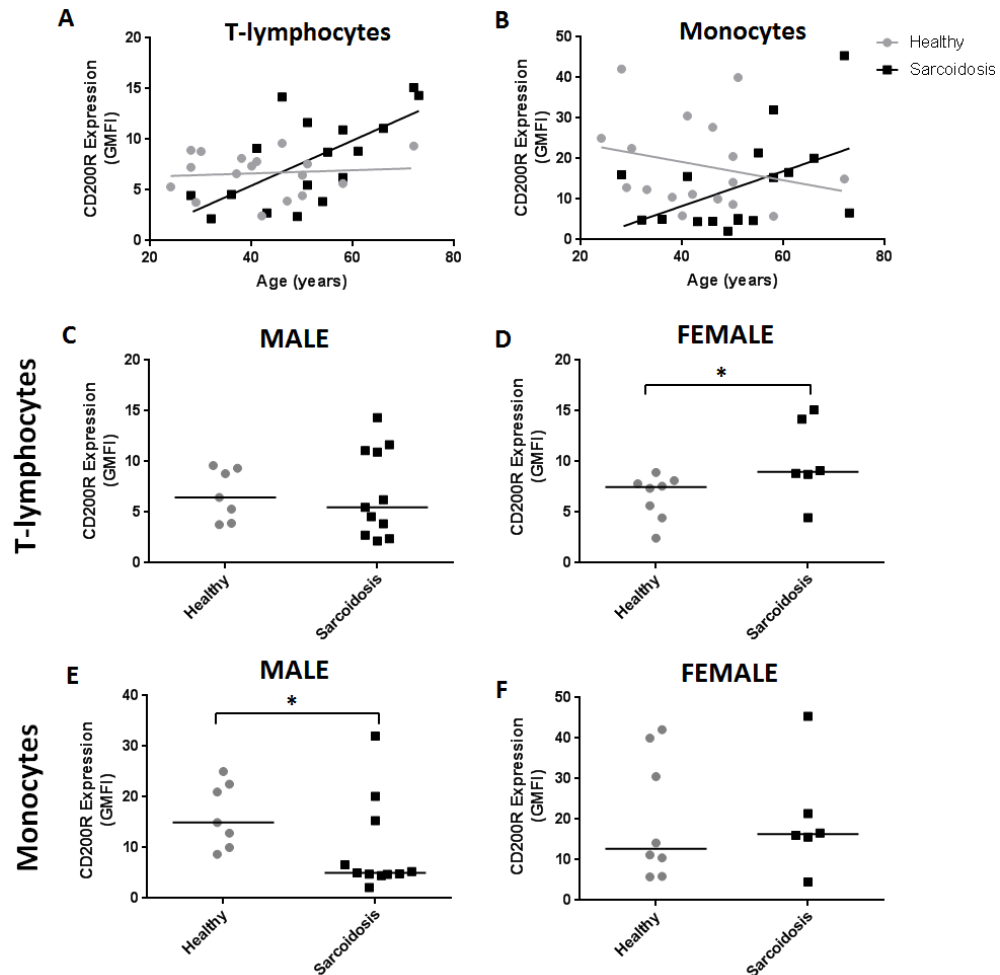
Figure 5.15: Correlation between T-lymphocyte and monocyte CD200R expression and age or gender in healthy controls and patients with sarcoidosis

Correlation between the age of patients (black squares) and controls (grey circles) with T-lymphocyte CD200R expression (GMFI). Black line, sarcoidosis linear regression (n = 17, ** p = 0.008, R^2 = 0.433); grey line, control linear regression (n = 17, p = 0.945, R^2 = 0.009). **B:** Correlation between the age of patients (black squares) and controls (grey circles) with monocyte CD200R expression (GMFI). Black line, sarcoidosis linear regression (n = 17, * p = 0.042, R^2 = 0.226); grey line, control linear regression (n = 17, p = 0.361, R^2 = 0.060). **C** and **D:** CD200R expression on T-lymphocytes in males and females respectively. Male controls and patients, p = 0.930; female controls and patients, * p = 0.043. Determined using the Mann-Whitney U test. **E** and **F:** CD200R expression on monocytes in males and females respectively. Male controls and patients, * p = 0.035; female controls and patients, p = 0.662. Determined using the Mann-Whitney U test. **C**, **D**, **E** and **F** data presented with median (black line).

5.3.5 CD200 Ligand Expression on PBMCs

CD200R expression on monocytes was demonstrably lower in a proportion of sarcoidosis patients, as was CD200R expression on T-lymphocytes in over a third of patients. The receptor's cognate ligand, CD200L, might also influence the release of pro-inflammatory cytokines in the assays previously used. The distribution of the ligand on peripheral blood leukocytes was characterised and the proportion of CD200L expression was also quantified on both healthy controls and patients with sarcoidosis in order to identify whether there were differences in the disease.

Expression of CD200L was absent on monocytes, but present on lymphocyte populations (Figure 5.16). CD200L was present on a subset of T-lymphocytes in both healthy controls and patients with sarcoidosis (Figures 5.16 A and B). The highest level of expression was observed on CD19$^+$ lymphocytes, B-cells, in which the overwhelming majority of population expressed the ligand (Figures 5.16 C and D). As T-lymphocytes were reduced in number in patients with sarcoidosis, the proportion of B-lymphocytes and other cells observed during flow cytometry were higher as a percentage of the PBMCs, though this translated to similar numbers of B-cells when this reduction was taken into account. The percentages of CD200L$^+$ T lymphocytes were similar in sarcoidosis patients and healthy subjects (Figure 5.17). However, because of the T cell lymphocytopenia previously mentioned (Section 3.3) there were significantly fewer T cells expressing CD200L in sarcoidosis whole blood (0.16 x10^6/ml ± SEM 0.03 compared with 0.39 x10^6/ml ± SEM 0.03, p = 0.0001). The identity of the subpopulation of T-lymphocytes expressing the ligand had not been identified in this study, whether it was attributed to CD4 or CD8 positive subsets, naïve or memory, another previously identified subset or a separate subset was unknown.

The relative expression of the ligand on these T-lymphocytes was similar between healthy controls (GMFI 12.45 ± SEM 1.12) and patients with sarcoidosis (GMFI 12.26 ± SEM 0.60). CD200L expression on B-lymphocytes was quantified on a limited number of patients and controls (Appendix A5.3), showing expression was not significantly different in this small sampling of individuals (p = 0.755), though a more comprehensive analysis would be

required to draw effective conclusions on B-lymphocyte CD200L expression in the disease.

There was no apparent correlation between the percentage of CD200L expressing T-lymphocytes and the level of CD200R expression on either monocyte or T cells in patients with sarcoidosis (Figure 5.17 D). Nor was there any association between the age of the patient, the number of T-lymphocytes in the blood or the number of monocytes and the percentage of CD200L$^+$ T-lymphocytes (Appendix A5.4); CD200L appeared independent of these factors. The CD200L was not expressed on cells to a greater or lesser degree in patients with sarcoidosis, yet the reduced overall T-lymphocyte number meant that there were fewer CD200L$^+$ cells in the blood of patients as an overall population, which may be a significant factor when considering earlier findings.
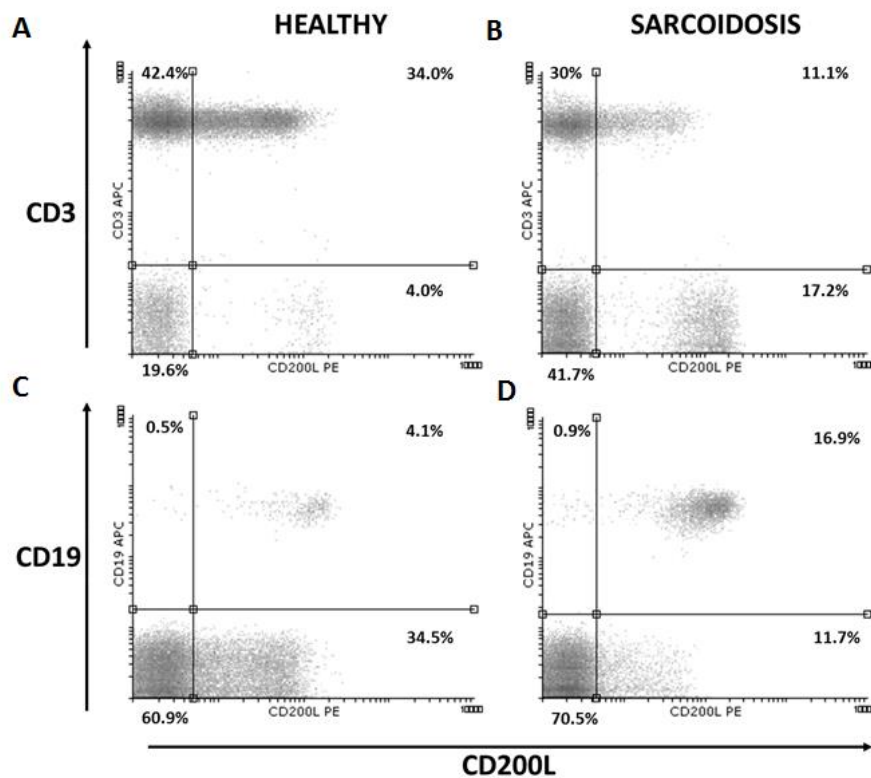
Figure 5.16: Representative density plots of CD200L expression on PBMCs from a healthy control and a patient with sarcoidosis, as detected by flow cytometry.

A and **B:** CD200L expression on PBMCs stained in combination with anti-CD3 antibodies in the healthy control and sarcoidosis patient respectively. Upper right quadrant represents CD3$^+$ CD200L$^+$ T-lymphocytes, lower right quadrant are CD3$^-$ CD200L$^+$ lymphocytes. Percentages represent the proportion of total PBMCs.
B and **C:** CD200L expression on PBMCs stained in combination with anti-CD3 antibodies in the healthy control and sarcoidosis patient respectively. Upper right quadrant represents CD19$^+$ CD200L$^+$ B-lymphocytes, lower right quadrant are CD19$^-$ CD200L$^+$ lymphocytes (T-lymphocytes). Percentages represent the proportion of total PBMCs.
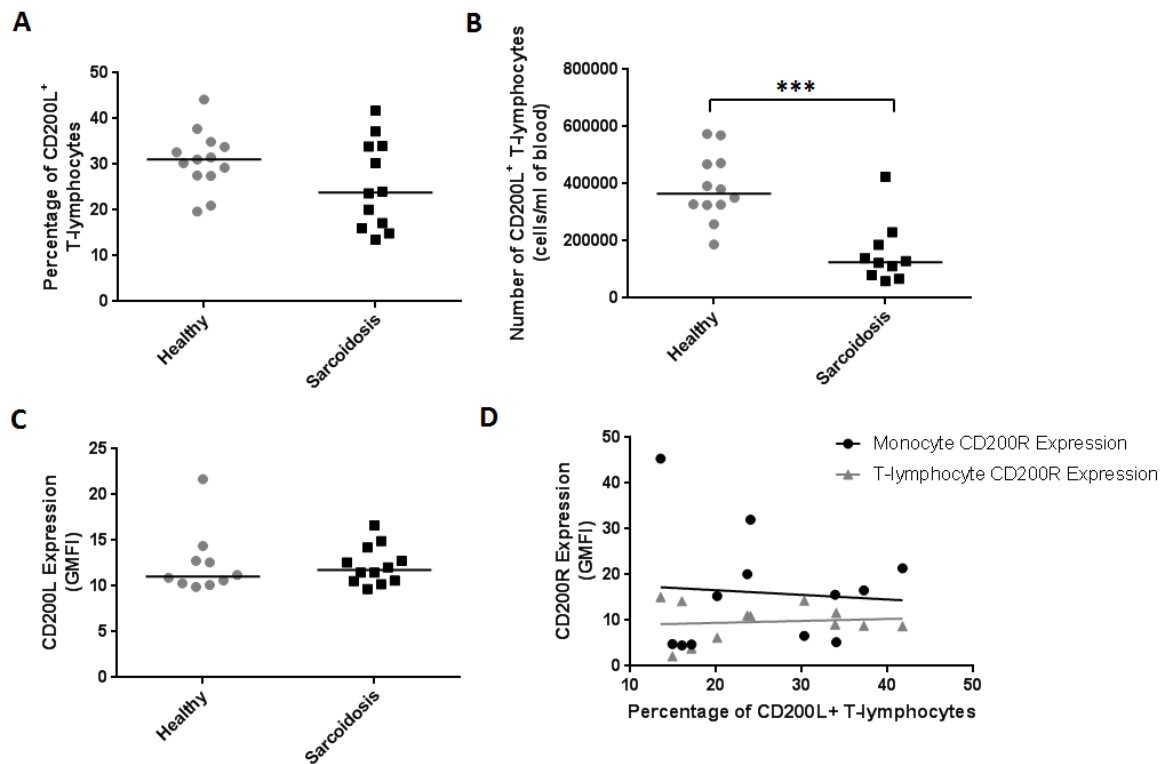
Figure 5.17: CD200L expression on T-lymphocytes from patients with sarcoidosis and healthy controls.

A-C: Healthy controls are represented by grey circles, patients with sarcoidosis by black squares. Black line represents the sarcoidosis linear regression, grey line represents the control linear regression. Data presented with median (black line). **A:** The percentage of CD200L+ cells within the T-lymphocyte population from PBMCs in patients with sarcoidosis (n = 12) or healthy controls (n = 13). No statistical significance, p = 0.116, as determined by Student's T test. **B:** The number of CD200L+ T-lymphocytes per ml of blood from patients with sarcoidosis (n = 10) and healthy controls (n = 12). *** p = 0.0001, as determined by Student's T test. **C:** CD200L expression (GMFI) on T-lymphocytes in patients with sarcoidosis (n = 12) and healthy controls (n = 10). The GMFI was taken from gated CD200L+ T-lymphocytes in exclusion of negative populations. No statistically significant difference between the populations (p = 0.771), as determined by Mann-Whitney U test. **D:** Correlation between monocyte and T-lymphocyte CD200R expression with the percentage of CD200L+ T-lymphocytes. Black line represents monocyte CD200R linear regression (n = 12, p = 0.457, R^2 = 0.006); grey line represents the T-lymphocyte CD200R linear regression (n = 12, p = 0.869, R^2 = 0.010).

5.3.6 Blocking or Activating CD200R/CD200L Signalling in Whole Blood Leukocytes and PBMCS

It has been demonstrated that CD200R was reduced on monocytes in sarcoidosis patients, and to a lesser degree, T-lymphocytes in some patients. Despite the positive correlations between this reduced expression and increased PHA induced pro-inflammatory TNF-α and IL-6 release from whole blood leukocytes, a more direct function demonstration of the link between reduced CD200R/CD200L signalling and pro-inflammatory cytokine release in response to PHA was required. To determine whether the CD200L/CD200R axis was important for regulating cytokine production in whole blood or PBMC preparations, blocking studies were performed using antibodies against CD200R or CD200L.

This was performed in healthy controls only. CD200R blocking was performed in PBMC cultures, as it was found that the polyclonal control antibody and CD200R antibody had a particularly high background activation in the whole blood system. CD200L blocking took place in the whole blood, which was more representative of the systems used previously. There was therefore no significant difference between PBS and the control IgG used in these experiments, therefore the IgG is represented here as the principle control. CD200R blockade increased cytokine release from isolated PBMCs from healthy subjects (Figure 5.18). TNF-α increased by 35.7 ± 29.2% (mean ± SEM; mean difference 1282 ± 427 pg/ml, p = 0.04), and IL-6 increased by 22.3 ± 19.4% (mean difference 14430 ± 8369 pg/ml, p = 0.16). Similarly, CD200L blockade in whole blood increased TNF-α and IL-6 release. TNF-α increased by 14.5 ± 7.4% (mean difference of 292 ± 93.6 pg/ml, p = 0.026) and IL-6 increased by 6.9 ± 3.1% (mean difference 8366 ± 3078 pg/ml, p = 0.042). IFN-γ and IL-8 also increased in response to CD200L blocking, though this was not statistically significant (Appendix A5.5). IFN-γ release was not increased in response to CD200R blocking in these experiments.

Having established that blocking the CD200R/CD200L interaction could increase the inflammatory response to PHA, there was a question over whether the CD200 receptor could be stimulated to diminish the inflammatory response. This is particularly important, as it could be used as a specific therapeutic target for intervention in inflammatory disease. A recombinant CD200L human protein Fc-bound chimera was obtained from a

commercial source (R&D), though it was acknowledged that this protein had not been validated for this kind of functional study, let alone used in a complex multi-celled whole blood assay with a stimuli such as PHA.

Anisomycin was included alongside PHA, as the former is a purported to be a MAPK activator, thus would give an indication as to whether the protein agonist was effectively working as required. As shown in Figure 5.19, the CD200L protein actually increased the inflammatory response to PHA for both IL-6 and TNF-α, as well as increasing the IL-6 release to anisomycin stimulation, though none of these reached statistical significance. Due to constraints in time and the amount of the protein available for this study, further optimising could not be performed using different experimental procedures, though the result was that the protein did not have the desired effect on this particular system.
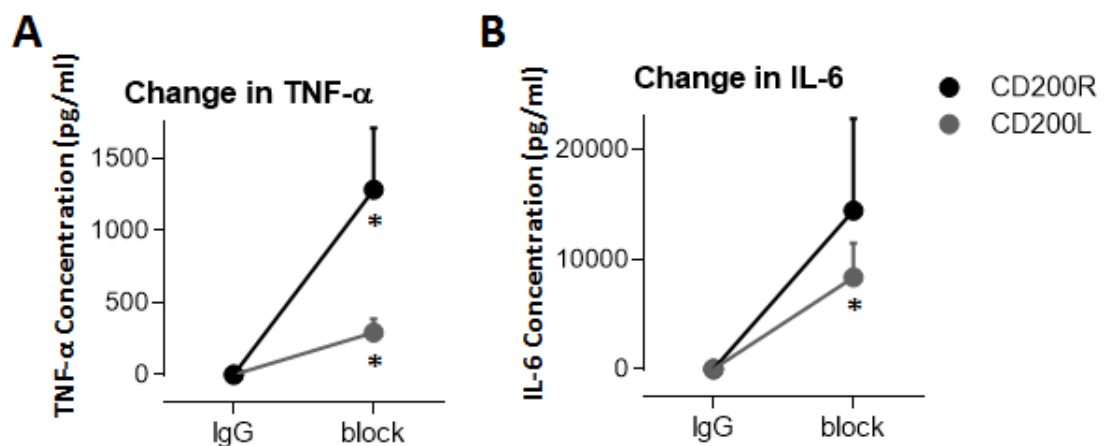
Figure 5.18: Blocking CD200R or CD200L in a PHA stimulated PBMC or whole blood assay in healthy controls.

CD200R blocking with an antibody took place in isolated PBMCs. CD200L blocking took place in a whole blood system. **A:** Change in TNF-α release (pg/ml) in PBMCs and whole blood after incubation with CD200R (n=5) or CD200L (n=6) antibodies compared with isotype control IgG. Cells were stimulated with 100μg/ml PHA for 16 hours **B:** Change in IL-6 (pg/ml) in PBMCs and whole blood after incubation with CD200R (n=5) or CD200L (n=6) antibodies compared with isotype control IgG. Cells were stimulated with 100μg/ml PHA for 16 hours. Results are presented as differences from isotype control IgG, with SEM error bars; *p < 0.05 using paired Student's T tests.
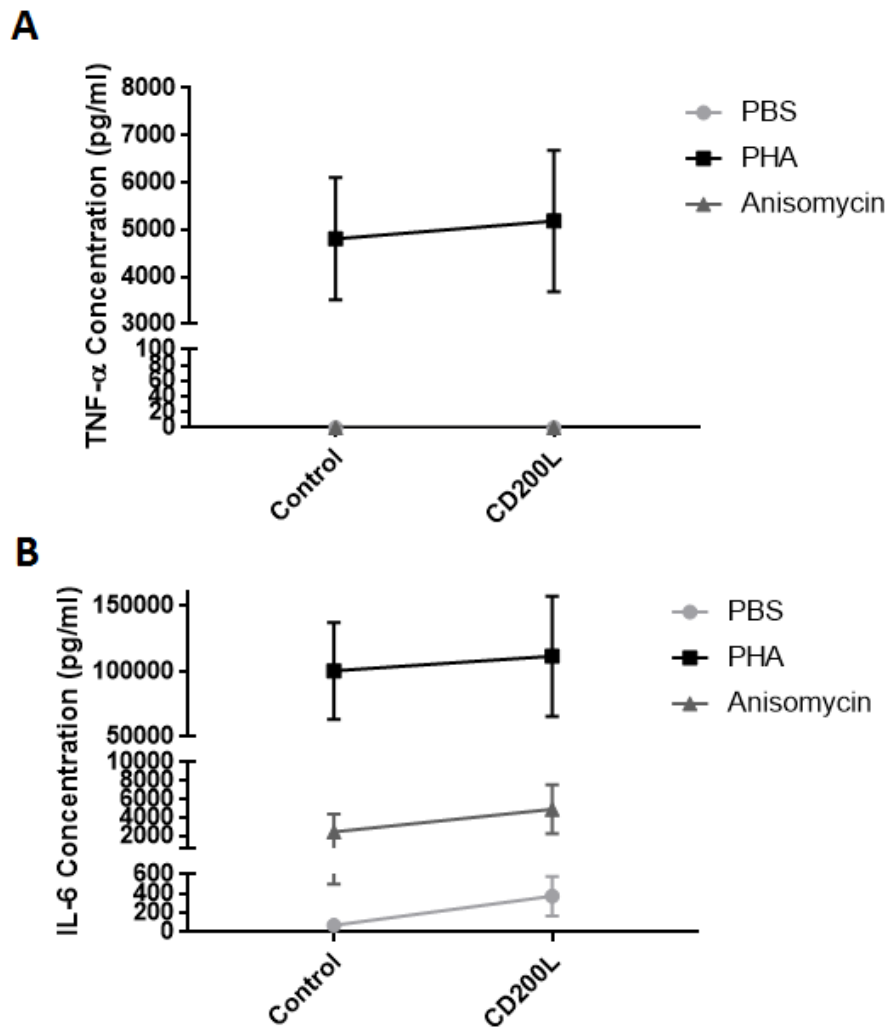
Figure 5.19: TNF-α and IL-6 release from whole blood after addition of PHA in the presence of a recombinant CD200L protein or a control Fc protein.

A: TNF-α release (pg/ml) from whole blood after incubation with PHA (100 µg/ml, n = 4), anisomycin (1 µg/ml, n = 3) or a PBS control (n = 4) for 16 hours. No statistically significant difference in TNF-α release for any of the stimuli ($p > 0.05$), as determined by paired Student's T test. **B:** IL-6 release (pg/ml) from whole blood after incubation with PHA (100 µg/ml, n = 4), anisomycin (1 µg/ml, n = 3) or a PBS control (n = 4) for 16 hours. No statistically significant difference in IL-6 release for any of the stimuli ($p > 0.05$). PHA was close to significance ($p = 0.074$), as determined by paired Student's T test.

5.3.7 CD200R and CD200L Expression on Cells in Granulomatous Tissue from Patients with Sarcoidosis

A key feature of sarcoidosis is the presence of non-necrotic/non-caseating granulomata, seen most commonly in lung tissue and associated lymph nodes [1]. CD200R had been detected on peripheral blood T-lymphocytes and monocytes, whereas expression of CD200L was present on peripheral T-lymphocytes and B-lymphocytes, but absent on circulating monocytes. Despite the majority of the focus of this work being on peripheral blood responses, it was necessary to identify the distribution of the receptor and ligand in tissue effected by the disease, as this had not been reported previously for the condition. Lymph node or lung biopsy samples from patients with sarcoidosis that been used in the diagnosis of the condition were obtained for this study. These were stained for the CD200 ligand and receptor, with a particular focus on granulomatous regions.

Haematoxylin and eosin (H & E) staining was used to identify regions with granulomata and cell distribution in tissue (Figure 5.20). This showed a morphology of granuloma consistent with non-caseating/non-necrotic forms observed in sarcoidosis, with a central core of histiocytes (monocyte/macrophage-derived cell types) and a peripheral 'ring' of fibroblasts. As shown in Figure 5.20, aside from those granulomata associated cells, lung/bronchial tissue contained collections of other cells, including lung epithelial cells, endothelial cells, smooth muscle cells, goblet cells, alveolar epithelial cells and other cell types distributed throughout. Without staining for definitive markers, a positive identification could not be established with absolute confidence for a lot of these cell types, so visual assessments were made based upon morphology.

Staining for CD200L showed widespread expression of the ligand across different cell types resident in the lung, particularly in alveolar epithelial cells, smooth muscle cells, endothelial cells and fibroblasts (Figure 5.21). As the antibodies used were polyclonal, there was a small amount of background staining by macrophage like-cells visible, so this would have to be accounted for when assessing expression. Granulomata are visible as regions of myeloid infiltration with peripheral fibroblast cells (Figures 5.21E and 5.21F). Under closer examination, the myeloid cells showed absence of CD200L expression, while the peripheral ring of fibroblasts expressed CD200L significantly (Figure 5.21F). This suggests that the inner core of the granulomata may have more limited contact with the

ligand by virtue of the myeloid cell (histiocyte) rich makeup of the sarcoidosis granuloma. This also aided in identifying granulomata in tissue, as a characteristic circular region of fibroblast CD200L$^+$ dark staining, contrast against a central region without stain, was relatively easy to recognise.

Similar patterns were observed in lymph nodes (Figure 5.22), except there appeared to be lower expression locally on resident cells, perhaps due to the cell types present within lymph nodes (leukocyte predominant) compared with lung tissue (resident lung cells, such as epithelial cells). Again, the presence of granuloma were easily identified using the staining of CD200L, and showed the absence of the ligand in the core of the granuloma, supporting peripheral blood findings that monocytes did not express the ligand and that macrophages and macrophage-derived cells were also negative for this ligand.

There were problems obtaining a clean CD200R staining of tissue with previously selected antibodies, thus requiring optimising and eventually selection of an alternative antibody. Due to time constraints, as this was at the very end of the project, there were some issues obtaining images for the CD200R, leading to microscope images that appeared darker than previous CD200L staining (Figures 5.23 and 5.24). Nevertheless, the staining showed that macrophages/histiocytes stained positively for CD200R, whereas other resident cells were negative for the receptor (Figure 5.23). This was essentially the opposite of the CD200L expression, where CD200R was expressed in the central core of the granuloma, but completely absent in the peripheral fibroblasts (Figure 5.24). Further work would be required in staining tissue from non-sarcoid granulomata to confirm whether the degree of expression is similar in other diseases such as tuberculosis. What can be concluded is that CD200L is widely expressed in sarcoidosis tissue, but absent in macrophage dominant regions, whereas CD200R appears to only be expressed within these cell types in tissue and is less thoroughly distributed, focussed mainly within the granulomata.
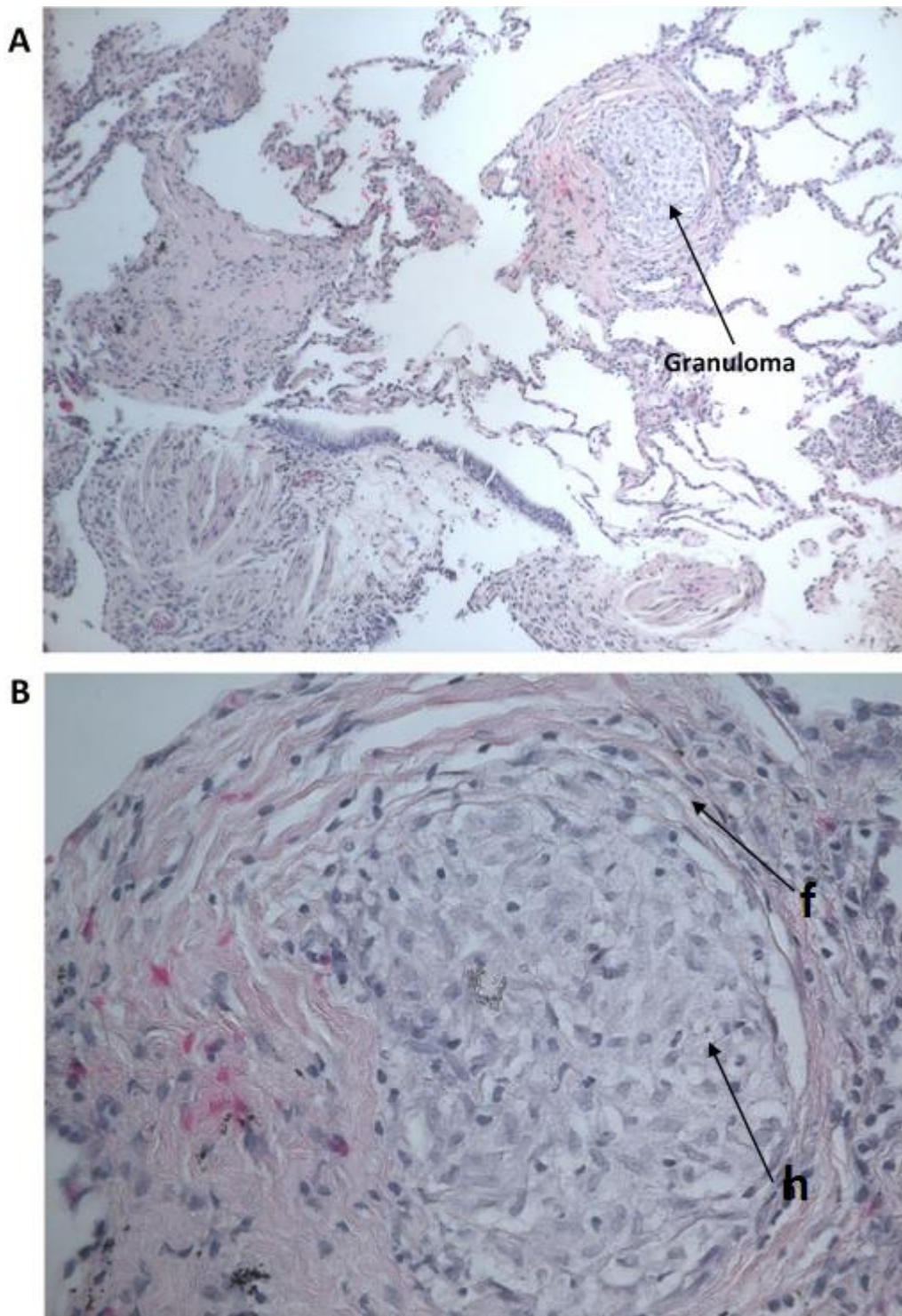
Figure 5.20: Haematoxylin and Eosin (H & E) staining of a lung biopsy from a patient with sarcoidosis displaying a non-caseating granuloma.

A: x100 magnification of tissue showing a variety of lung resident cells. Granuloma labelled. **B:** Non-caseating granuloma under x400 magnification. Fibroblasts labelled **f** and histiocytes (myeloid cells, monocyte/macrophage like cells) labelled **h,** which make up the central core of the granuloma.
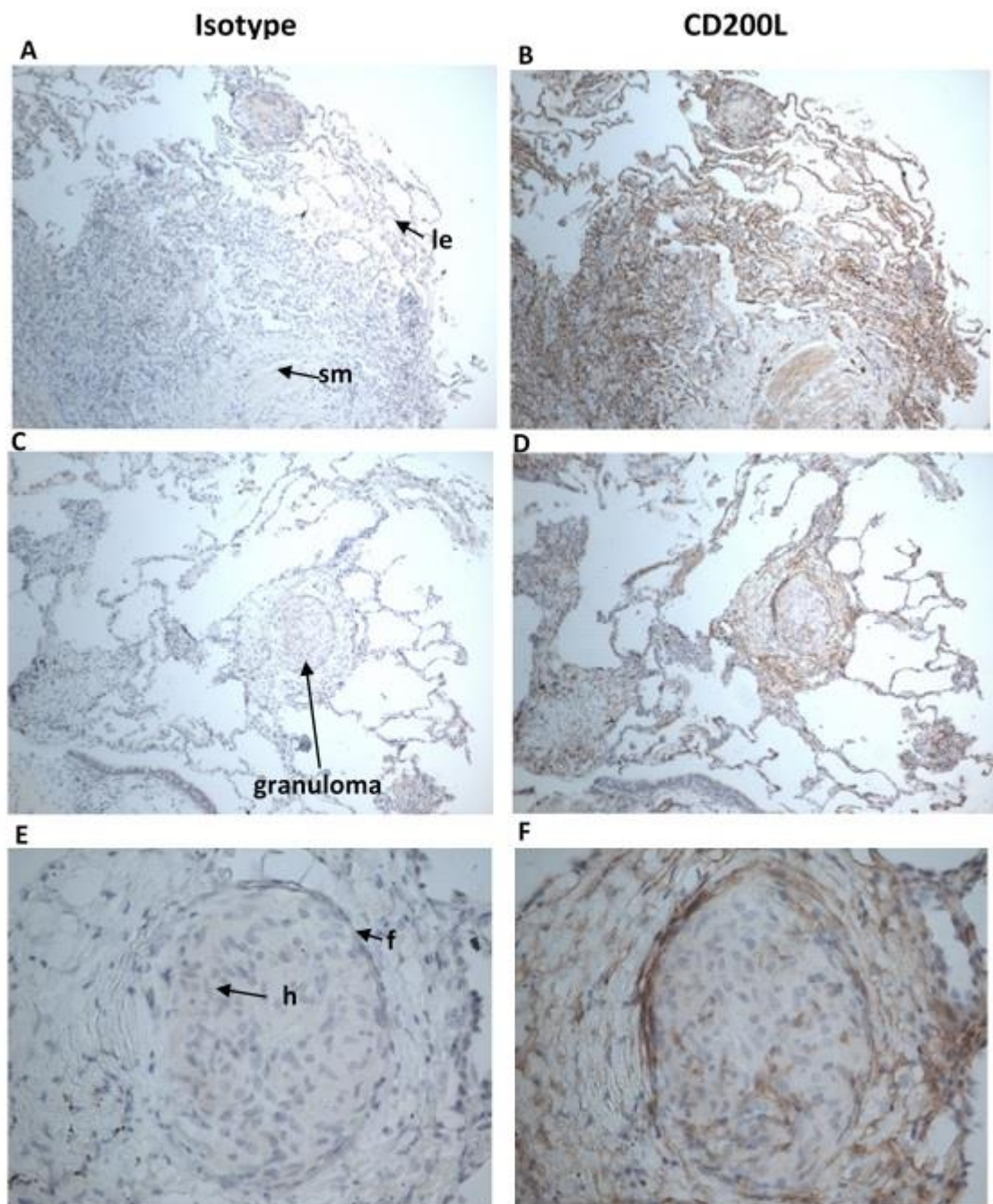
Figure 5.21: CD200L expression in lung tissue of patients with sarcoidosis detected by immunohistochemistry.

A-**D**: Lung biopsy samples from 2 patients stained using a control antibody (polyclonal goat IgG) and anti-CD200L polyclonal antibody. Magnification x100. **le**: lung epithelial cells and **sm**: smooth muscle cells. Granuloma highlighted in **C,** present in **D** in approximately the same position. **E** and **F**: Granuloma viewed at x400 magnification stained with a control antibody and CD200L. Central myeloid like cells, macrophage/histiocytes (labelled **h**) are CD200L⁻, whereas surrounding fibroblasts (**f**) express the ligand.
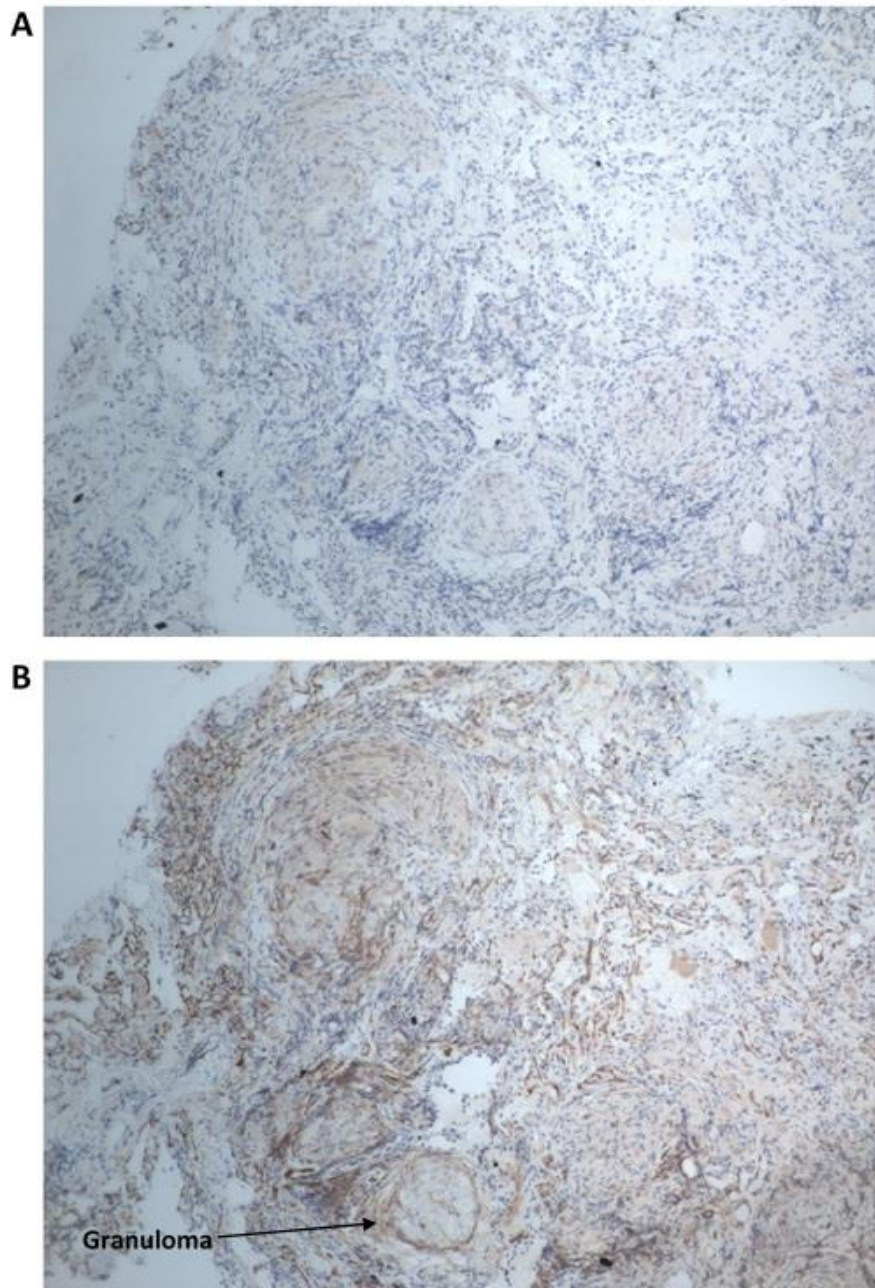
Figure 5.22: CD200L expression in lymph node tissue of a patient with sarcoidosis, detected by immunohistochemistry.

Staining for CD200L by IHC viewed at x100 magnification. **A:** Isotype control shows light background staining in myeloid cells. **B**: CD200L staining shows a granuloma labelled with negative expression in the core and peripheral fibroblast positive expression.
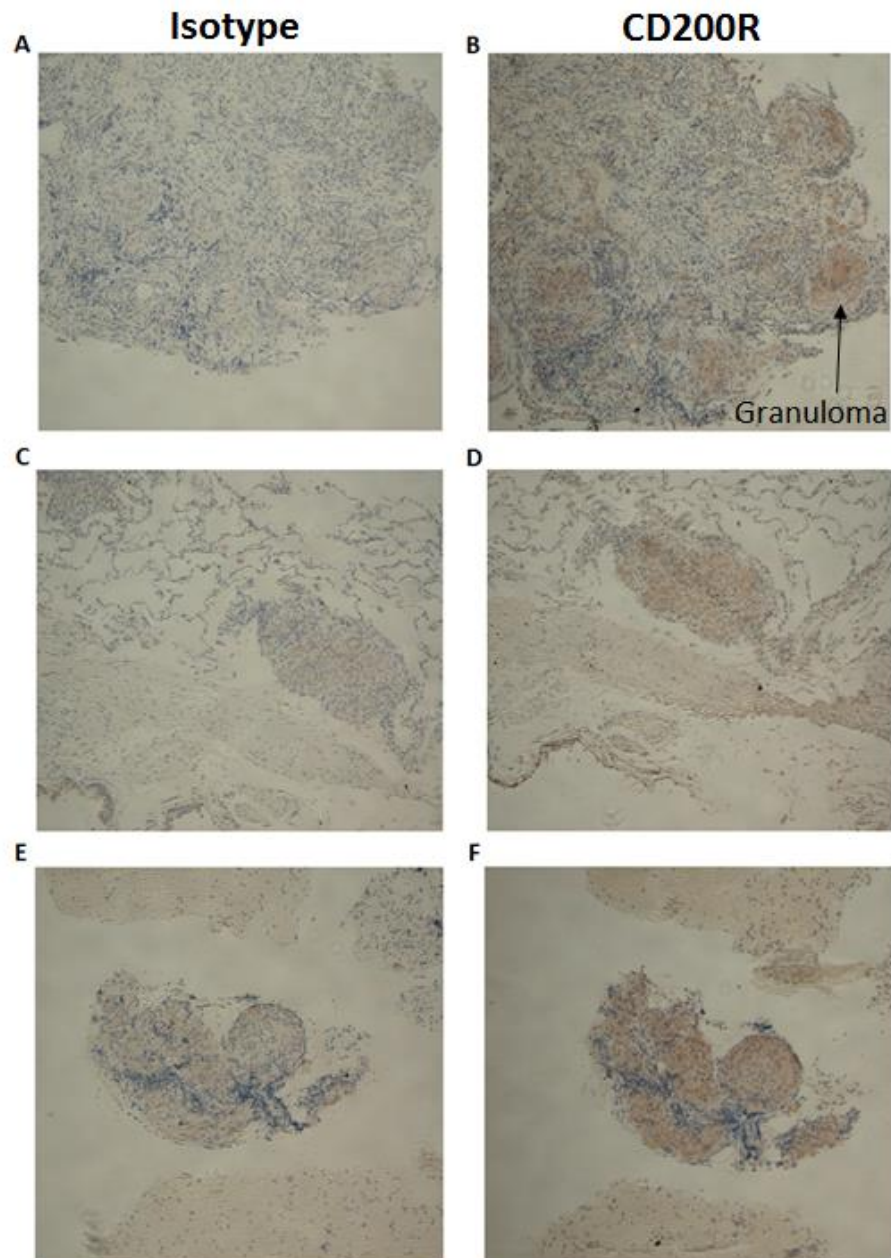
Figure 5.23: CD200R expression in lung and lymph node tissue of patients with sarcoidosis.
All slides presented at a magnification x100. **A** and **B**: Lymph node biopsy sample from a patient
stained using a control antibody (**A**) and an anti-CD200R antibody (**B**). **C-F:** Lung biopsy samples
from 2 separate patients with sarcoidosis, displaying control slides (**C** and **E**) and CD200R
expression on macrophage like cells (**D** and **F**). An example granuloma is labelled in **B.**
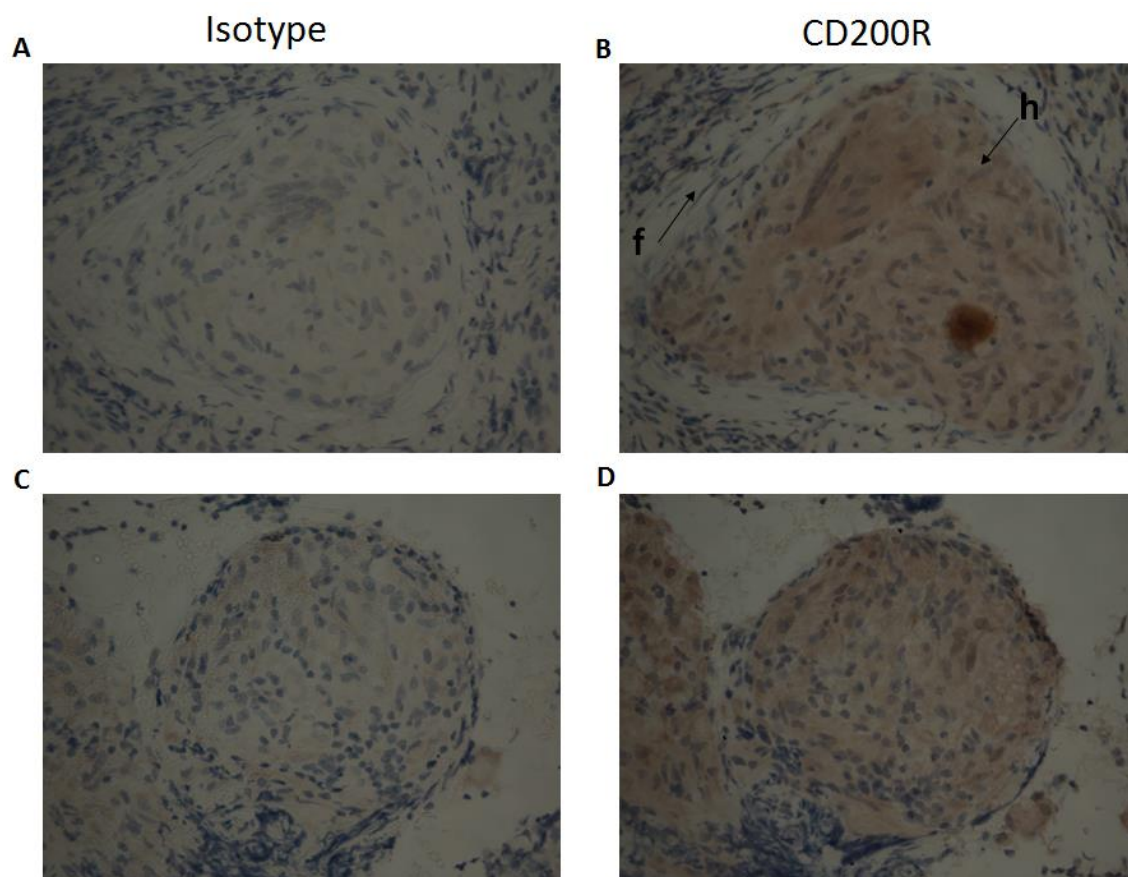
Figure 5.24: CD200R expression in granuloma in biopsy samples of patients with sarcoidosis.
Lymph node biopsy (**A** and **B**) and lung biopsy (**C** and **D**) granuloma stained with isotype control antibody (**A** and **C**) and anti-CD200R antibody (**B** and **D**). Magnification is x400. Histiocytes (**h**) and fibroblasts (**f**) are labelled in **B.**

5.3.9 Associations between CD200R/CD200L and the Clinical Features of Patients with Sarcoidosis

As had been established previously (Section 3.3), a number of clinical features were present within the cohort of patients with sarcoidosis that were either traditionally associated with the staging of the disease (Scadding CXR stage) or markers of the degree of inflammation (C-reactive protein/CRP and plasma viscosity/PV) or granulomatous burden (serum ACE). Having identified populations of patients with specific CD200R expression profiles, it is important to discover whether this is associated with any of these clinical features, or whether CD200R possibly represents a different way to stratify patient populations.

Looking at serum ACE levels, the classical marker of sarcoidosis despite the limitations it has in diagnosis and prognosis, CD200R expression on T-lymphocytes and monocytes did not correlate with the level of sACE (Figure 5.25, $p = 0.496$, $R^2 = 0.045$ and $p = 0.958$, $R^2 = 0.015$ respectively). This was also the case for CRP levels, though monocyte CD200R expression inversely correlated with plasma viscosity levels (Figure 5.25 F, $p = 0.015$, $R^2 = 0.163$), suggesting that there is possibly an association between inflammatory markers and CD200R. The chest radiographic staging showed a trend towards lower CD200R expression in both T-lymphocytes and monocytes relating to higher stages in the disease (Figures 5.26 A and B), though the limited numbers meant that this was not statistically significant. The number of organs involved and whether patients had extrapulmonary involvement was also not significantly associated with either high or low CD200R expression in either monocytes or T cells (Figures 5.26 C – F). Using previously established phenotypes which stratified patients based on CD200R T-lymphocyte and monocyte expression, the profiles of pulmonary restricted and extrapulmonary organ involvement show that there is not a significant difference between CD200R phenotypes and the type of organ involvement (Appendix A5.6). The majority of patients were CD200R$^{+/low}$ or CD200R$^{+++/high}$ in both pulmonary and extrapulmonary groups, without a significant skew towards one group. Whether or not there would be some correlation with a larger number of patients has yet to be determined, but based on this data, the expression of CD200R does not appear to be acutely tied to the current clinical features used to classify patients with sarcoidosis.
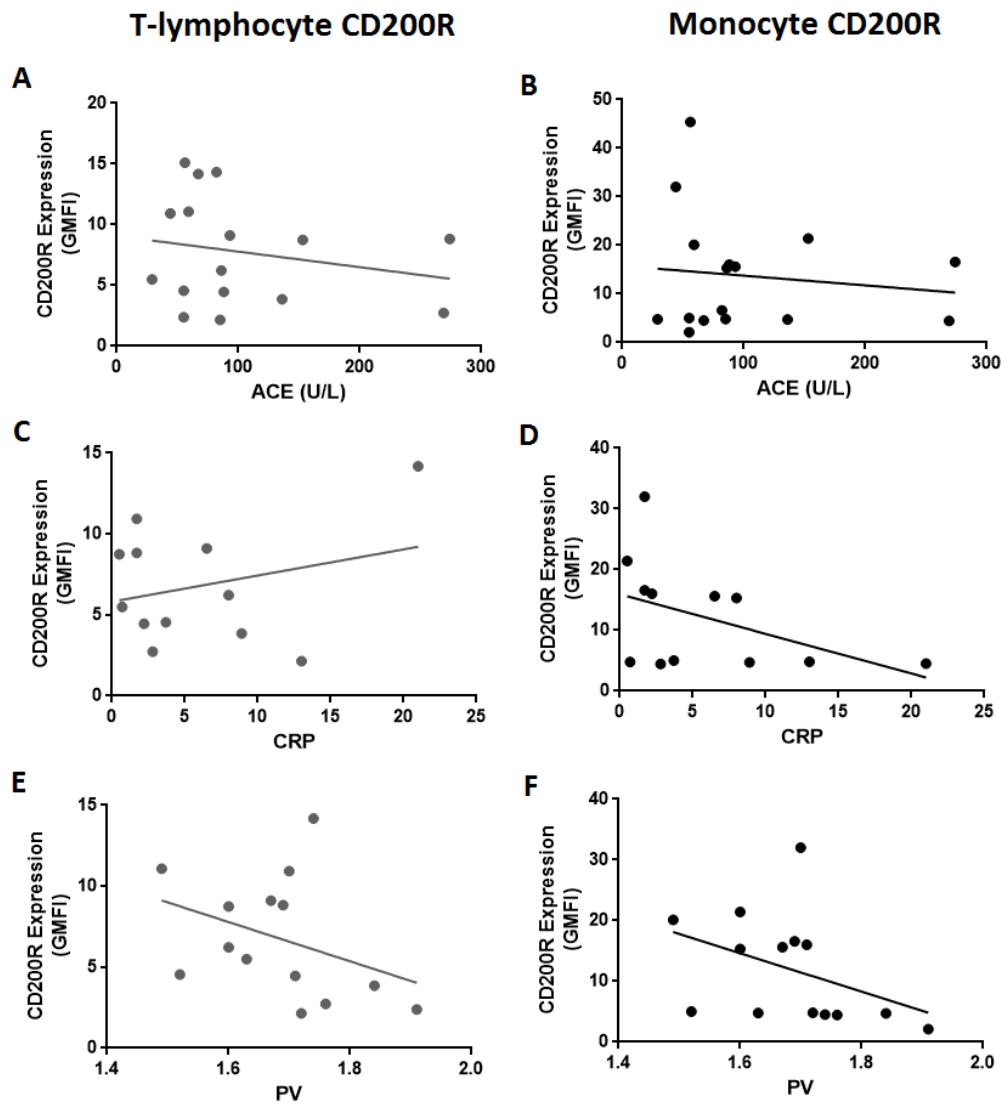
Figure 5.25: CD200R expression correlated with serum ACE, C-Reactive Protein (CRP) and Plasma Viscosity (PV) in patients with sarcoidosis.

T-lymphocyte CD200R expression (**A**, **C** and **E**) and monocyte CD200R expression (**B, D** and **F**) were correlated with serum ACE (**A** and **B**), CRP (**C** and **D**) and PV (**E** and **F**). There was no statistically significant correlation between serum ACE and CD200R expression; **A:** p = 0.496, R^2 = 0.045, **B:** p = 0.958, R^2 = 0.015). Neither did the CRP correlate with T-lymphocyte CD200R (**C:** p = 0.630, R^2 = 0.074) or monocyte CD200R expression (**D:** p = 0.054, R^2 = 0.202). PV correlations with CD200R T-lymphocyte expression was not statistically significant (**E:** p = 0.078, R^2 = 0.136), however, monocyte CD200R did correlate with PV (**F:** * p = 0.015, R^2 = 0.163)**.** Black/grey line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.
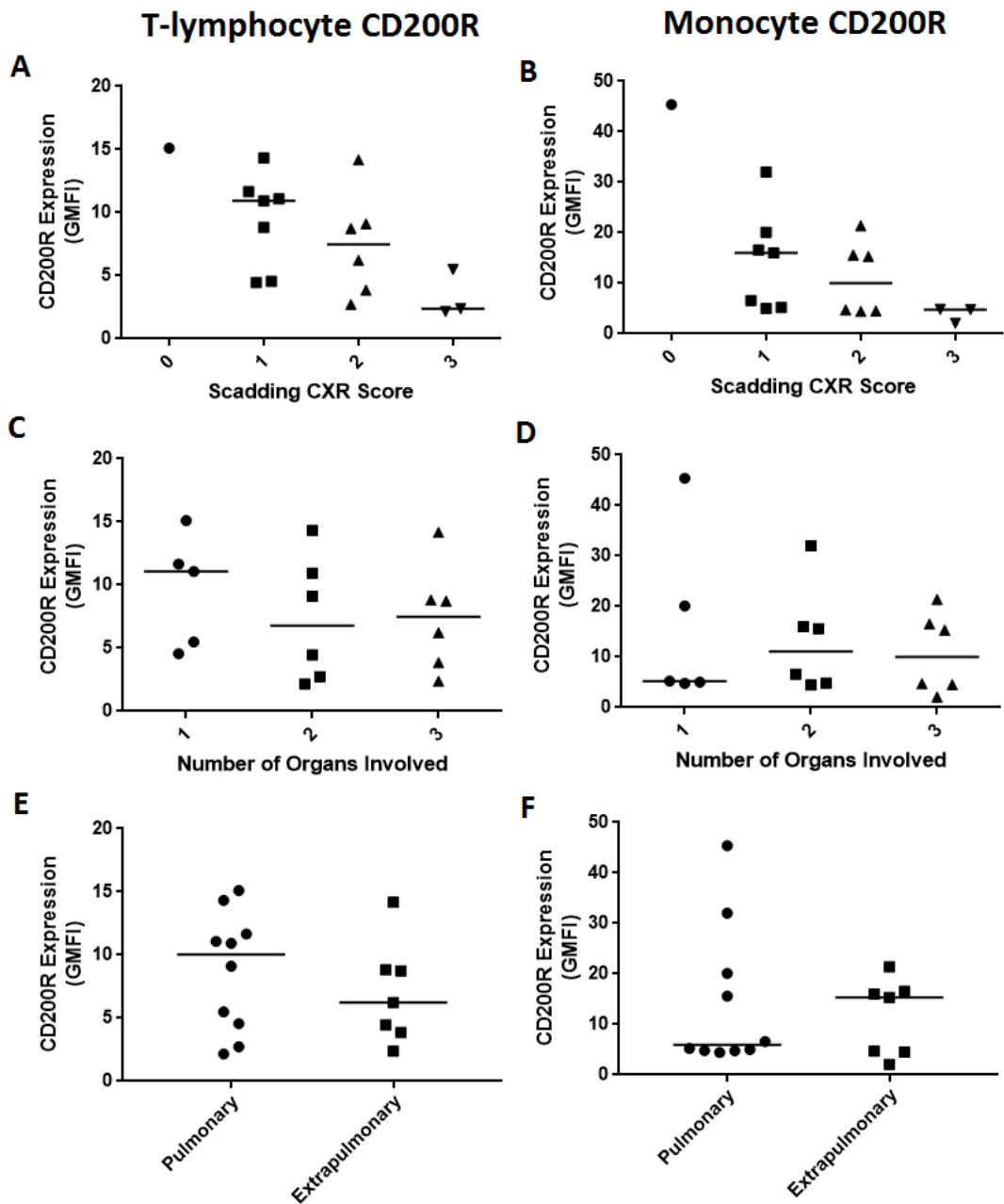
Figure 5.26: CD200R expression correlated with organ involvement and radiological staging in patients with sarcoidosis.

T-lymphocyte CD200R expression (**A**, **C** and **E**) and monocyte CD200R expression (**B, D** and **F**) were correlated with the Scadding CXR stage (**A** and **B**), number of organs patients had involved (**C** and **D**) and whether patients had pulmonary restricted or extrapulmonary organ involvement (**E** and **F**). There was no statistically significant correlation between any of the features presented (p > 0.05), determined using One-way ANOVA (**A** – **D**) and Mann-Whitney U tests (**E** and **F**). Data presented with median (black line).

5.4 Discussion

Sarcoidosis has been associated with enhanced tissue Th1 immune responses [280, 339, 831], yet diminished peripheral blood pro-inflammatory responses [22, 23], leading some to research the role of regulatory responses in contributing to this apparent anergy [23]. Despite this, there does not appear to be a previously documented comprehensive study on the role of some recently identified regulatory immune receptors in the disease, such as SIRP-α and the CD200 receptor (CD200R). The research undertaken in this study presents a characterisation of the expression of these receptors on peripheral blood mononuclear leukocytes in patients with sarcoidosis, along with the IL-10 receptor. It has also characterised the expression of the ligands for CD200R and SIRP-α on these leukocytes: CD47 and CD200 ligand (CD200L). Fundamentally, it shows that patients with sarcoidosis do not have significantly enhanced IL-10R, SIRP-α or CD47 expression on peripheral leukocytes when compared with controls to support the previously documented peripheral blood anergy in other reports. This research showed that CD200R expression in patients with sarcoidosis is reduced on monocytes and the reduced expression of the receptor is associated with enhanced IL-6 and TNF-α release from PHA induced whole blood. This could be a contributing factor in the enhanced IL-6 and TNF-α responses observed previously in this report (Chapter 3) and raises the possibility that CD200R may play a role in the immunopathogenesis of the disease.

5.4.1 The IL-10 Receptor and Cytokine

To the best knowledge of the author, this is the first report to characterise the expression of IL-10R on peripheral blood mononuclear cells in sarcoidosis. Patients with sarcoidosis were not significantly different in their expression of the receptor from healthy controls, on either T-lymphocytes or monocytes. This is broadly supported by the data previously obtained showing that there was no significant difference in IL-10 release in whole blood from patients in response to either PHA or SEA (Section 3.3).

The IL-10 receptor is of interest pathologically, as loss of function polymorphisms in the gene have been associated with early onset inflammatory bowel disease (IBD) [832, 833],

as well as IL-10 cytokine gene polymorphisms being associated with both ulcerative colitis (UC) [834] and Crohn's Disease [835] (both IBDs). Whilst the expression of the receptor has been reported to be reduced on the surface of T-lymphocytes in a subset of patients with systemic lupus erythematosus (SLE) displaying lupus nephritis [836], another study found no difference in expression between patients with SLE, rheumatoid arthritis and controls [837]. The data for patients with sarcoidosis suggests that the receptor is not subject to large differences in expression as a result of the disease, though functional studies were not performed to identify any differences in signalling. A study of the IL-10 gene in a limited number of Japanese patients did not discover any associations between IL-10 polymorphisms and the disease [838], therefore it is possible that the receptor is neither functionally deficient or under-expressed in sarcoidosis.

The expression of the receptor was not associated with leukocyte number in blood, but patient IL-10R expression on monocytes inversely correlated with IL-6 release from the blood. IL-10R expression on T-lymphocytes was associated more with serum ACE levels in patients. This receptor can regulate inflammation predominantly through potently inhibiting accessory cell inflammatory cytokine release [839, 840]. IL-10R signalling through JAK-STAT can reduce TNF-α cytokine release from monocytes and macrophages, though the role of IL-10 in the immunopathogenesis of sarcoidosis has yet to be fully elucidated [315]. The association between elevated monocyte IL-6 release to PHA and reduced IL-10R expression is worthy of note. IL-6 can also signal through the JAK-STAT pathway [841], so there is a possibility that the inverse correlation arises due to increased signalling from IL-6 modifying IL-10R expression. That being said, the elevated CRP concentrations from individuals with lower monocyte IL-10R suggests that both IL-6 and CRP may increase in response to reduced IL-10 regulation of inflammation in those individuals, or vice versa. Care needs to be taken not to assume a correlation is equal to causation (post hoc ergo propter hoc), therefore functional studies would be required to define this association.

It has been demonstrated by some groups that patients with sarcoidosis have higher concentrations of serum IL-10 when compared with healthy controls [26, 842], however, others have reported no significant difference in IL-10 concentrations in patient serum [644, 843]. This suggests that there is some variation between studies, often a feature of

research into a disease such as sarcoidosis with heterogeneous patient populations. The analysis in these studies take basal levels from fresh blood, whereas the study presented in the current work used non-stimulated blood after 16 hours to determine that there was no statistically significant difference in IL-10 release after this time between patients and controls. Additionally, the assessment of IL-10 release was determined using PHA or SEA stimulation in a multi-celled whole blood system, which may differ from other research performed with specific cell types or different stimuli.

Cranshaw *et al* reported that sarcoidosis monocytes had reduced IL-10 production [822]. The choice of methods for cell isolation and stimulation could explain the differences in findings observed with the current study, since Cranshaw *et al* studied monocytes that had been isolated by magnetic bead negative selection and stimulated with lipopolysaccharide [822]. A similar study isolating alveolar macrophages and monocytes showed that LPS induced IL-10 release from monocytes was significantly lower in patients with sarcoidosis compared with controls, and monocyte IL-10 was greater than alveolar macrophage release of the cytokine [315]. Interestingly, another study showed that the mRNA expression of IL-10 in PBMCs was not significantly different between healthy controls and patients with sarcoidosis, though this was performed with mixed leukocytes and not an isolated cell type [829]. The assays performed in this study focused on mixed cell populations, therefore the analysis of single celled populations, whilst informative, may not relate directly to the findings presented here.

Based on the data in this present study, it cannot be demonstrated that the IL-10 regulatory axis was dysfunctional in sarcoidosis. Further functional assays would be required to discover whether the IL-10R/IL-10 regulatory axis had greater impact on the immunopathogenesis of the disease in peripheral blood. Neither was it specifically observed that the receptor was differentially expressed based upon the number of organs involved, extrapulmonary organ involvement or chest X-ray staging, though a more comprehensive study with more patients would be necessary to ascertain whether this is actually a marker of disease activity.

5.4.2 Signal Regulatory Protein Alpha (SIRP-α) and CD47 Expression

SIRP-α is a receptor notable for being myeloid restricted, whilst the CD47 receptor, its cognate ligand, is ubiquitously and highly expressed on cells [844]. Unlike IL-10, CD47 is bound to the cell surface and not soluble, which therefore means the interaction requires an element of cell to cell contact for engagement with SIRP-α. CD47 has been given the interesting title of a "don't eat me" signal [844], so called for being a ligand to SIRP-α that prevents phagocytosis of host cells [845]. This pairing was of interest in sarcoidosis for a number of reasons; principally because SIRP-α and CD47 have been associated with the formation of giant multinucleated cells from macrophages (which are features of granulomata) [846, 847], hence the alternative name for SIRP-α of 'macrophage fusion receptor'. It should be noted that CD47 is itself a receptor and can bind to other proteins, such as integrins and thrombospondin [848, 849], to serve other roles separate from the interaction with SIRP-α. There is also evidence that CD47 is not the only ligand for SIRP-α, surfactant proteins A and D have also been shown to bind to the receptor to induce an anti-phagocytic signal [850].

Supporting the findings by other groups that SIRP-α expression is mainly limited to myeloid cells [851, 852], this research found that SIRP-α was confined to monocytes within the PBMCs. However, no significant difference was observed in the expression of the receptor between patients with sarcoidosis and healthy controls.

SIRP-α is absent on myeloid cells from patients with myeloid leukaemia [852], whereas CD47 is overexpressed on myeloid leukaemia cells [853, 854]; this points to a dichotomy wherein myeloid leukaemia cells both avoid regulatory control (through SIRP-α reduction/removal) and ensure their own survival by preventing phagocytosis from non-cancerous phagocytes (through increasing CD47 expression). As no significant difference was observed in either monocytes or lymphocytes in terms of the CD47 expression, it would suggest that, at least in peripheral leukocytes, neither SIRP-α nor CD47 are likely to be the reason for the enhanced responses of blood leukocytes to PHA stimulation in the disease.

This comes with a few caveats however. As the total number of leukocytes present in sarcoidosis blood is reduced as a result of the T cell lymphocytopenia, it would be a possibility that there are fewer CD47 regulatory proteins overall for monocytes to engage with, which could reduce regulatory signalling. Whilst this cannot be excluded, the overabundance of cells expressing CD47, i.e. all of the leukocytes, would suggest that this is unlikely to be a major factor. Additionally, the functional capabilities of the two proteins were not tested directly, therefore it remains possible that there is a dysfunction in the signalling that has not been identified by purely determining expression levels. Lastly, the role for the pair in multinucleated giant cell fusion might suggest that they play a much more significant role in tissue which was not studied in this research. Future study of the receptors in sarcoidosis tissue would be of particular interest, as it is a rare display of myeloid accumulation within well-defined granulomata set against surrounding tissue resident cells, therefore detection of SIRP-α could be easily detected on histiocytes if it is expressed. In a study of Crohn's disease by Baba *et al* [855], the presence of dendritic cells positive for SIRP-α (and HLA-DR) were localised to regions affected by the disease and spontaneously released TNF-α and IL-1β. The group used a recombinant CD47 protein to reduce pro-inflammatory cytokine release, which not only decreased TNF-α and IL-1β, but also other cytokines such as IL-6 and IFN-γ. If cells displaying SIRP-α expression are present in tissue, it is possible these could be targeted for therapeutic intervention in a similar manner if there were parallels between the diseases. This would be a possible area for future work.

5.4.3 The CD200R/CD200L Regulatory Axis in Sarcoidosis

This study has shown that patients with sarcoidosis have distinct alterations in the expression of the CD200 receptor compared with healthy controls. Unlike IL-10R and SIRP-α, CD200R expression was reduced on monocytes in patients, the majority of the population belonging to a group with low expression of the receptor on this cell type, termed CD200R^{low}. Not only that, but whereas healthy controls displayed a moderate level of T-lymphocyte CD200R expression, CD200R^{++} , patients with the disease tended to display either high or low T cell CD200R, CD200R^{+} or CD200R^{+++} respectively. By contrast, CD200L expression was not significantly altered in expression or in terms of the

percentage of T-lymphocytes expressing the ligand. It is perhaps in keeping with the heterogeneous nature of sarcoidosis that a single expression profile is not present across all individuals with the disease. Whilst most patients will express low T-lymphocyte and monocyte CD200R, or high expression of both, there are notably individuals expressing high T cell CD200R with low monocyte expression of the receptor. This indicates that there are specific profiles present in CD200R expression; interestingly this data highlights that no healthy controls expressed low expression of the receptor on both cell types, whereas this was a profile displayed by patients.

The expression was not correlated with the percentage of intermediate monocytes within patients, though this is an indirect measure rather than an analysis of CD200R expression on each of the monocyte subsets. Reports have highlighted that this monocyte subset is expanded in patients with sarcoidosis [336], that CD16$^+$ monocytes (intermediate and non-classical) release more TNF-α than CD16$^-$ monocytes (classical) upon mycobacterial antigen challenge [856] and intermediate monocytes express more TNFR1 (tumour necrosis factor receptor 1) than other subsets [336]; suggesting that non-classical and intermediate monocytes might be more pro-inflammatory than non-classical monocytes. A comparative analysis of the expression of CD200R individual subsets was not completed as part of this current study, though this is clearly an area for future work that requires addressing by the use of multicolour flow cytometry using more fluorophores than were used in the current study. The question as to whether CD200R is lower on intermediate or non-classical monocytes, and whether this is lower on patients with sarcoidosis, would add to the current understanding of the role of these cells in the immunopathogenesis of the disease.

Overall, low CD200R on monocytes was associated with elevated IL-6 release from whole blood in response to PHA. This was more pronounced for CD200R$^+$/low patients compared with high expressers in both cell types, where both TNF-α and IL-6 are elevated. It should be noted that, in of itself, it appears that monocytes CD200R expression may be a greater influence on the total release of these cytokines, as CD200R$^{+/high}$ were not significantly associated with elevated IL-6 or TNF-α responses. This provided an association, yet was an indirect link between the PHA-induced release of these cytokines and CD200R. By blocking the receptor or ligand using an antibody in

healthy controls and observing an increased release of TNF-α and IL-6, it was demonstrated that the CD200R/CD200L interaction was capable of regulating PHA induced activation of mixed peripheral leukocytes. Although the whole blood assay used in the present study has an advantage in minimally perturbing cells such as monocytes that are highly pleiotropic and respond rapidly to changing environments on isolation and in tissue culture, the assay does have some limitations. For example, due to T cell lymphocytopenia, fewer CD200L+ lymphocytes will be present in sarcoidosis blood for potential ligation of monocyte CD200R, though the interactions in this regard cannot be determined at present for a complex multi-celled system such as the blood. This may also contribute to the elevated TNF-α and IL-6 responses in whole blood stimulated with PHA from patients; regulation via the receptor may be reduced as a result of the reduced availability of the ligand.

CD200R has been identified to act by reducing MAPK (mitogen-activated protein kinase) signalling [857, 858], as summarised in Figure 5.27. MAPK signalling is comprised of three pathways: ERK (extracellular signal regulated kinase), JNK (c-Jun N-terminal kinase) and p38 (P38 mitogen-activated protein kinase), responsible for a diverse range of activity including cell proliferation, cell migration, apoptosis and inflammatory processes [794]. There is a degree of overlap between JNK and p38 pathways in terms of the upstream activators and pathway components, though all three can be activated independently or in unison depending on the type of signals being initiated (Figure 5.28). MAPK pathways can be activated by a variety of receptors, though one example relating to inflammation relates to the activation of Toll-like receptors (TLRs). Specific TLRs can be stimulated (for example, TLR4 with LPS), which leads to activation of MYD88 (myeloid differentiation primary response gene 88) dependent activation of MAP3K (MAP kinase kinase kinase), which subsequently leads to activation of the JNK, p38 and ERK signalling [764, 794]. These signalling pathways can ultimately activate transcription factors, including NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), which leads to the transcription and translation of pro-inflammatory cytokines [764]. There are a variety of different activation pathways involved in TLR signalling and other ways MAPK signalling can be activated, therefore these systems have developed refined mechanisms of both activation and control.

It has been demonstrated that, in addition to T cell receptor interactions, PHA can activate a variety of TLRs, including TLR2, 4, 5 and 6; predominantly extracellular receptors [699]. The whole blood assay was notable for the release of enhanced TNF-α and IL-6 in response to PHA in patients with sarcoidosis; both of these cytokines can be released as a result of MAPK signalling events, particularly p38 [859-861], but also ERK and JNK [862-864]. The blocking of CD200R and CD200L led to increases in both of these cytokines in response to PHA, consistent with increased MAPK signalling events. CD200L engagement of CD200R has been shown to reduce MAPK signalling in all pathways in mast cells [857], occurring as a result of the phosphorylation of Dok2 (docking protein 2) and the subsequent recruitment of RasGAP (Ras p21 protein activator 1/Ras GTPase activating protein) which is involved in the MAPK pathway [857, 858]. Whilst RasGAP is most commonly associated with the ERK pathway in signalling [865], it has been shown that it influences other MAPK pathways such as p38 [866]. It should be noted that the CD200R receptor also recruits SHIP-1 (Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1) via Dok1 phosphorylation in an indirect mechanism dependent upon Dok2 and RasGAP [858], though this has a limited negative effect on the inhibition through CD200R, as SHIP-1 is itself responsible for inhibition of MAPK signalling (therefore recruitment to the receptor reduces this regulation) [867].

More needs to be established functionally for the signalling in sarcoidosis cells to be characterised fully. Attempts to agonise the receptor with a recombinant CD200L protein were not effective within the PHA whole blood assay. This might relate to a number of factors, including the experimental set up, the order in which the peptide was added (post or pre stimulation) or other undetermined issues. When Zhang *et al* [857] attempted to inhibit degranulation of mast cells using a soluble CD200L peptide, the group did not observe reduced degranulation; they only managed this when the receptor was overexpressed in mouse cells. The authors suggest that this may be the result of the density of CD200L required for activation, which might be naturally greater on cell surfaces and also allow other interactions that a soluble form lacks. A similar lack of efficacy on mouse mast cells under normal conditions with CD200 peptide was observed in another study, suggesting that this is not an isolated issue [868]. Research using human PBMCs highlighted that agonists of CD200R also required cross-linking for any effect to be observed, which would naturally occur between cell surface interactions but be absent

for soluble peptide [869]. If this were the case, the results obtained within the current study would be consistent with these findings. Other groups have demonstrated soluble engagement with peptides in a murine system are capable of inducing a negative signal however [870], therefore it is possible that it may relate to the kind of experiment being performed; PHA or a multi-celled *ex vivo* system may have confounding effects that are not fully understood for this interaction, though the evidence suggests addition factors are required for a soluble peptide to inhibit CD200R under normal conditions.

Despite the focus of this research on peripheral blood leukocytes, identifying whether the CD200 receptor and ligand were expressed in the tissue of patients with sarcoidosis was important to provide a link between the observed expression in peripheral blood and the cells in granulomatous lungs and lymph nodes. Despite aesthetic issues relating to the darkness of the images (relating to time constraints), the staining showed that CD200R was expressed on histiocytes within the granulomata, but absent from the majority of lung resident cells, supporting the findings of the peripheral blood studies where the receptor was expressed on monocytes, both myeloid cell types. There appears to be limited published examples of human tissue staining of CD200R by immunohistochemistry; during the experiments there were technical difficulties in obtaining positive staining with a different antibody that might relate to the use of formalin-fixed paraffin-embedded tissue (FFPE). Macrophages have displayed CD200R expression by IHC in neural tissue (frozen, not FFPE) , and human mast cells in skin have been shown to express the receptor by use of fluorescent microscopy [868]. It is clear that the tissue expression of the receptor is limited to specific cell types. By contrast, CD200L was heavily expressed by a range of different cell types in granulomatous tissue and was particularly high on the fibroblasts surrounding the peripheral region of the distinct granulomata. This suggests that there is CD200L expression in tissue of patients with sarcoidosis, though the absence of CD200L within the core of the granulomata may be significant, as histiocytes may not therefore gain significant regulatory signals in this pathway as a result. Whether this is the case is unknown, but further work on the role of the receptor and ligand in granulomata may merit further investigation. The CD200L ligand has been identified in various tissue and organ systems previously, showing widespread distribution on cells in support of the findings of this study [827, 871, 872]. There were clear limitations on the type of information that could be obtained from the

tissue staining, as this was only on patients with sarcoidosis and had no additional point of reference in healthy tissue. For ethical reasons, healthy controls are not available for study and would likely not present with the kind of immune infiltrates present in diseased tissue. Tuberculosis granulomatous tissue would be a reasonable comparison, though these were not available at the time this study was performed. It would therefore be an area for future research to compare the levels of CD200R and CD200L in tissue between patients with TB and sarcoidosis to see whether the expression was similar between the two.

It should be noted that the current study focussed on PBMCs for characterising the expression of CD200R, though it has been demonstrated that the receptor is heavily expressed on granulocytes (neutrophils) and dendritic cells, more so than even monocytes [873]. The role of neutrophils and dendritic cells has not been studied in this current report, but given the preliminary findings of this work, it may be prudent to study these cell types in terms of the CD200 receptor for sarcoidosis in the future. Additionally, this study highlighted that a subset of T-lymphocytes expressed the CD200L receptor, though did not identify the subset responsible. It has been shown by another group that by activating T cells with a stimuli, there is an increase in the proportion CD200L expressing cells [874]. In any future work relating to CD200L, staining with activation markers together might be pertinent, though this was not included in the current study.

How the CD200 receptor relates to the clinical features of the disease is more complex. There was a demonstrable association between plasma viscosity and CD200R expression on monocytes, though this was not the case for CRP. Nor was there any correlation between T-lymphocyte CD200R and these inflammatory markers, suggesting that the expression of the receptor is unlikely to simply be a function of elevated inflammatory action in patients. This would be supported by the IL-10, IL-10R and SIRP-α data, none of which showed a generalised reduction in expression/concentration consistent with a systemically reduced regulatory mechanism. The CD200R data also did not significantly correlate with the typical clinical findings used to stratify patients, such as Scadding CXR stage or organ involvement. There were trends, though a more comprehensive study with more patients would aid in determining whether the CD200R expression relates to these factors; based on this preliminary data, it appears that CD200R does not directly associate

with these, though it appears to relate more to the differences observed from whole blood. The significance of this expression in a clinical setting would require a longitudinal study, to determine whether low expression relates to prognostic outcomes in patients or represents another feature of the disease not covered by current descriptors. It is interesting that CD200R^{low} on monocytes was predominantly male patients, as this was not replicated in healthy controls. This could relate to the gender difference in disease manifestation in sarcoidosis mentioned previously (Chapter 1 and Chapter 3), which would be worthy of investigating further in any subsequent studies alongside areas not covered in this research. Whether or not CD200R has utility as a potential biomarker remains to be seen, as this is only a pilot study with a limited number of patients at one time point. Much would depend on whether expression of the receptor changes over time and whether differential expression represents particular prognostic outcomes. As a preliminary study, with no existing work on the CD200 receptor in sarcoidosis previously, there were limits to what could be investigated given limits in time and patient samples, though a great deal has been completed to allow future work to expand upon these initial findings.

5.4.4 Conclusion

This study is the first to characterise the expression of the regulatory receptor CD200R and its cognate ligand CD200L on peripheral blood mononuclear cells in sarcoidosis. It has demonstrated that distinct CD200R^{low} and CD200R^{high} monocyte subpopulations exist in patients with sarcoidosis, and low monocyte CD200R expression was associated with heightened TNF-α and IL-6 production. T-lymphocyte CD200R also displayed a bimodal distribution of expression, predominantly CD200R^{+} and CD200R^{+++}, whereas the vast majority of patients had medium expression on T cells, CD200R^{++}. Blockade of CD200R-CD200L interactions led to increased TNF-α and IL-6 secretion by monocytes, recapitulating the hyper-activated monocyte state seen in sarcoidosis. No association was found between monocyte CD200R expression and serum ACE activity, suggesting that CD200R is not simply a marker of granuloma burden. Other studies have reported reduced CD200R expression on monocytes in rheumatoid arthritis [826], but this current work found no associations between CD200R expression and serum C-reactive protein,

with some association to plasma viscosity, signifying that the CD200R^{low} phenotype is not merely a manifestation of systemic inflammation in sarcoidosis. Further studies are required to assess its value in predicting disease progression and response to anti-inflammatory therapy. Whether a similar hyper-responsive phenotype is exhibited by local tissue macrophages in sarcoidosis has yet to be determined. Murine models suggest that in lung inflammation, monocytes actively contribute to resident macrophage populations [290]. Lung macrophages are potent producers of TNF-α and IL-6 [296, 341] which contribute to the formation of sarcoid granulomata [593].

CD200R/CD200L is implicated in the pathology of many inflammatory or degenerative diseases via reduced or decreased protein expression on leukocytes, including Alzheimer's disease [827], rheumatoid arthritis [826], Parkinson's disease [875, 876] and different types of cancer [828, 877, 878]. The present study provides evidence that the CD200R/CD200L axis is also important in modulating pro-inflammatory cytokine release in sarcoidosis, and raises the possibility that disease progression could be propagated by reduced monocyte CD200R expression. CD200 recombinant proteins and agonistic antibodies have been reported to reduce macrophage activation and promote immunological tolerance in human cells *in vitro* and in mice *in vivo* [868-870, 879] . There is a need for improved treatment options for patients with sarcoidosis, and the CD200L/CD200R axis is potentially tractable for future therapy [880]. Investigating the possible therapeutic and prognostic value of CD200R in sarcoidosis might be useful in ultimately providing improved clinical outcomes in patients with the disease.

Chapter 6
General Discussion and Conclusions

6.1 General Discussion

Sarcoidosis is a disease of contradictions, not least in the often repeated paradigm that the condition displays elevated tissue inflammatory responses alongside reduced peripheral blood immune responses. The disease is heterogeneous in manifestation, variable in how it progresses or resolves and difficult to precisely characterise in terms of its pathogenesis. If a researcher selected two patients at random from a cohort defined as having sarcoidosis, it is possible that they might display radically different clinical and immunological features which might otherwise suggest they had different diseases. The challenges in researching this disease cannot be understated, as the protean presentation of patient symptoms and immunological inconsistencies in sarcoidosis means results can be equally variable. It still remains a condition without an aetiology, a poorly understood immunopathogenesis, variable and often invasive diagnostic procedures, inadequate prognostic markers and limited therapeutic options [1].

The findings of this research reflect the contradictions in immune responses and variability in the disease; patients with sarcoidosis displayed enhanced pro-inflammatory responses to the lectin PHA in peripheral blood, yet reduced responses to a different immune activator SEA. The T-lymphocytes present in the blood are greatly reduced in number in patients with sarcoidosis, intermediate monocytes are expanded as a population within monocytes in the disease and yet the total number of monocytes and other mononuclear leukocytes are unaffected. Patient immune responses to specific innate immune ligands were not elevated, yet there is clearly a demonstrable reduction in the regulatory receptor CD200R on monocytes that is associated with enhanced inflammatory cytokine release. Even the expression of CD200R on T-lymphocytes represents the immunological dichotomy present in sarcoidosis, with patients displaying either high or low expression, compared with the medium level exhibited on healthy controls. This theme of contradiction runs through the results obtained from the study, and perhaps represents the inherent complexity of the disease that is termed 'sarcoidosis'. To the best knowledge of the author, this is the first study to characterise the expression of CD200R and CD200L on peripheral leukocytes in sarcoidosis, showing that the regulatory axis in sarcoidosis might be altered. There are a number of implications of this finding, yet it also leaves a number of questions to be answered. The

findings from this study as a whole provide numerous avenues for discussion, as well as possible future directions for research based on the preliminary work presented.

6.1.1 Summary of the Key Findings

Patients with sarcoidosis were a well-defined cohort assessed through a multidisciplinary team that required consensus for a positive diagnosis from a respiratory physician with a particular interest in ILDs like sarcoidosis, a thoracic radiologist and a thoracic pathologist. All presented with some form of pulmonary organ involvement, whether mediastinal lymph node or parenchymal lung inflammation, and 43% of the cohort had identified extrapulmonary organ involvement. The majority of patients were Scadding CXR stage I or II, 76.6%; there were no stage IV patients present in this cohort. 60% of patients had serum ACE levels above the normal range, which was associated with an increased number of organs involved in the disease and sACE was higher in patients with extrapulmonary organ involvement ($p = 0.01$). The plasma viscosity and C-reactive protein levels were elevated in 26% and 27% of patients respectively, and were correlated with each other, but not sACE levels.

Patients with sarcoidosis had a significantly lower number of peripheral blood mononuclear cells in their blood compared with controls, 1.21×10^6 cells/ml compared with 2.03×10^6 cells/ml ($p < 0.0001$). This was attributable to a T cell lymphocytopenia; patients had a mean of 0.66×10^6 cells/ml compared with 1.43×10^6 cells/ml in controls ($p < 0.0001$). Both $CD4^+$ and $CD8^+$ subsets of T-lymphocytes were reduced in number in sarcoidosis. The number of monocytes, B-lymphocytes, and natural killer cells were not significantly altered in patients with sarcoidosis. CD25 was present on a greater proportion of the T-lymphocyte population in patients with sarcoidosis and was more highly expressed on their T cells, though the number of $CD25^+$ cells was significantly reduced due to the T cell lymphocytopenia. The Intermediate monocyte subset ($CD14^{++}$ $CD16^+$) made up a greater proportion of the monocyte population in sarcoidosis compared with controls (13.6% compared with 9.3% respectively, $p < 0.05$), matched by a reduction in Classical monocytes in the disease. PHA induced cytokine release was greater in whole blood assays than isolated PBMCs. After 16 hours incubation with PHA, patients

with sarcoidosis released more IL-6 and TNF-α in response to high concentrations of the lectin than healthy controls ($p < 0.001$ and $p < 0.05$ respectively with 100 µg/ml of PHA); these two cytokines were strongly correlated with each other in PHA induced whole blood release ($p = 0.001$, $R^2 = 0.56$). Intracellular TNF-α and IL-6 accumulation in PHA and Brefeldin A treated PBMCs suggest that the predominant initial source of these cytokines are monocytes; the increase in both cytokines in monocytes were elevated in patients with sarcoidosis. Unlike PHA, SEA induced a significantly lower concentration of IL-6 release from whole blood in patients compared with controls ($p < 0.0001$). PHA induced IL-6 and TNF-α release in patients may correlate with some clinical features to a small degree, but neither correlated with serum ACE levels.

Whole blood was treated with a range of ligands and cytokines for 16 hours: LPS, MDP, IE-DAP, β-glucan, Poly I:C, TNF-α, IFN-γ, HMGB1, S100B, S100A9 and S100A12. No statistically significant differences were observed in cytokine release (TNF-α, IFN-γ, IL-6 or IL-10) between patients with sarcoidosis and healthy controls with any stimuli used in this system. A number of trends were observed, though none of these were significant enough to accurately conclude differences exist between responses in the cohorts. RAGE expression was not detected to any significant degree on cell surfaces on lymphocytes or monocytes using the method employed. Intracellular RAGE was detected in both cell types, yet there was no significant difference in expression between controls and patients. There was also no significant differences in the change in expression in response to a variety of RAGE ligands (S100 proteins and HMGB1) or other features ligands/cytokines. NOD2 expression inside cells was also not significantly different between patients and controls at baseline, however, exposure to MDP and TNF-α led to an increase in NOD2 expression in healthy controls, but a reduction in the expression of NOD2 in patients with sarcoidosis ($p < 0.05$).

IL-10R expression was detected on both T-lymphocytes and monocytes in peripheral blood leukocytes and was not significantly different in expression between healthy controls and patients with sarcoidosis. Low IL-10R expression on T cells was associated with elevated serum ACE in patients with sarcoidosis. The myeloid specific SIRP-α and its cognate ligand CD47, the latter being ubiquitously expressed on the PBMCs, were present

in patients with sarcoidosis, but were not significantly different in the levels of expression compared with controls.

The regulatory receptor CD200R and its cognate ligand CD200L were also characterised on peripheral blood leukocytes. CD200R was expressed on monocytes and T-lymphocytes, CD200L was expressed on a subset of T-lymphocytes and heavily expressed on B-lymphocytes. CD200R expression on monocytes was lower in patients with sarcoidosis relative to healthy controls (p = 0.046). Individuals could be defined as either CD200R^{low} or CD200R^{high} based upon their monocyte expression; 52.9% of patients were CD200R^{low} compared with only 11.7% of healthy controls (p < 0.0001). CD200R expression on T cells in healthy controls tended to be a medium level (CD200R^{++}), whereas patients tended to be either low (CD200R^{+}) or high (CD200R^{+++}) for CD200R expression. CD200R^{low} was associated with higher IL-6 release from PHA induced whole blood assays (p = 0.018) and blocking the interaction between CD200R and CD200L increased the IL-6 and TNF-α release from healthy controls in PHA stimulated whole blood. The percentage of CD200L positive T-lymphocytes was not significantly different between healthy controls and patients with sarcoidosis, though the number of CD200L^{+} T cells was lower in patients due to the T cell lymphocytopenia (p = 0.0001). CD200R was present on histiocytes and other myeloid cells in tissue from patients with sarcoidosis, localised particularly in the core of the granulomata present in lymph nodes and lungs affected by the disease. CD200L was highly expressed and widely distributed in tissue; the ligand was present on a range of lung cell types including fibroblasts, alveolar epithelial cells and smooth muscle cells. Granulomata displayed a peripheral ring of fibroblasts expressing the ligand with a core containing CD200L negative cells, the histiocytes, which is consistent with the findings that monocytes do not express the ligand. Despite some association with plasma viscosity, CD200R expression on monocytes did not correlate with traditional markers used to assess sarcoidosis, such as serum ACE and CRP levels, suggesting that CD200R expression may relate to different features of the disease pathology than existing markers.

6.1.2 Wider Implications

The key novel findings presented in this report relate to the expression of CD200R on peripheral blood mononuclear leukocytes in sarcoidosis, the first study to characterise the CD200R/CD200L regulatory axis in the disease. The results showing a reduced level of expression of the receptor on monocytes and its association with enhanced pro-inflammatory responses to PHA are potentially exciting, as it can direct future study in sarcoidosis to investigate a regulatory mechanism that may potentially have an important role in the immunopathogenesis of the disease (see Section 6.4).

The overall results presented here, in one sense, serves as a microcosm representing the paradox often associated with study into the immunology of the disease. As previously mentioned, research into peripheral immune responses have previously either reported similar responses to a healthy population [26], an increased peripheral response [16-20] or a reduced/anergic peripheral immunity [21-25]. Within this study, all of the above has been demonstrated depending on the conditions of the experiment. PHA stimulation at high concentrations led to increased peripheral blood responses in patients, which would be consistent with the work published indicating an increased responsiveness can exist in sarcoidosis. Responses to SEA were the opposite, supporting the often cited peripheral anergy concept [23]. For a large number of the other ligands and cytokines used in the whole blood assays, there was no significant difference observed between healthy controls and patients (though with some variability), supporting the idea of a relatively unperturbed peripheral system. The co-existence of these different responses would of course appear perfectly feasible as a concept; not all stimuli in any pathology will invoke the same responses. It is however an often repeated concept, based primarily on limited numbers of reports highlighting and reinforcing a fallacious concept that there is an inherent 'peripheral anergy' that exists in sarcoidosis [23, 24]. So often is this interpreted as a global feature of peripheral blood immune responses, that review articles can cite it as a feature of the disease [4]. The research presented here for the immune responses supports a more nuanced and less 'all encompassing' description of peripheral blood immunity. It could be posited that there exists specific and distinct alterations in immune responses in sarcoidosis; a protean system characterised by specific responsiveness to some immune stimuli and diminished responsiveness to other stimuli in the disease.

Whilst this continues to support the concept of an inherent paradox present in the disease, the work presented in this study suggests that the peripheral blood system itself is also subject to contradictions that have not been reported with equal vigour as a clearer, but less precise, narrative relating to anergy.

This is important, as it goes to the heart of the concept of the disease. The research collated and presented as part of the introduction to this work is extensive and should indicate that sarcoidosis is best thought of as a disease with a systemic component (Chapter 1). Multiple organ systems are often involved and there are serum markers, such as sACE, that reflect the downstream impact of local tissue inflammatory responses [47, 232, 242-244]. The T cell lymphopenia observed in this study, and similar reports by other groups [257, 258, 715], suggests that peripheral leukocytes are not merely inactive bystanders in the disease. Additionally, the elevated CD25 expression also observed on T cells in the current work also points to systemic alterations in leukocyte populations that might actively contribute to the immunopathogenesis of the disease. Broadly speaking, the findings suggest that a number of peripheral blood leukocyte populations are altered in sarcoidosis and could be considered for both clinical and possible prognostic value.

Sarcoidosis has a degree of overlap with other similar diseases in the end pathology, namely that diseases like Crohn's Disease, TB, Berylliosis and a variety of infectious conditions can display granulomata (Section 1.8). There is a question regarding how related each of these are to sarcoidosis, as diseases such as TB have a defined infective cause and a better understood immunopathogenesis related to the infection itself [619], whereas the aetiology of sarcoidosis remains unknown [1]. Despite the differences, some of the basic processes underlying granuloma formation are believed to be similar across these diseases [260], therefore sarcoidosis may represent an opportunity to understand the immune mechanisms of a disease which could provide insight into understanding other related conditions. Without a direct comparison between individuals with different conditions, there are limits to how much this can inform other studies. However, having identified that peripheral monocytes in sarcoidosis display a reduced CD200R expression, it would perhaps be useful to look at these related diseases to see whether the same presentation is a feature of all granulomatous diseases (see Chapter 6). After all, it has been shown that other inflammatory conditions also display reduced CD200R , whereas it

has been shown in cancer that CD200L is increased in terms of expression [828]. Both of these diametrically opposed manifestations of CD200R/CD200L modulation show that this regulatory axis may be important in a variety of other conditions. It should also be noted that, whilst the results are very preliminary, the difference in NOD2 receptor responses to stimulation with its cognate ligand MDP and TNF-α between sarcoidosis patients and healthy controls may provide some additional details relevant to Crohn's Disease. NOD2 polymorphisms are considered a risk factor in Crohn's Disease [429, 430]; the preliminary findings that TNF-α and MDP actual decrease NOD2 expression in sarcoidosis T cells and monocytes when healthy controls display an increase in expression upon stimulation is an interesting discovery. Early onset sarcoidosis and Blau syndrome are believed to be associated with 'gain of function' NOD2 mutations [881], though Crohn's Disease has typically been associated with 'loss of function' mutations [882]. This does not necessarily translate to reduced expression of NOD2 in Crohn's patients; in general TNF-α increases NOD2 expression, but a defective response to TNF-α is observed in Crohn's [795]. It is therefore surprising to see a reduction in NOD2 expression in patients with sarcoidosis in this study after stimulation, though further investigation might provide insight into the mechanisms underlying this and how it relates to Crohn's Disease.

Therapeutic strategies for sarcoidosis are currently limited, corticosteroids remain the drug of choice for patients with progressive disease, though the side-effects of this treatment can be distressing to patients and is associated with higher rates of relapse [390, 592]. Developing alternatives is difficult however, as there are currently no accepted, widely used animal models of the disease, despite some attempts to simulate the granulomatous inflammation observed in sarcoidosis in mice [883]. Any identified target for possible therapeutic intervention, such as CD200R, would likely need to demonstrate functional efficacy in alternative granulomatous inflammatory models (such as TB models) and safety in such systems prior to wider examination for sarcoidosis. At a later stage, should CD200R/CD200L become a potential target of interest, these issues would have to be addressed in earnest.

Ultimately, this work further highlights that a great deal still has to be understood about sarcoidosis. These findings cannot support a specific aetiological theory; none of the

ligands used to stimulate whole blood led to significantly increased pro-inflammatory cytokine release when compared with healthy controls. The cause therefore remains unknown [1]. Nor does this research fully elucidate the immunopathogenesis of the disease, though the important impact of the work is that it indicates that regulatory pathways may be important in the disease. The extent to which this applies would require further investigation; it is hoped that identifying a specific reduction in CD200R expression, rather than a generalised reduction of regulatory receptors that included IL-10R and SIRP-α, might aid in focusing future work on this particular regulatory pathway and help in the development of much needed therapies for patients with sarcoidosis [591, 592].

6.1.3 Limitations of the Work

A number of issues with specific experimental procedures and results have been mentioned previously (see Sections 3.4, 4.4 and 5.4), therefore the focus of this discussion will be on broad limitations and areas for improvement in subsequent work that could expand upon this. To allow for examination of limitations arising from this research specifically, rather than any kind of research, issues broadly relating to limitations such as financial considerations and overall time permitted for the research will not be discussed in detail. It should be noted however that there was clearly a limit to the kind of experiments and resources/materials that could be employed based on both financial considerations and time. Therefore, with greater investment and time available, some areas could be expanded upon in the future that could not be addressed during this study.

The first and most important consideration relates to the availability of patients with sarcoidosis. As mentioned previously, the incidence of sarcoidosis is estimated to be approximately 11 to 36 per 100,000 [95], though a UK study suggested that this could be as low as 5 per 100,000 people in this country [382]. It is fair to conclude that sarcoidosis is not a 'common' disease, therefore the potential pool of donors for a disease such as this is low. This was discussed in detail in Section 3.4.1.1, though it bears repeating as it impacted on a large number of factors present in the study. Although the number of

individuals recruited for this study was very good, 30 patients in total, the fact remains that there were limits to what could be done with a cohort of this size when multiple streams of research were being undertaken. Although blood was used in various different experimental procedures from each donor, only a limited selection could be performed for each patient from each sampling. Additionally, as some aspects of the research were expanded, the priority of experiments in other areas had to be reduced to accommodate newer areas where there were promising initial results. An example of this would be that significant amounts of the data from CD200R was obtained later in the project, whilst the ligand stimulation of whole blood and RAGE/NOD2 staining on leukocytes were being performed. In order to put research into order of priority, more blood was apportioned to regulatory receptor work and the continuation of whole blood assays with PHA/SEA stimulation, whilst less resources were placed into ligand stimulations and RAGE/NOD2 work (Chapter 4) which had shown less significant results. This clearly limits what can be concluded from some aspects of work, though this is considered a necessary feature of an exploratory study such as this, where some areas will invariable display more initial promise. With a larger number of patients and more time and resources, these areas could be investigated more thoroughly.

Another issue relating to the patients recruited is that, due to the local demographic in the region, the majority of patients were Caucasian. It has been demonstrated that racial differences exist in sarcoidosis in terms of organ involvement and clinical course of the disease [48, 95]; therefore any conclusions obtained from this study are specific to Caucasian patients and further study with other racial groups would be necessary to provide a rounded conclusion about the findings obtained in this current study. Additionally, although latent TB patients were initially recruited for the study, there is a question regarding how relevant these results are when comparing them to the sarcoidosis and healthy cohorts. Only a limited number were included, of varying ages and racial groups, therefore this would not necessarily be a good comparison. This relates to limitations in obtaining blood from latent TB patients; existing latent TB patients were typically receiving medication for the condition and could not be used in this study. Newly diagnosed patients were found to be difficult to recruit as many did not consent and there were significant language barriers in clinic. The result was a cohort too varied and limited in number, therefore any future work would need to either find a different patient

group for comparisons or utilise a more effective method for obtaining samples matched more closely to the sarcoidosis patient cohort.

One of the other primary limitations of the current study relates to the use of a single sample from patients. Many of the findings presented in this research, such as the reduced CD200R expression on monocytes, have been obtained from patients at a specific stage in the disease. It has been demonstrated that features present in sarcoidosis can change over time [78, 95], it is therefore possible that the observations presented for patients might also be dynamic, though this cannot be established in this kind of preliminary study. As a 'temporal snapshot' of the disease, it has been suggested in this research that findings might relate to existing factors, like comparing serum ACE levels to the number of organs a patient has involved. This establishes whether a finding, such as increased IL-6 and TNF-α release from whole blood or the CD200R phenotype on monocytes and T cells, might relate to prognosis; a study over a longer period charting changes in these factors might be useful (see Section 6.5). The findings presenting in this report are to be considered preliminary, part of a pilot/exploratory study that can subsequently be investigated in the future.

The value and limitations of the use of a whole blood assay have been covered previously in detail (Chapter 3), though it is acknowledged that if there had been more time, patients and resources, a more comprehensive study looking at the immune response from individual leukocytes would have been of value. This was defined initially by the decision to use PHA as an activator of immune responses, which was demonstrably more suited to whole blood (Section 3.3.6) and requires multiple cell interactions for optimum activity [699]. Whilst it would have been possible to test purer cell populations in some of the subsequent ligand work, the limited resources available (blood in particular) meant that consistency and a direct comparison to other work with the lectins and mitogens was considered preferable to utilising a different assay system for those experiments (Chapter 4). The main issue would invariably be that the precise reason for any differences observed in whole blood would be more difficult to ascertain; however, looking at the whole blood using this holistic approach would be more physiologically relevant than isolated PBMCs or a single cell type response. The precise mechanisms underlying the

differences observed from these assays could be investigated in subsequent work in the future.

6.1.4 Future Research

With a diverse report spanning a multitude of different research areas, there are a large number of potential projects that can be investigated further or in more detail based on the preliminary data obtained here. This cannot cover every conceivable research possibility; a number of possible future experiments have been suggested previously (Sections 3.4, 4.4 and 5.4), and therefore only primary areas of interest for future work are discussed.

The whole blood assay was an effective holistic tool for assessing *ex vivo* cytokine release from peripheral leukocytes with PHA stimulation. It was clear that latent TB patients were not necessarily suitable as a comparison cohort alongside sarcoidosis patients and healthy controls. Other reports comparing sarcoidosis to other diseases have opted for related but particularly distinct diseases; a favourite for comparison is tuberculosis, owing to the pulmonary granulomatous inflammation common to both diseases [643, 644, 884]. This can be a bit of a double edged sword however, as some transcriptomics data has suggested that the two diseases are very similar [513, 884], whereas a profile of peripheral blood and BAL suggested that the responses in the former were different between the two diseases [644]. This is not necessarily a negative, as it is important to test how specific the findings observed in sarcoidosis are, whether from whole blood tests or CD200R experiments. For example, it has been demonstrated that CD200R expression on leukocytes is also reduced in patients with Alzheimer's disease and Rheumatoid Arthritis [826, 827], therefore a comparison between other inflammatory diseases is appropriate. There are a large number of diseases with immunological components in their pathogenesis; a functional proteomics analysis selected patients with sarcoidosis and included a comparison with diseases including systemic sclerosis and idiopathic pulmonary fibrosis [885]. This leaves open the possibility that distinguishing features of sarcoidosis can be tested against other pathologies for a robust study and comparison.

One of the primary areas that would be necessary for future study is the expansion of the existing preliminary data to feature a longitudinal study on how CD200R and CD200L change in patients over time. As the existing data was obtained from patients at only a single point during the disease, it is not easy to place the observations of either CD200R expression or the PHA whole blood assays in context. The principle question that would need answering would be to address how (or if) the CD200R expression profiles on patients and the whole blood assay results relate to disease prognosis and progression. Studies which follow patients over a longer period taking different readings at different times can provide invaluable insights into the dynamic changes in the disease [48, 53, 55, 95, 886]. Retrospective analyses would also be required for this to be effective, as patient outcomes could be associated with either CD200R expression or whole blood assay responses.

The observation that intermediate monocyte populations are increased in sarcoidosis has been noted by Hijdra *et al* [336, 734]; it is possible that these CD16 expressing monocytes are more pro-inflammatory in nature [336, 734, 856]. Whilst the raw percentages of this subset could be correlated with CD200R expression on monocytes and other experiments (such as the whole blood assay), this was not a direct association that can be examined more thoroughly. In their report, Hijdra *et al* elegantly showed how different monocyte subsets differentially expressed TNFR1 and TNFR2, each subset displaying different expression profiles [336]. It can therefore be clearly demonstrated that there is a distinction between these subsets. Applying this to future work relating to CD200R, if would be of great value to stratify monocyte subsets in patients and characterise the difference in CD200R expression on individual subsets in the disease. The hypothesis would relate to whether intermediate monocytes display reduced CD200R expression and contribute to the ongoing pathogenesis of the disease. This would be an important next step in any future study into CD200R/CD200L in sarcoidosis.

The 'elephant in the room', so to speak, is that sarcoidosis is regularly identified as a disease of pronounced local tissue inflammatory responses [1], yet this current study has characterised only peripheral blood responses. For sarcoidosis, bronchoalveolar lavage cells are a useful source of leukocytes from the local tissue which display enhanced inflammatory cytokine release and activity [18, 280]. Future work would, as a necessity,

be required to move to BAL cells and characterise the expression of the CD200 receptor and ligand on both patients with sarcoidosis and suitable controls. It is of course difficult to obtain BAL from truly 'healthy' controls, for ethical reasons. However, a comparison of BAL from patients with another suitable group undergoing BAL, such as IPF, would still provide a comparison to provide the link between the results observed in this study and the local tissue responses in active leukocytes. The immunohistochemistry performed as part of this current study had shown that both CD200L and CD200R are expressed in tissue of patients with sarcoidosis. Another necessary future experiment requires a semi-quantitative comparison of these results with other similar tissue from another disease, such as TB, to see whether the CD200R/CD200L expression levels shown are specific to sarcoidosis or a general feature of granulomatous inflamed tissue.

The failure to demonstrate an agonistic response for CD200R has been discussed previously (Section 5.4), but logically a reasonable direction for future work would be to develop a suitable agonist for the receptor and demonstrate the effectiveness of this in modulating human CD200R. Others have reported success in developing these in mice [870, 880], therefore it is possible that a designed agonist would have immunosuppressive properties that might prove effective in controlling sarcoidosis inflammatory responses. This is hindered a little by the lack of a mouse model for sarcoidosis; though others have developed apparently similar model of the granulomatous inflammation in murine models that might mimic the disease [883]. The first step would be to demonstrate that a peptide or antibody therapy could work on *in vitro* cell models, transitioning to more complex systems after this. Such work would be initially difficult, though ultimately useful in developing possible therapeutics for a number of inflammatory diseases including sarcoidosis.

6.2 Final Conclusion

Sarcoidosis is a disease of varied peripheral immune responses. This study has demonstrated that patients with sarcoidosis are capable of providing a robust inflammatory response when treated with specific stimuli, such as PHA. In other circumstances, such as that observed with SEA stimulation, the response from patients can be reduced relative to healthy controls. Patients exhibited a reduction in peripheral T-lymphocyte number that may impact this response negatively, thus explaining why many reports identify a 'peripheral anergy' in immune responses for the disease. To the best of the author's knowledge, this is the first time CD200R has been characterised in sarcoidosis. This study shows that there was reduction in CD200R regulatory receptor expression on monocytes in the disease and that CD200R^{low} was associated with increased pro-inflammatory responses from whole blood leukocytes in PHA stimulations. This may be a possible contributing factor explaining how increased TNF-α and IL-6 are released in response to PHA in sarcoidosis whole blood. There is potential for the targeting of this CD200R/CD200L axis for therapy in patients with sarcoidosis and the expression of the receptor may also be important in further understanding the immunopathogenesis of this complex disease.

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Appendix

Appendix Contents

A2.1 – Research participant consent form, research participant information sheet and research participant debriefing sheet used for healthy controls for this study.

Centre Number:

Study Number:

CONSENT FORM

Title of Project: The Immunology of Sarcoidosis

Please initial box

Name of Researcher: Simon David Fraser

1. I confirm that I have read and understand the information sheet dated 20.5.13 (version 1) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

3. I understand that I am under no obligation to take part and that, if I agree to take part, I am free to change my mind at any time.

4. I understand that I will not benefit financially if this research leads to the development of a new treatment or test.

5. I agree to give up to 30ml of blood.

6. I agree that any excess samples not used in this study may be stored and used in future research.

7. I understand that these samples will be stored anonymously and I consent to the information derived from them to be analysed by a computer.

8. I hereby freely give my consent to take part in this study.

_____   _____   _____
Name of Volunteer   Date   Signature

_____   _____   _____
Name of Person   Date   Signature
taking consent

When completed, 1 for patient; 1 for researcher site file;

Version No 1.1 (20.5.13)

Research Participant Information Sheet

The Immunology of Sarcoidosis

You are being invited to participate in a research study as a healthy volunteer. Before you decide whether to participate, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and feel free to ask us if you would like more information or if there is anything that you do not understand. Please also feel free to discuss this with your friends, relatives and GP if you wish. We would like to stress that you do not have to accept this invitation and should only agree to take part if you want to. You are free to withdraw at anytime without explanation and without incurring a disadvantage.

What is the purpose of this study?
We are interested in how people develop different types of lung diseases. One disease in particular called sarcoidosis is associated with alterations in the immune system which can be measured in a blood sample. We are studying the immunological and genetic differences of white blood cells (leukocytes) in patients with sarcoidosis. As well as blood samples from patients with sarcoidosis, we also need samples from healthy volunteers (you) for comparison.

Why Have I been chosen?
As well as blood samples from patients with sarcoidosis, we will take blood samples from healthy volunteers (you) for comparison.

Who is organising the study?
Dr Simon Hart who is a clinical academic doctor at Hull York Medical School.

What will happen to me if I take part?
We would like to take a 30 ml blood sample (equivalent to about 6 teaspoons).

Do you have to take part?
No. You are under no obligation to agree to the study. It is on a voluntary basis. If you prefer not to take part you do not have to give a reason.

What are the possible risks of taking part?
There are no risks in taking part. Blood sampling is mildly uncomfortable.

What are the possible benefits of taking part?
There are no specific benefits for you by taking part in the study. The information we get may help us treat future patients.

Who will see your records and know about your taking part?
The information collected about you during the course of the study will be kept confidential. Any information about you that leaves the medical school will be anonymous so that you cannot be recognised from it.

Details of participation
If you take part, we will take a 30 ml sample of your blood. Samples will be taken at a mutually convenient time at the University of Hull or at Castle Hill Hospital. Your participation in the study will then be finished. Your blood sample will be processed and be stored prior to analysis.

Consent
If you decide to take part in the study, we will ask you to sign a study consent form.

Further information
Please feel free to ask any questions about the study at any time.
Should you have complaints or issues regarding this study, please contact Dr Simon Hart: simon.hart@hyms.ac.uk.
If you require any further details, please contact Simon Fraser on 01482 461889

Thank you for taking the time to read this information sheet.

Version No 1.1 (20.5.13)

Research Participant Debriefing Sheet

The Immunology of Sarcoidosis

What Now?

Thank you for participating in the research. The sample you have donated will be given an anonymous identifying number and the whole blood, leukocytes, plasma and serum either used immediately or stored at -80ºC for later use. Should you have any questions regarding these procedures, ask one of the research team and they can answer these. Your contribution may be anonymously used in research material as part of a larger group control.

Again, many thanks for volunteering.

If there are any problems:

The risks associated with taking blood are minimal, in some cases a small bruise may appear where the sample was taken from.

Further information
If you require any further details, please contact Simon Fraser on 01482 461889

Thank you for taking the time to read this information sheet.

Version No 1 (8.2.13)

A2.2 – Patient information sheet and patient consent form sheet used for patients with sarcoidosis for this study.

Subject Information Sheet and Informed Consent Form *(27.03.13 - version 1.2)*

1. *Study title*   **Immunology of lung fibrosis**

2. *Invitation paragraph*
You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

3. *What is the purpose of the study?*
In many lung diseases the lungs are damaged by inflammation and scarring. Often no cause can be identified, but there is evidence that the body's own immune system may attack the lungs. We would like to look for an abnormal immune response in the lungs using samples of blood, sputum, lung fluid, and lung biopsies that you would routinely have performed as part of your medical investigations.

4. *Why have I been chosen?*
We will be taking samples from about 100 patients with scarred lungs, and also 100 patients with other lung diseases.

5. *Do I have to take part?*
It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign a consent form, and will be given this information sheet and a signed consent form to keep. If you decide to take part you are still free to withdraw at any time and without giving a reason. If you decide to withdraw we would still like to use any samples we have already taken for our research. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of medical care you receive.

6. *What will happen to me if I take part?*
You will be invited to allow us to use some of the samples that will be taken as part your routine medical care. These samples may include (a) blood – an extra **30mls (6 teaspoons)** will be taken; (b) lung lavage(washing) – if you have this test performed as part of your medical care, we would like to use a small amount of the sample (20mls) for research; (c) sputum(phlegm) –you will be invited to submit a specimen of sputum after inhaling a saline (salty) solution; (d) exhaled breath condensate – you will be invited to provide a specimen by breathing into a tube; (e) pleural fluid – if your doctor needs to tap excess fluid from your chest, a small amount of that sample (20mls) will be used for research; (f) **lung and lymph node biopsy** – if you have a lung biopsy performed as part of your medical care, we would request to take a small surplus portion ($<0.5cm^3$) for research.
You will not need to have any of these procedures performed unless they are required as part of your standard medical care, except (c) induced sputum and (d) exhaled breath condensate.

7. *What is the aim of this research?*
We will use the samples to answer some key questions about what causes lung scarring. In particular we will look for evidence of an abnormal immune response in the lungs. In the blood sample we will look for naturally occurring variations in a number of genes that may be important in determining who gets lung fibrosis.

8. *What will happen to any samples I give?*
Samples will be preserved under secure, controlled storage conditions until used for the testing required in this study.

9. *What are the possible disadvantages and risks of taking part?*
Participating in this study will have no implications for you or your treatment, or for future insurance. It is possible that our research may highlight something unexpected in a sample, for example, a lung infection. In this case, you will be contacted by a doctor to discuss the result, and treatment may be offered.

10.   Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential and will not be recorded in your medical records. Any information about you which leaves the hospital will have your name and address removed so that you cannot be recognised from it.

11.   What will happen to the results of the research study?

The results will be presented at scientific meetings and published in scientific journals.

12.   Who has reviewed the study?

The Hull & East Riding Research Ethics Committee has reviewed the study.

13.   Contacts for Further Information

If you have any questions about this research study you should contact Dr S. Hart, Division of Cardiovascular and Respiratory Studies, Castle Hill Hospital, Cottingham HU16 5JQ. Tel.: 01482 624067. email: s.hart@hull.ac.uk

If you have any questions regarding your rights as a research subject or any complaints in relation to this study, you may contact the Patient Advice and Liaison Service, Hull Royal Infirmary on 01482 623065.

Centre Number:
Study Number:
Patient Identification Number for this study:

CONSENT FORM *(28.3.13 – version 1.2)*

Title of Project: Immunology of lung fibrosis

Name of Researcher: Dr Simon Hart, Division of Cardiovascular and Respiratory Studies, Castle Hill Hospital, Cottingham HU16 5JQ. Tel.: 01482 624067. email: s.hart@hull.ac.uk.

Please initial the boxes

I confirm that I have read and understood the information sheet for the above study (**27.3.13**) and have had the opportunity to ask questions. ☐

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

I understand that sections of my medical notes may be looked at by doctors involved in the study where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

I allow a sample of blood to be used for this research study. ☐

I allow a sample of bronchoalveolar lavage (BAL) to be used for this research study. ☐

I allow a sample of **lung and/or lymph node biopsy** to be used for this research study. ☐

I allow a sample of pleural fluid to be used for this research study. ☐

I agree to give a sample of induced sputum. ☐

I agree to give a sample of exhaled breath condensate. ☐

I allow a genetic analysis to be performed. ☐

I allow these samples to be stored for use in future research studies. ☐

_____   _____   _____
Name of Patient   Date   Signature

_____   _____   _____
Name of Person taking consent   Date   Signature
(if different from researcher)

_____   _____   _____
Researcher   Date   Signature

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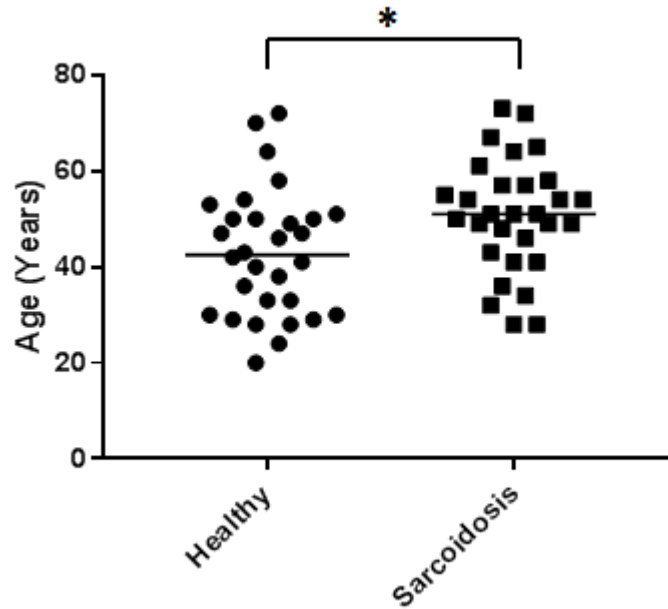
Figure A3.1 – Age of sarcoidosis patients and healthy controls within the cohorts. Median age of healthy controls was 42.5 years and sarcoidosis patients median age was 51 years, represented with a black line. * P = 0.019, Student T Test.
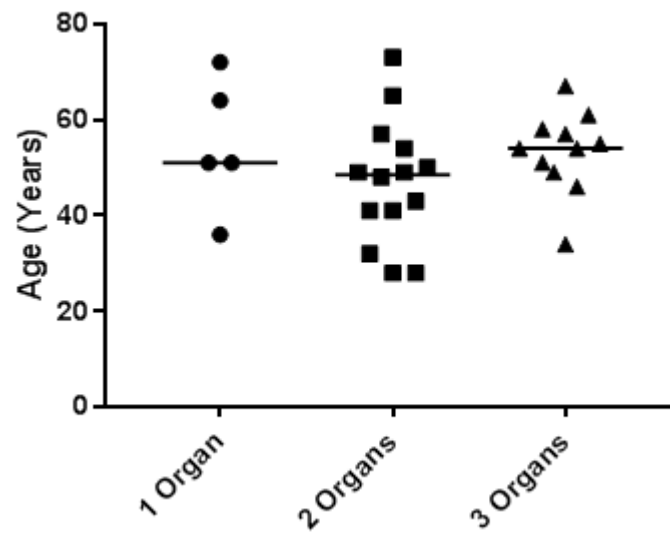
Figure A3.2 – Age of sarcoidosis patients correlated with the number of organs involved. Data presented with median (black line), total n = 30 patients. Median values for 1, 2 or 3 organs were 51, 48.5 and 54 respectively. No statistical significance between groups (p > 0.05), as determined using One-way ANOVA.
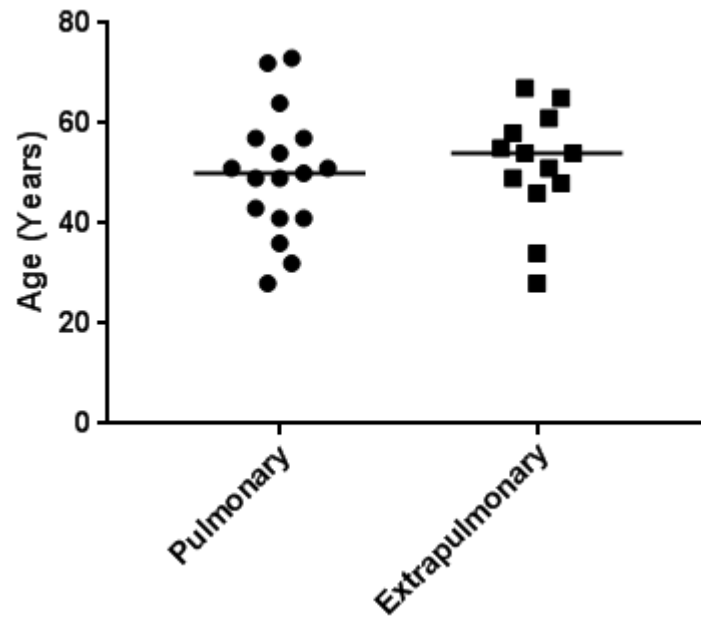
Figure A3.3 – Age of sarcoidosis patients with pulmonary restricted or extrapulmonary organ involvement. Data presented with median (black line), total n = 30 patients. Median values for pulmonary and extrapulmonary were 50 and 54 respectively. No statistical significance between groups (p = 0.711), as determined using Student T Test.
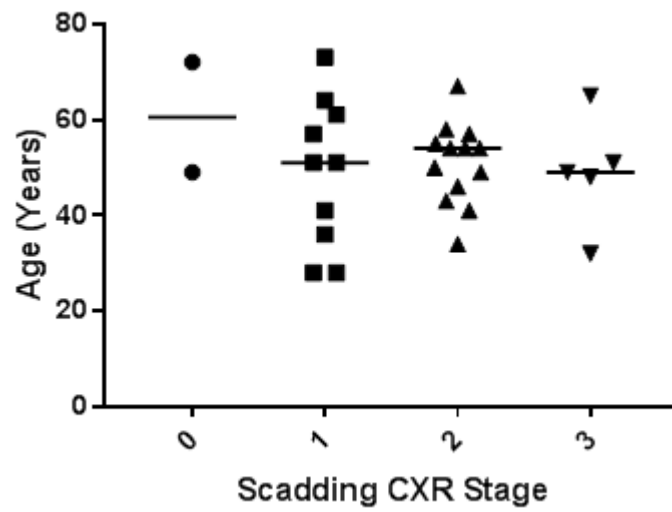
Figure A3.4 – Age of sarcoidosis patients correlated with the Scadding CXR stage. Data presented with median (black line), total n = 30 patients. Median values for stages 0, 1, 2 and 3 were 60.5, 51, 54 and 49 respectively. No statistical significance between groups (p > 0.05), as determined using One-way ANOVA.
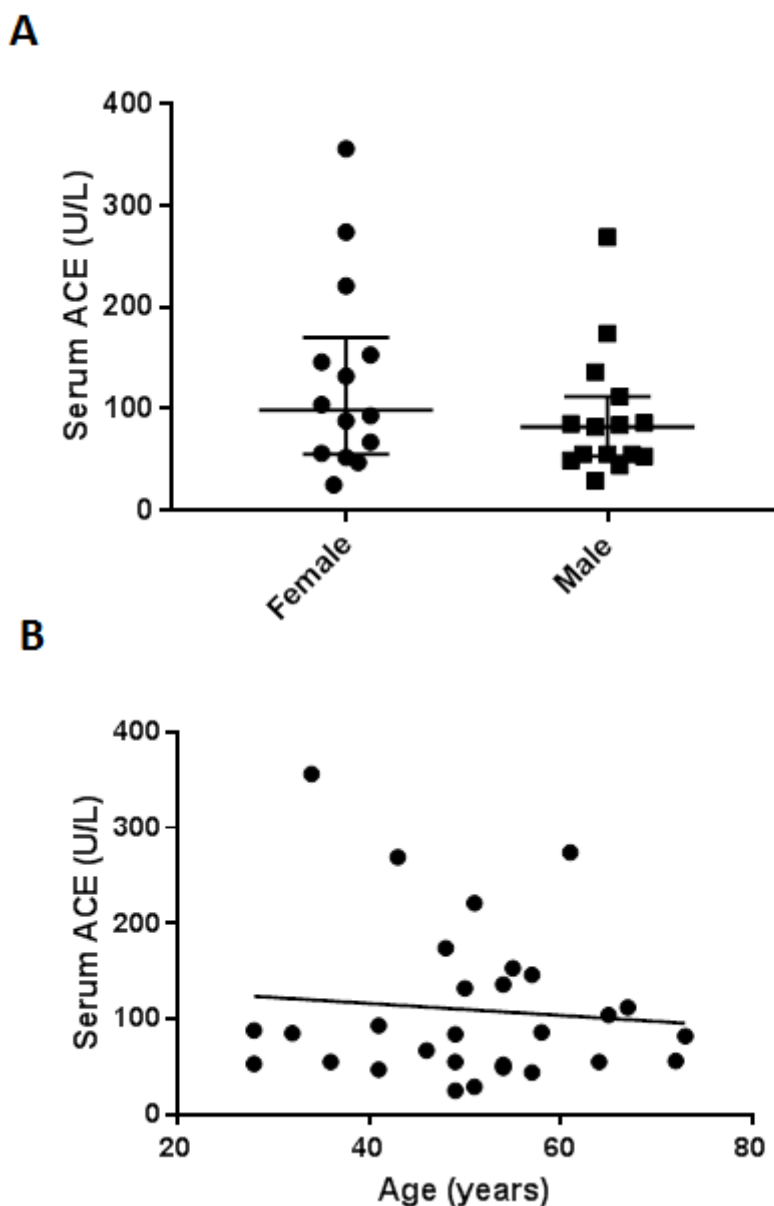
Figure A3.5 – Serum ACE correlated with age and gender of patients. **A:** Serum ACE in male and female patients. Represented with median and interquartile range (IQR). Median serum ACE was 98.5 U/L and 82.0 U/L in female and male patients respectively. No statistical significance between male and female patients (p = 0.217), as determined using Mann-Whitney U Test. **B:** Correlation between serum ACE concentration and the age of patients. Line represents linear regression (R^2= 0.009). No statistically significant correlation (p = 0.869) using Spearman's Rank Correlation Test.
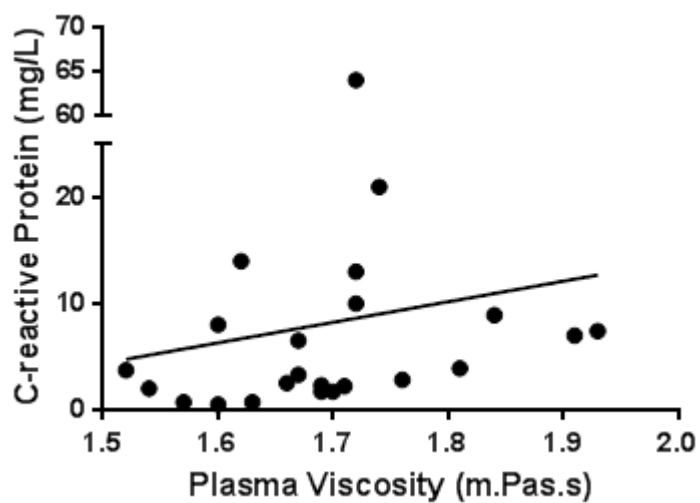
Figure A3.6 – Correlation between levels of the inflammatory markers C-reactive protein (CRP) and Plasma Viscosity (PV) in patients with sarcoidosis. Line represents linear regression (R^2= 0.024). There was a statistically significant correlation (p = 0.026), determined using Spearman's Rank Correlation Test. Y axis segmented for outlying CRP value.
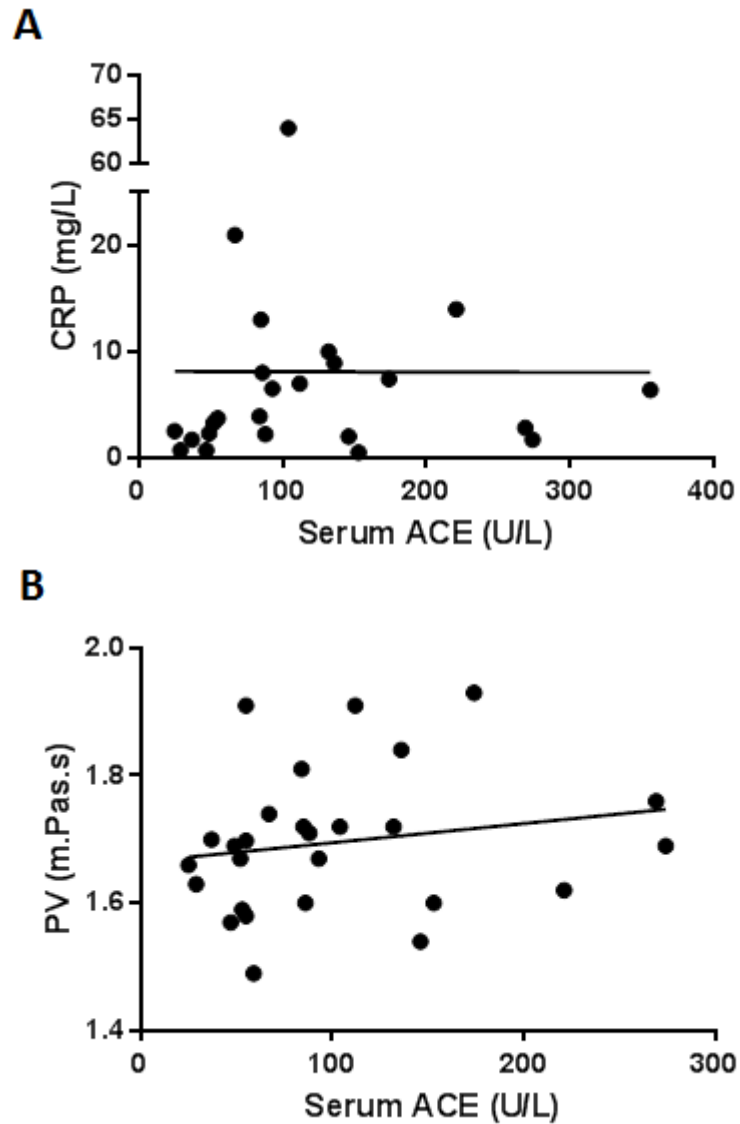
Figure A3.7 – Correlating patient inflammatory markers with serum ACE levels. **A:** There was no significant correlation between levels of C-reactive protein and serum ACE (p = 0.264, R^2 < 0.001). **B:** The association between plasma viscosity (PV) and serum ACE was also low (p = 0.361, R^2 = 0.0033). Data presented with linear regression line and statistical interpretation performed using Spearman's Rank Correlation Test.
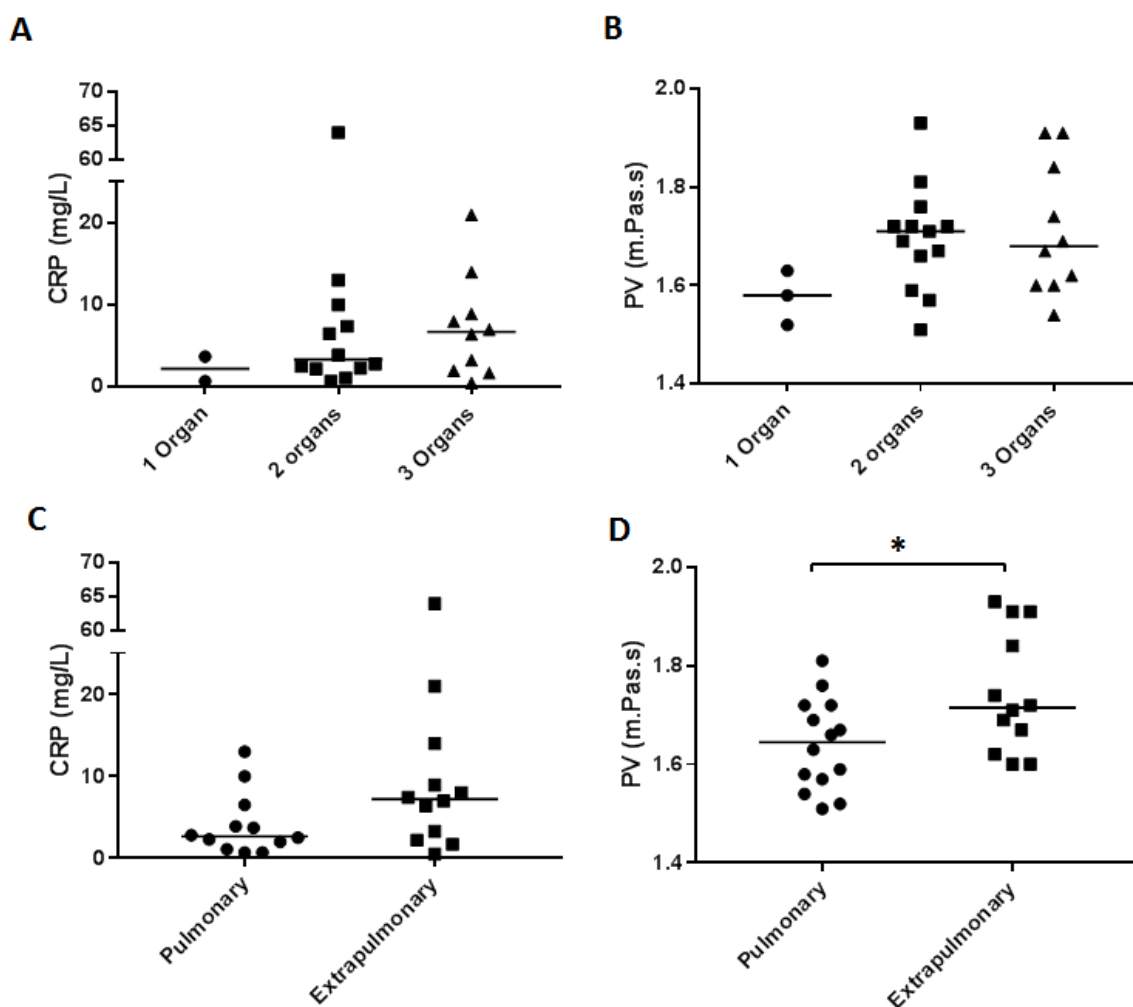
Figure A3.8 – Differences in CRP and PV levels depending on sarcoidosis patient organ involvement. **A:** CRP levels in patients with 1, 2 and 3 organs involved in the disease. **B:** PV levels in patients with 1, 2 and 3 organs involved. **C:** CRP levels in the blood of sarcoidosis patients with pulmonary restricted and extrapulmonary organ involvement. **D:** Elevated PV in patients with extrapulmonary organ involvement. Data presented with median (black lines). * p = 0.03, as determined using Mann–Whitney U test.
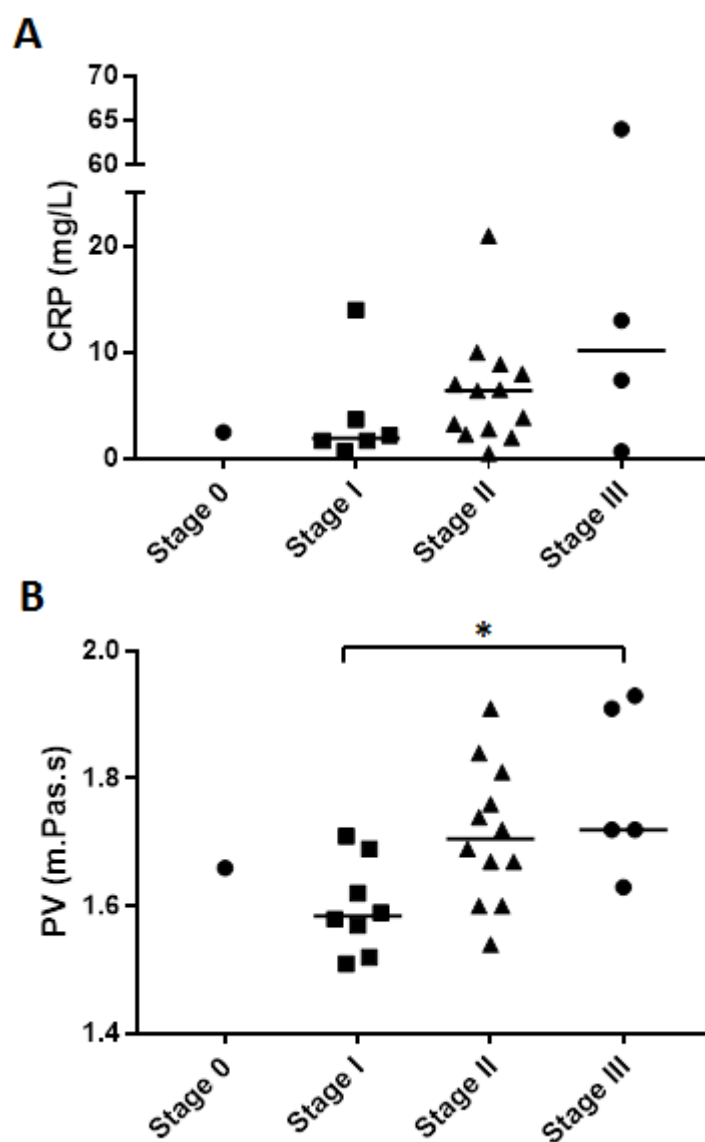
Figure A3.9 – CRP and PV measurements in sarcoidosis patients with different radiographic Scadding CXR stages. **A:** C-reactive protein levels in patients with increasing stages, the median CRP is greater in stages II and III, though is not statistically significant (p > 0.05), as determined by One-way ANOVA. **B:** Plasma viscosity measurements in patients with increasing stages, the median PV is significantly higher in stage III compared with stage I patients. * p = 0.024, as determined by One-way ANOVA. Data presented with median (black lines).
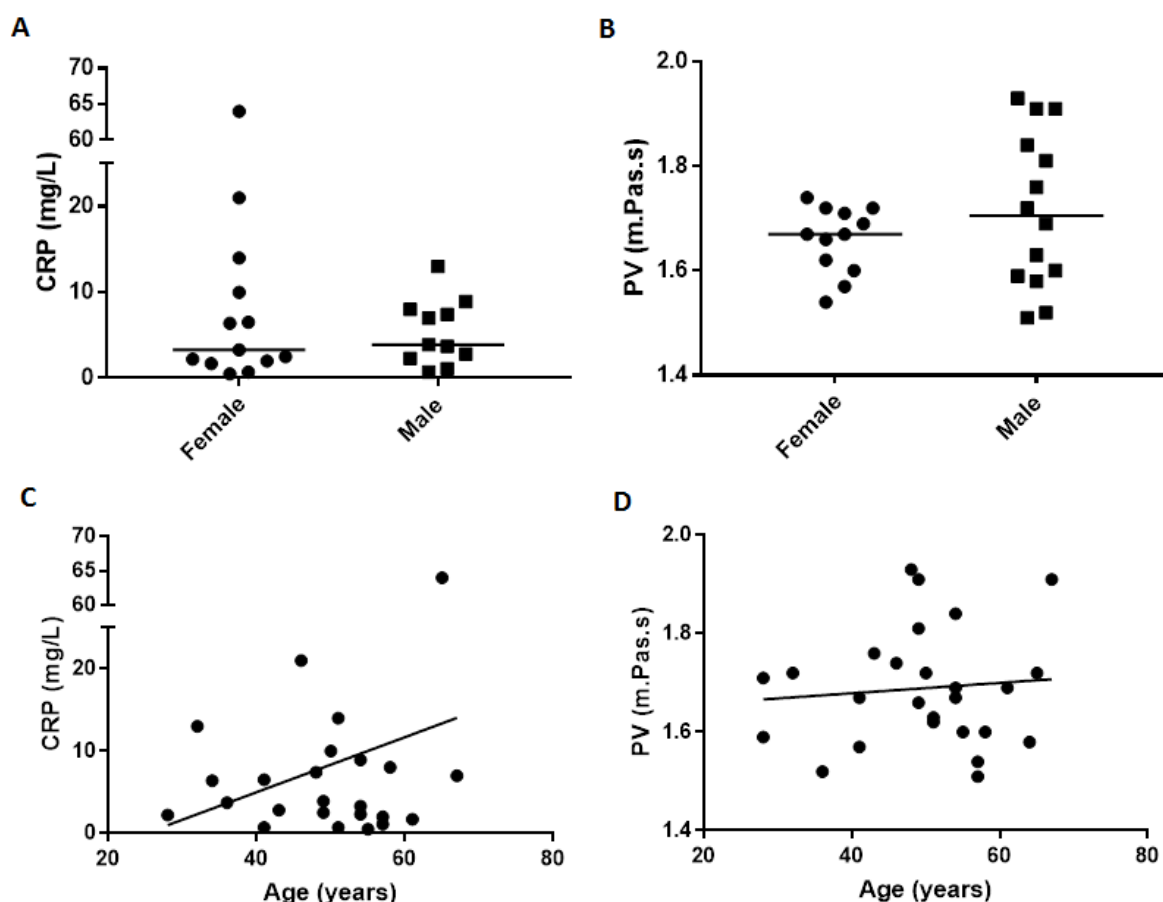
Figure A3.10 – CRP and PV measurements in sarcoidosis patients associated with gender and age. **A:** C-reactive protein levels in male and female patients. **B:** Plasma viscosity levels in male and female patients. Data presented with median (black lines, A and B). No statistical significance in A and B, as determined by Mann-Whitney U test. **C:** Correlation between CRP levels and the age of patients. No statistically significant correlation present (p= 0.805, R^2 = 0.068). **C:** Correlation between PV and the age of patients, not statistically significant (p= 0.765, R^2 = 0.009). Data in C and D presented with linear regression line and statistical interpretation performed using Spearman's Rank Correlation Test.

Appendix A3.11
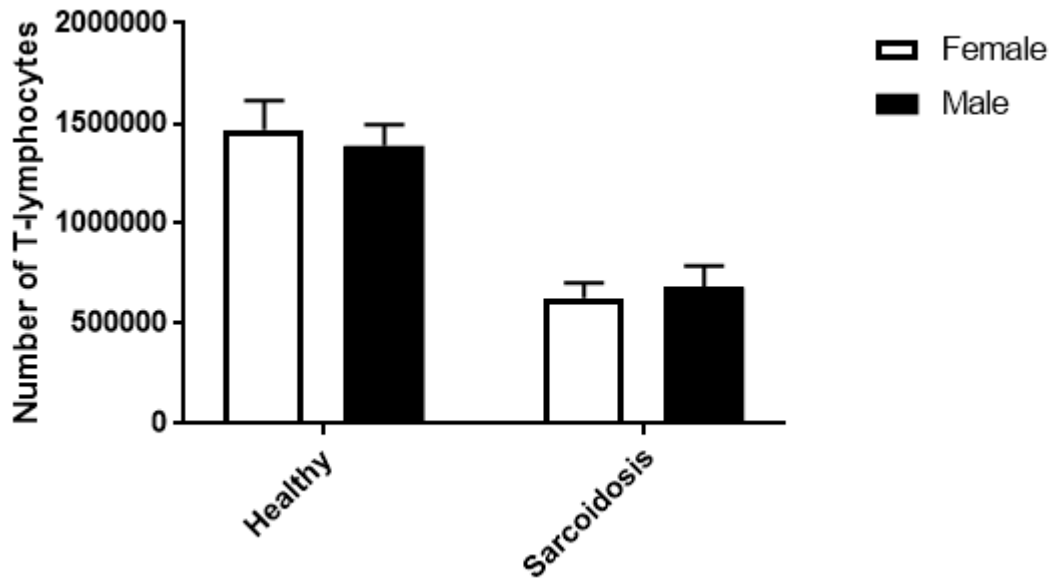
Figure A3.11 – The number of T-lymphocytes in patients with sarcoidosis and healthy controls separated by gender.

Male (black bars) and female (white bars) sarcoidosis patients and healthy controls presented with mean ±SEM. No statistical significance as determined by a Two-way ANOVA test (p = 1.00). n = min 9, max 13.

Appendix A3.12
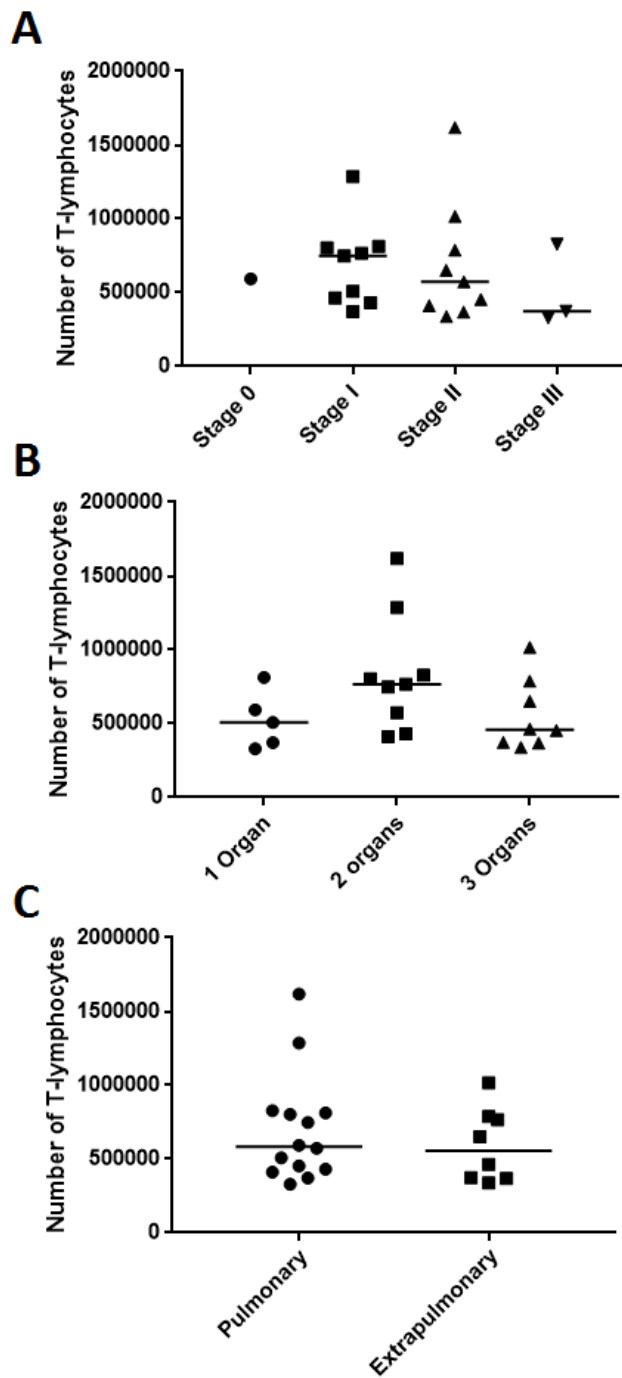
Figure A3.12 – The number of T-lymphocytes in patients with sarcoidosis correlated with the Scadding CXR stage, the number of organs involved and extrapulmonary organ involvement.

Data presented with median (black lines). **A:** T-lymphocyte number in patients with different Scadding stages. (p > 0.05, Kruskal-Wallis test). **A:** T-lymphocyte number in patients with different numbers of organs involved (p > 0.05, Kruskal-Wallis test). **A:** T-lymphocyte number in patients with extrapulmonary or pulmonary restricted organ involvement (p = 0.570, Mann-Whitney U test).

Appendix A3.13
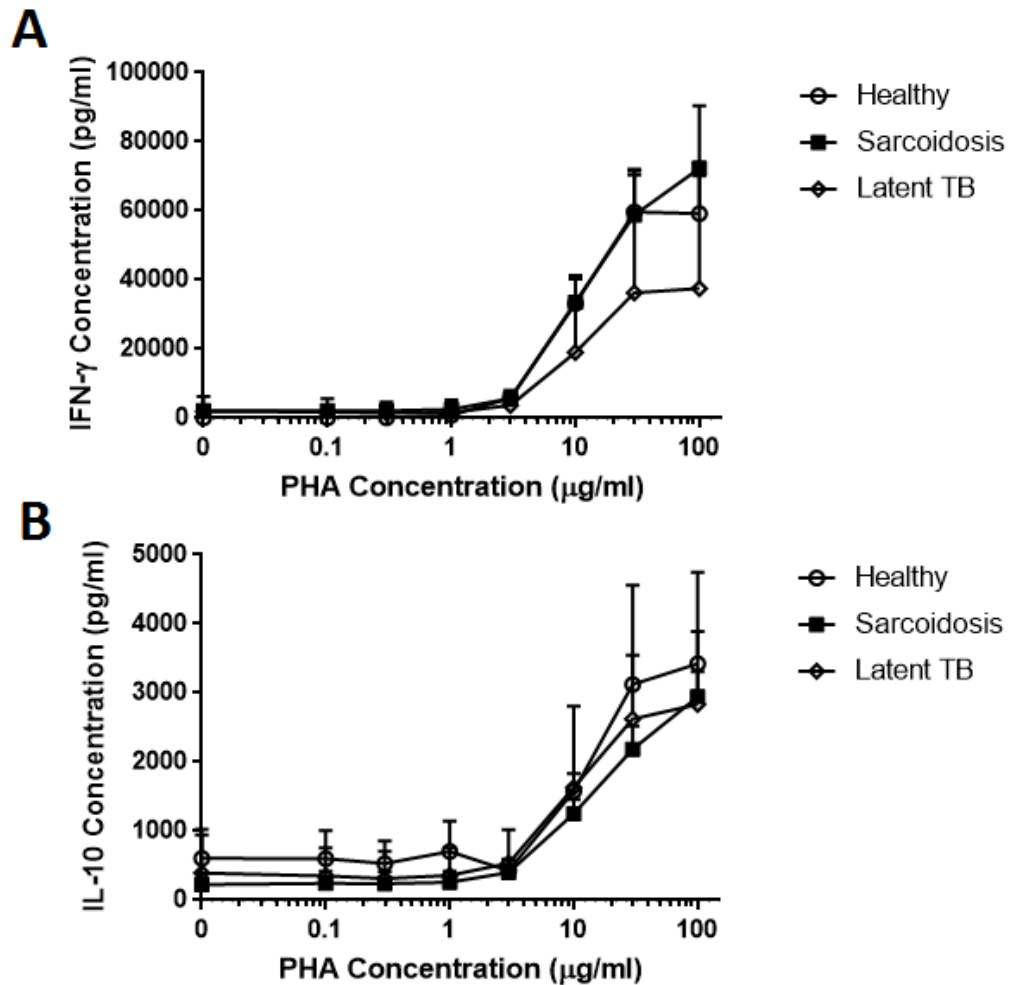
Figure A3.13 – Whole blood IFN-γ and IL-10 cytokine release in response to PHA in patients with sarcoidosis, latent TB patients and healthy controls.

A dose response to PHA was undertaken for 16 hours at concentrations of 0 µg/ml to 100 µg/ml of PHA in sarcoidosis, latent TB and control whole blood. Plasma was analysed by ELISA for **A:** IFN-γ and **B:** IL-10. There was no statistically significant difference in any of the cytokines tested at any concentration of PHA stimulation. Data presented with mean ± SEM (one sided) and analysed by Two-way ANOVA. IFN-γ n = 21, n= 18 and n = 5 and IL-10 n = 17, n = 11 and n = 5 for healthy controls, patients with sarcoidosis and latent TB patients respectively.

Appendix A3.14
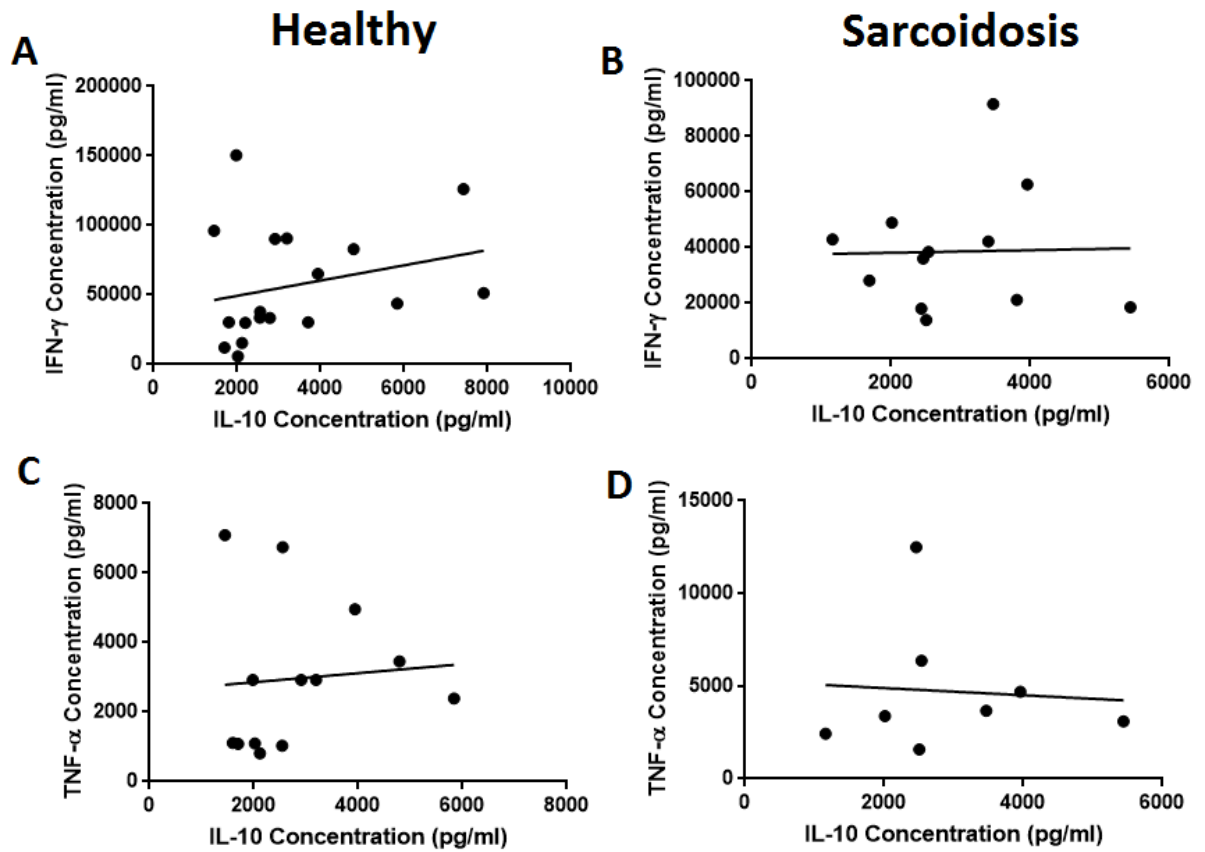
Figure A3.14 – Correlation between pro-inflammatory cytokines and IL-10 released from PHA stimulated whole blood in patients with sarcoidosis and healthy controls.

IFN-γ, TNF-α and IL-10 release were correlated for healthy controls (A, C) and sarcoidosis patients (B, D) **A** and **B:** Correlation between IFN-γ and IL-10, healthy controls (p = 0.152, R^2 = 0.067), sarcoidosis patients (p = 0.939, R^2 = 0.001). **C** and **D:** TNF-α correlation with IL-10, healthy controls (p = 0.529, R^2 = 0.007), sarcoidosis patients (p = 0.703, R^2 = 0.005). Black line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.

Appendix A3.15
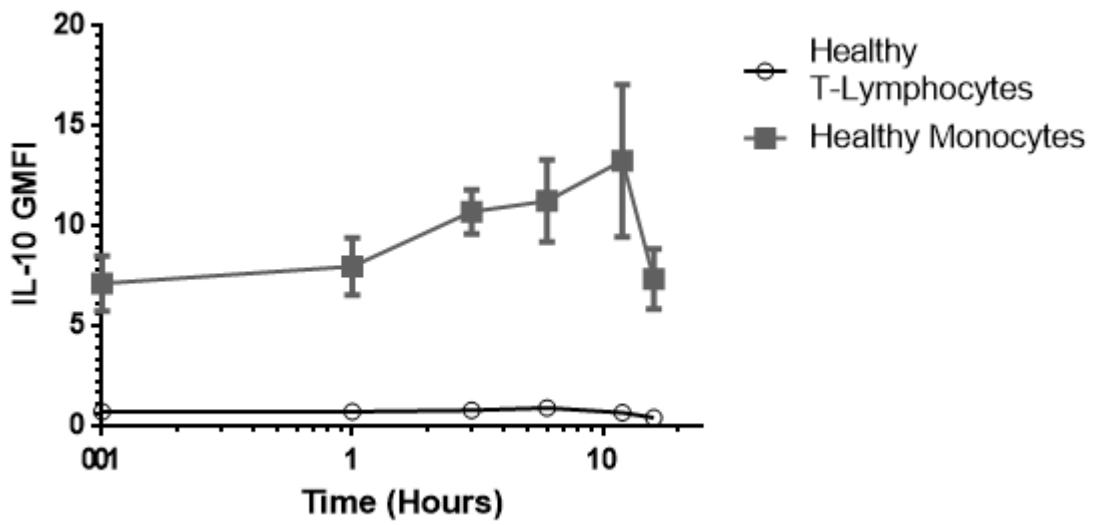
Figure A3.15 – Intracellular IL-10 accumulation in response to Brefeldin A treated PHA stimulated PBMCs in healthy controls.

Intracellular IL-10 accumulation in T-lymphocytes (grey squares) and monocytes (clear circles) in response to PHA (10 µg/ml) in healthy controls (n = 3). Data presented as mean ± SEM.

Appendix A3.16
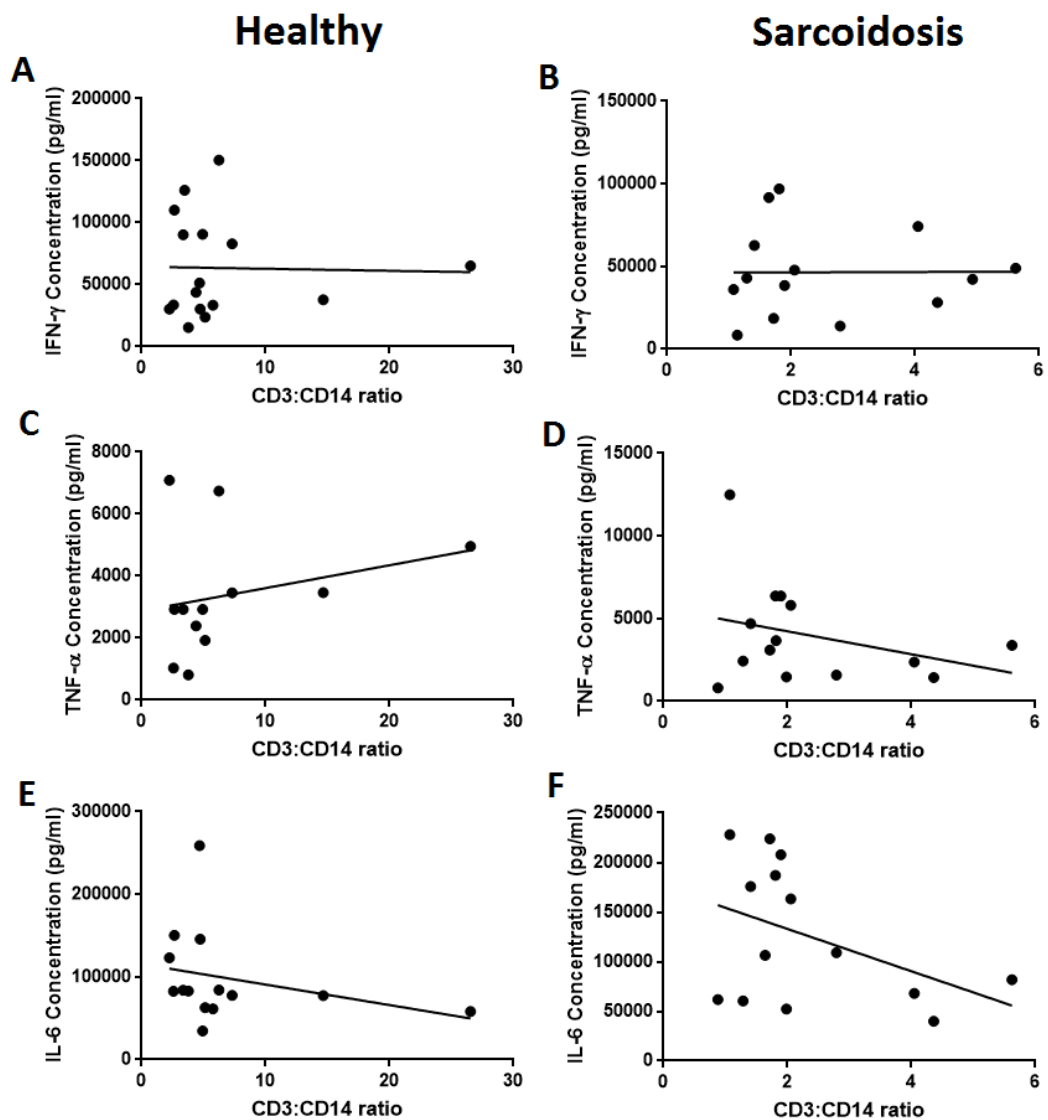
Figure A3.16 – Correlation between pro-inflammatory cytokine release from PHA stimulated whole blood and the CD3:CD14 ratio in PBMCs from patients with sarcoidosis and healthy controls.

IFN-γ, TNF-α and IL-6 release were correlated for healthy controls (A, C and E respectively) and sarcoidosis patients (B, D and F respectively). **A** and **B:** Correlation with IFN-γ, healthy controls (p = 0.788, R^2 = 0.001), sarcoidosis patients (p = 0.671, R^2 < 0.001). **C** and **D:** Correlation with TNF-α, healthy controls (p = 0.297, R^2 = 0.068), sarcoidosis patients (p = 0.454, R^2 = 0.098). **E** and **F:** IL-6 correlation, healthy controls (p = 0.342, R^2 = 0.081), sarcoidosis patients (p = 0.303, R^2 = 0.187). Black line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.

Appendix A3.17
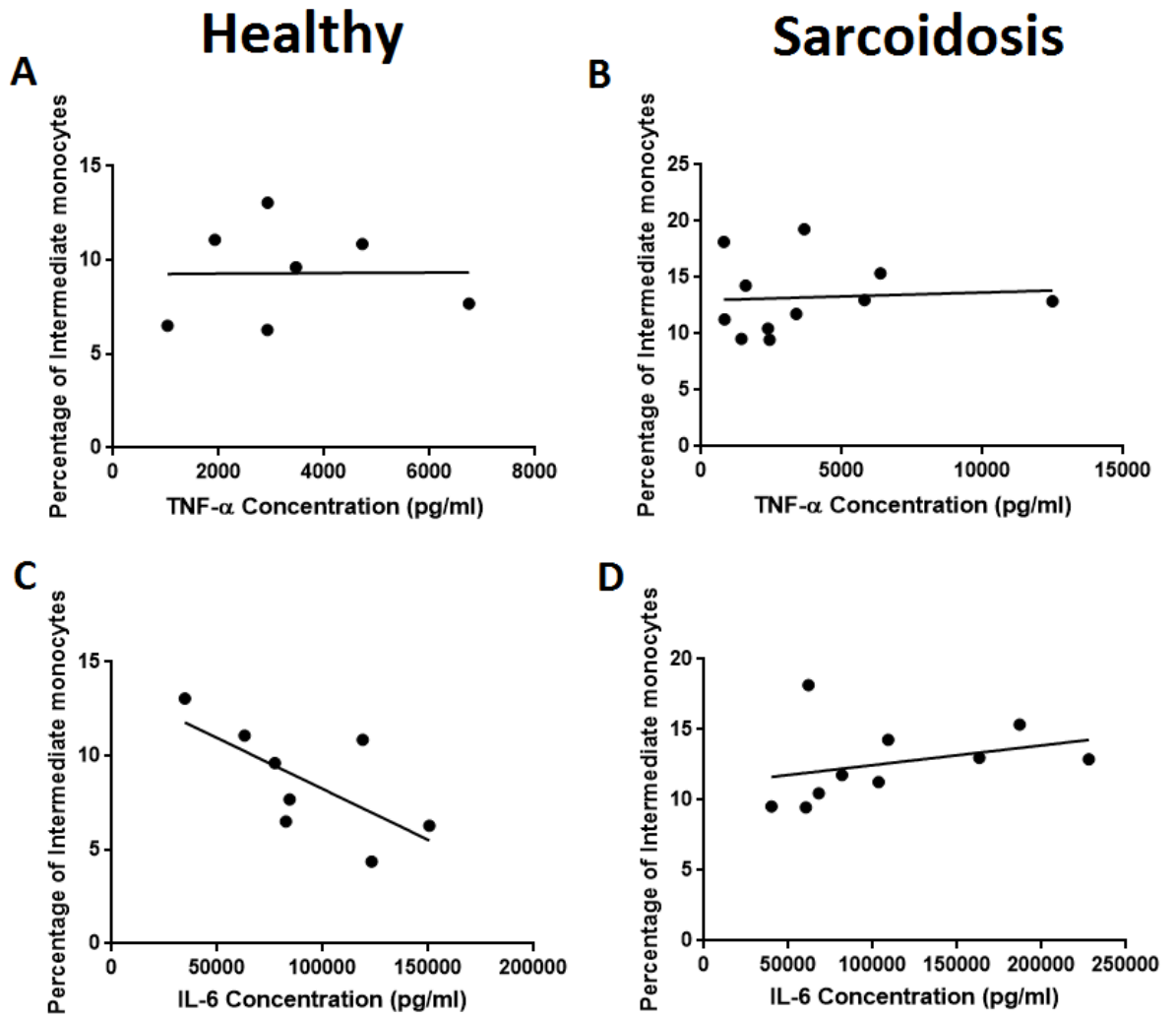
Figure A3.17 – Correlation between PHA induced TNF-α or IL-6 release and the percentage of intermediate monocytes within the population.

A: Healthy controls TNF-α (p = 0.783, R^2 < 0.001). **B:** Patients with sarcoidosis TNF-α (p = 0.539, R^2 = 0.005). **C:** Healthy controls IL-6 (* p = 0.022, R^2 = 0.468). **D:** Patients with sarcoidosis IL-6 (p = 0.105, R^2 = 0.101). Black line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.

Appendix A3.18
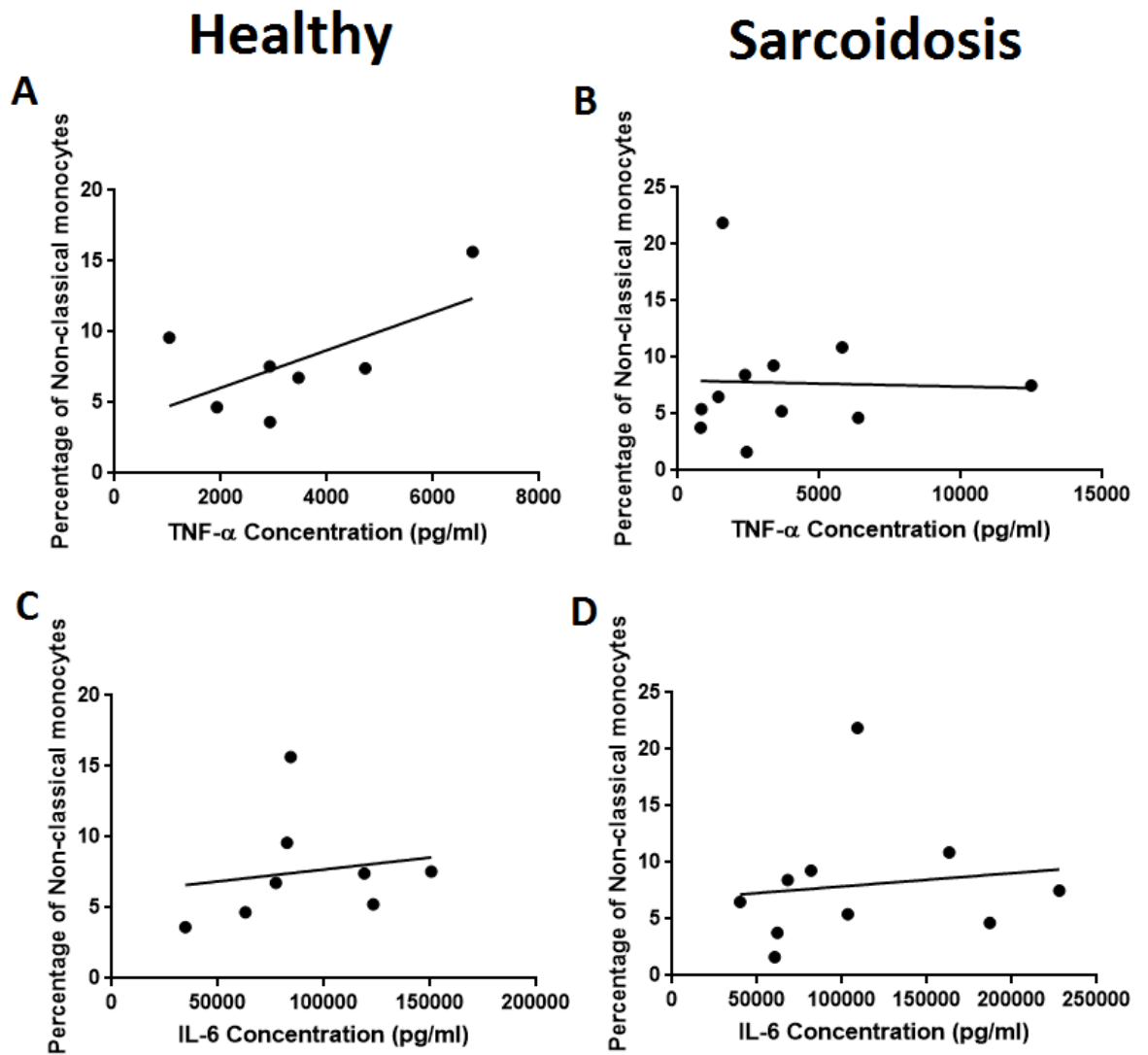
Figure A3.18 – Correlation between PHA induced TNF-α or IL-6 release and the percentage of Non-classical monocytes within the population.

A: Healthy controls TNF-α (p = 0.713, R^2 = 0.402). **B:** Patients with sarcoidosis TNF-α (p = 0.673, R^2 = 0.001). **C:** Healthy controls IL-6 (p = 0.197, R^2 = 0.028). **D:** Patients with sarcoidosis IL-6 (p = 0.279, R^2 = 0.017). Black line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.

Appendix A3.19
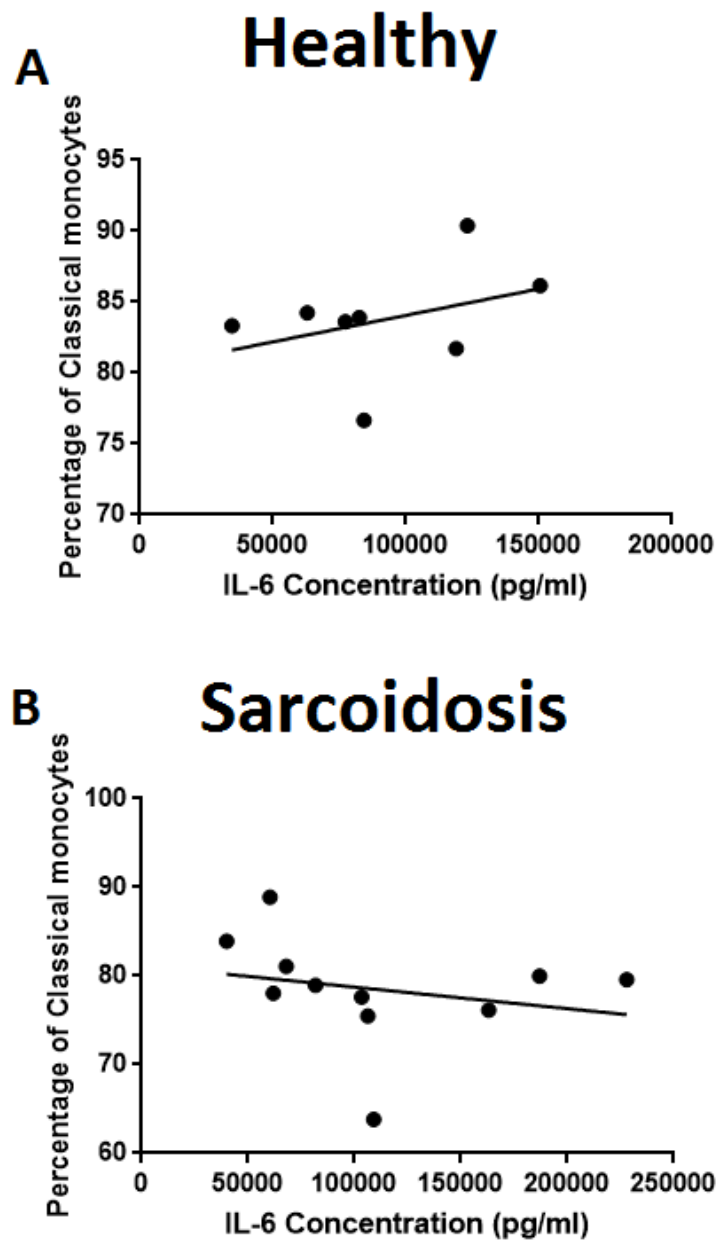
Figure A3.19 – Correlation between PHA induced IL-6 release and the percentage of Non-classical monocytes within the population.

A: Healthy controls (p = 0.428, R^2 = 0.128). **B:** Patients with sarcoidosis (p = 0.138, R^2 = 0.054). Black line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.
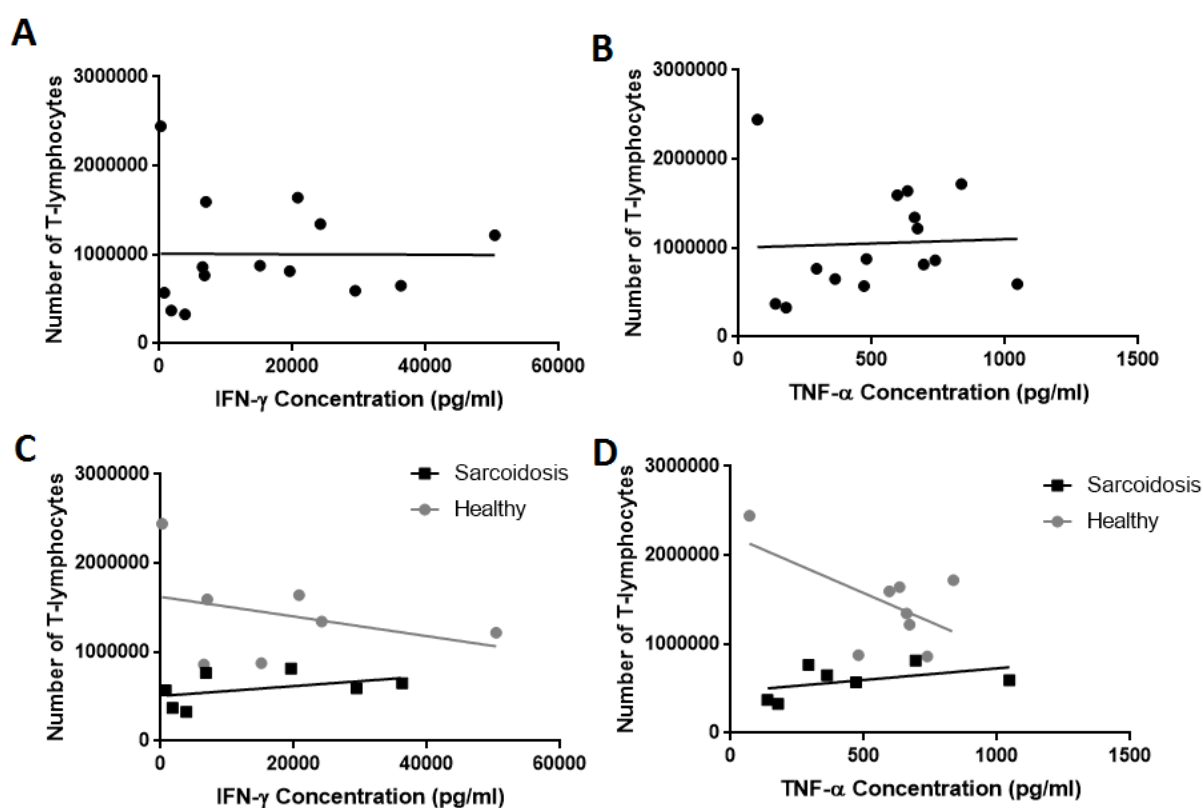
Figure A3.20 – Correlation between IFN-γ and TNF-α release from SEA stimulated whole blood and the number of T-lymphocytes in patients with sarcoidosis and healthy controls.

IFN-γ release (**A, C**) and TNF-α release (**B, D**) were correlated with the number of T-lymphocytes in blood in healthy controls (grey circles), patients with sarcoidosis (black squares) or pooled together for analysis (**A, B**). **A:** Pooled data from both healthy controls and sarcoidosis patients IFN-γ release correlated with T-cell number. p = 0.532, R^2 < 0.001. **B:** Pooled data from both healthy controls and sarcoidosis patients TNF-α release correlated with T-cell number. p = 0.375, R^2 = 0.002. **C:** IFN-γ correlated with T-lymphocyte number in healthy controls (p = 0.713, R^2 = 0.115) and sarcoidosis patients (p = 0.200, R^2 = 0.193). **D:** TNF-α correlated with T-cell number in healthy controls (p = 0.582, R^2 = 0.343) and sarcoidosis patients (p = 0.236, R^2 = 0.211. Black/grey line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.
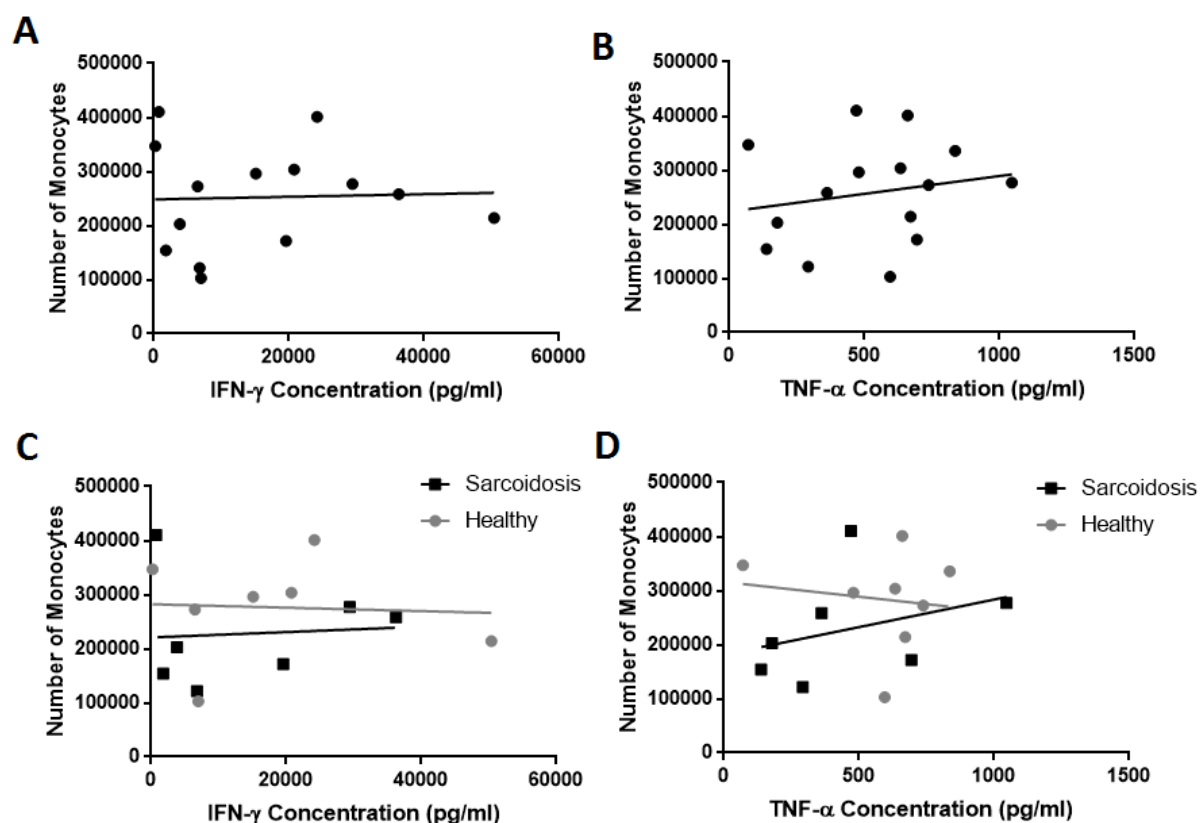
Figure A3.21 – Correlation between IFN-γ and TNF-α release from SEA stimulated whole blood and the number of monocytes in patients with sarcoidosis and healthy controls.

IFN-γ release (**A, C**) and TNF-α release (**B, D**) were correlated with the number of monocytes in blood in healthy controls (grey circles), patients with sarcoidosis (black squares) or pooled together for analysis (**A, B**). **A:** Pooled data from both healthy controls and sarcoidosis patients IFN-γ release correlated with monocyte number. p = 0.916, R^2 = 0.001. **B:** Pooled data from both healthy controls and sarcoidosis patients TNF-α release correlated with monocyte number. p = 0.567, R^2 = 0.036. **C:** IFN-γ correlated with monocyte number in healthy controls (p > 0.999, R^2 = 0.003) and sarcoidosis patients (p = 0.964, R^2 = 0.005). **D:** TNF-α correlated with monocyte number in healthy controls (p = 0.882, R^2 = 0.019) and sarcoidosis patients (p = 0.200, R^2 = 0.113). Black/grey line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.
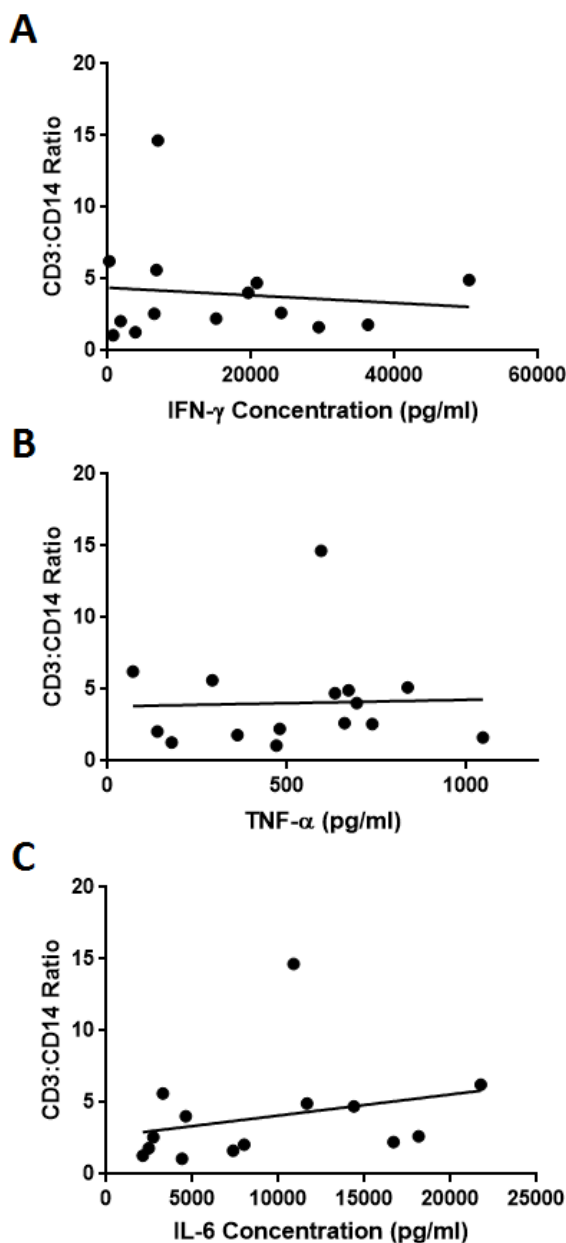
**Figure A3.23 – Correlation between IFN-γ and TNF-α release from SEA stimulated whole blood and the CD3:CD13 ratio in PBMCs for patients with sarcoidosis and healthy controls.**

IFN-γ, TNF-α and IL-6 release were correlated with the PBMC CD3:CD14 ratio in blood from healthy controls and patients with sarcoidosis. Data from donors pooled together (black circles). **A:** IFN-γ correlated with CD3:CD14 ($p = 0.832$, $R^2 = 0.013$). **B:** TNF-α correlated with CD3:CD14 ($p = 0.873$, $R^2 = 0.001$). **C:** IL-6 correlated with CD3:CD14 ($p = 0.067$, $R^2 = 0.074$). Black/grey line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.
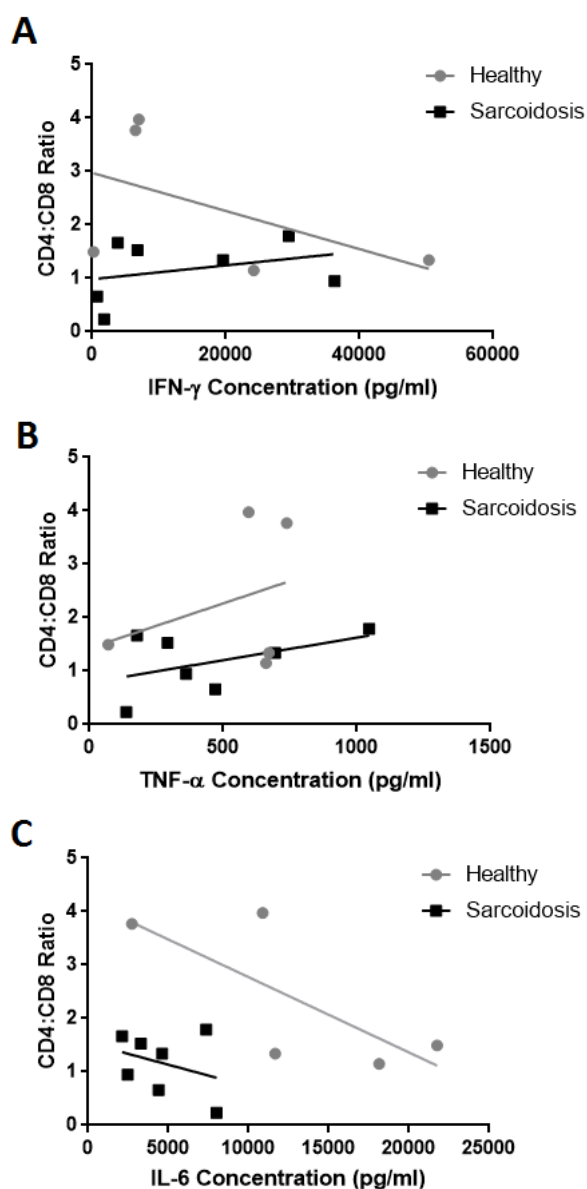
Figure A3.23 – Correlation between IFN-γ and TNF-α release from SEA stimulated whole blood and the T-lymphocyte CD4:CD8 ratio in patients with sarcoidosis and healthy controls.

IFN-γ, TNF-α and IL-6 release were correlated with the CD4:CD8 T-lymphocyte ratio in blood from healthy controls (grey circles) and patients with sarcoidosis (black squares). **A:** IFN-γ correlated with CD4:CD8 in healthy controls (p = 0.450, R^2 = 0.269) and sarcoidosis patients (p = 0.302, R^2 = 0.106). **B:** TNF-α correlated with CD4:CD8 in healthy controls (p = 0.950, R^2 = 0.109) and sarcoidosis patients (p = 0.397, R^2 = 0.223). **C:** IL-6 correlated with CD4:CD8 in healthy controls (p = 0.350, R^2 = 0.545) and sarcoidosis patients (p = 0.556, R^2 = 0.107). Black/grey line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.
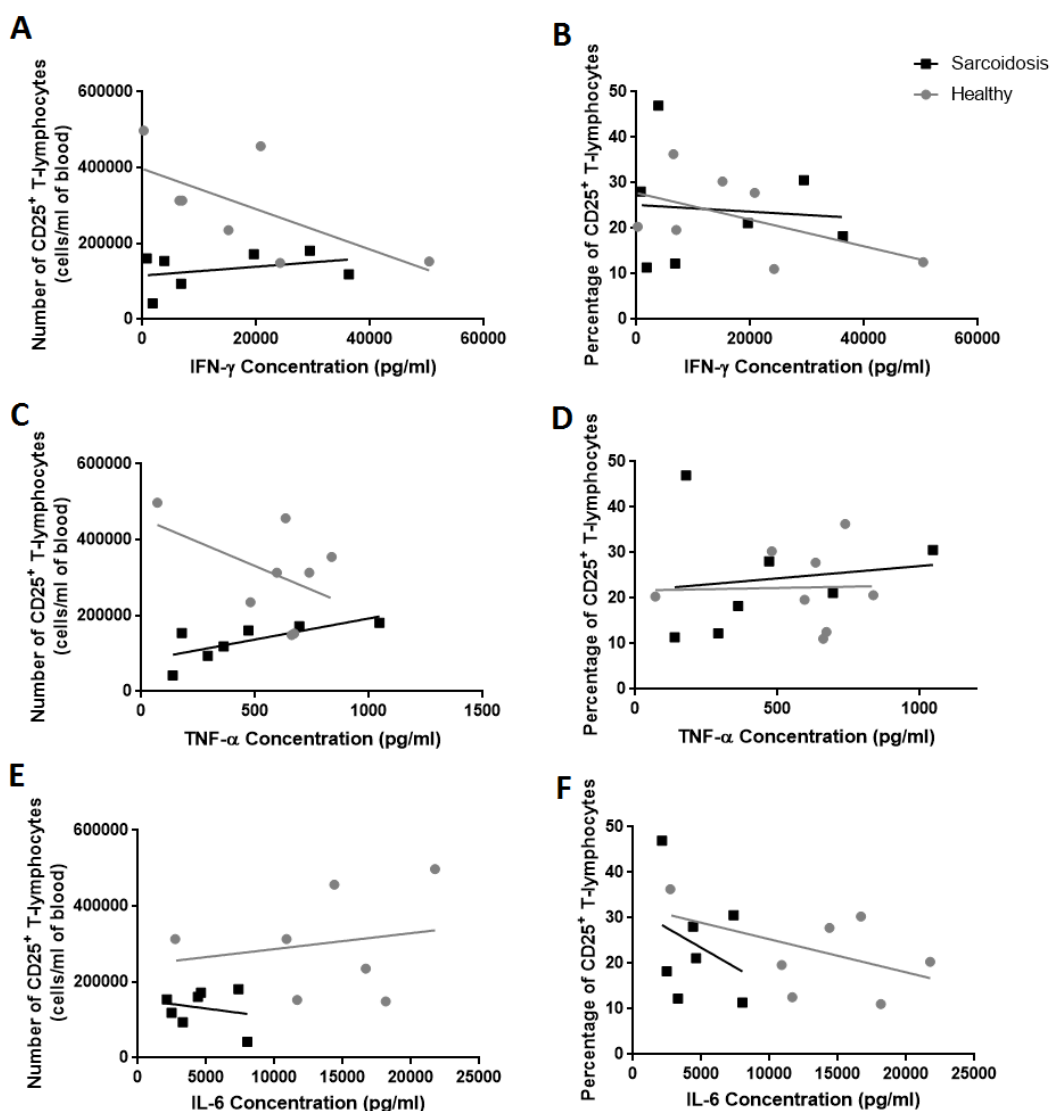
Figure A3.24 – Correlation between pro-inflammatory cytokine release from SEA stimulated whole blood and T-lymphocyte CD25 in patients with sarcoidosis and healthy controls.

The number of CD25^{+} T-lymphocytes (**A, C** and **E**) and the percentage of CD25^{+} T-lymphocytes (**B, D** and **F**) were correlated with IFN-γ, TNF-α and IL-6 release in healthy controls (grey circles) and patients with sarcoidosis (black squares). **A:** IFN-γ in healthy controls (p = 0.088, R^2 = 0.419) and sarcoidosis patients (p = 0.556, R^2 = 0.113). **B:** IFN-γ in healthy controls (p = 0.200, R^2 = 0.275) and sarcoidosis patients (p = 0.964, R^2 = 0.007). **C:** TNF-α in healthy controls (p = 0.501, R^2 = 0.210) and sarcoidosis patients (* p = 0.012, R^2 = 0.510). **D:** TNF-α in healthy controls (p = 0.935, R^2 < 0.001) and sarcoidosis patients (p = 0.357, R^2 = 0.020). **E:** IL-6 in healthy controls (p = 0.906, R^2 = 0.036) and sarcoidosis patients (p = 0.840, R^2 = 0.048). **F:** IL-6 in healthy controls (p = 0.498, R^2 = 0.229) and sarcoidosis patients (p = 0.444, R^2 = 0.103). Black/grey line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.
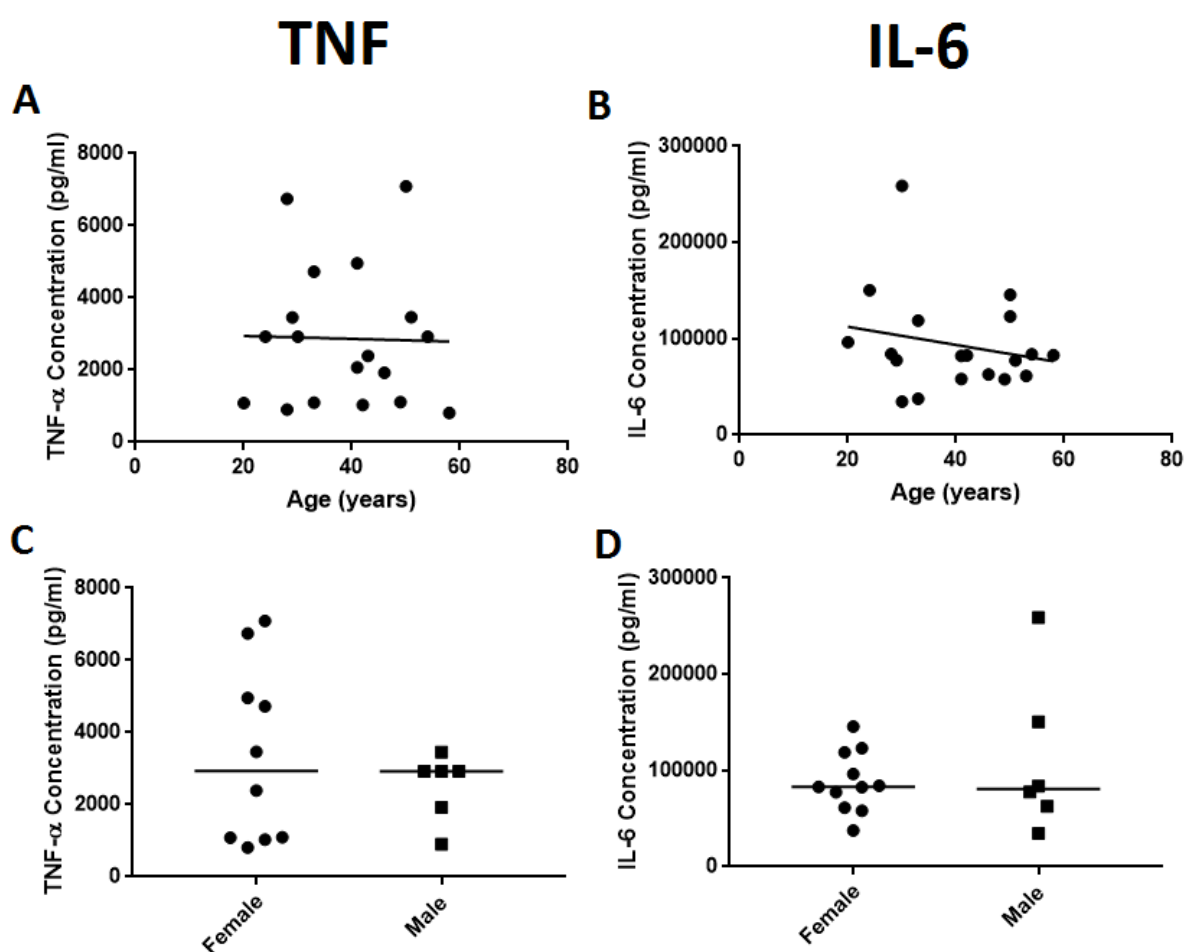
Figure A3.26 – Correlation between pro-inflammatory cytokine release from PHA stimulated whole blood and the age and gender of healthy controls.

The age (**A, B**) and gender (**C, D**) of healthy controls were correlated with TNF-α (**A** and **C**) and IL-6 (**B** and **D**) release. **A:** TNF-α correlated with age (p = 0.887, R^2 = 0.001). **B:** IL-6 correlated with age (p = 0.488, R^2 = 0.044). Black line = linear regression, statistical significance determined using Spearman's Rank Correlation Test. **C:** TNF-α correlated with gender (p = 0.635). **D:** IL-6 correlated with gender (p = 0.887). Black line = median, statistical significance determined using the Mann-Whitney U test.
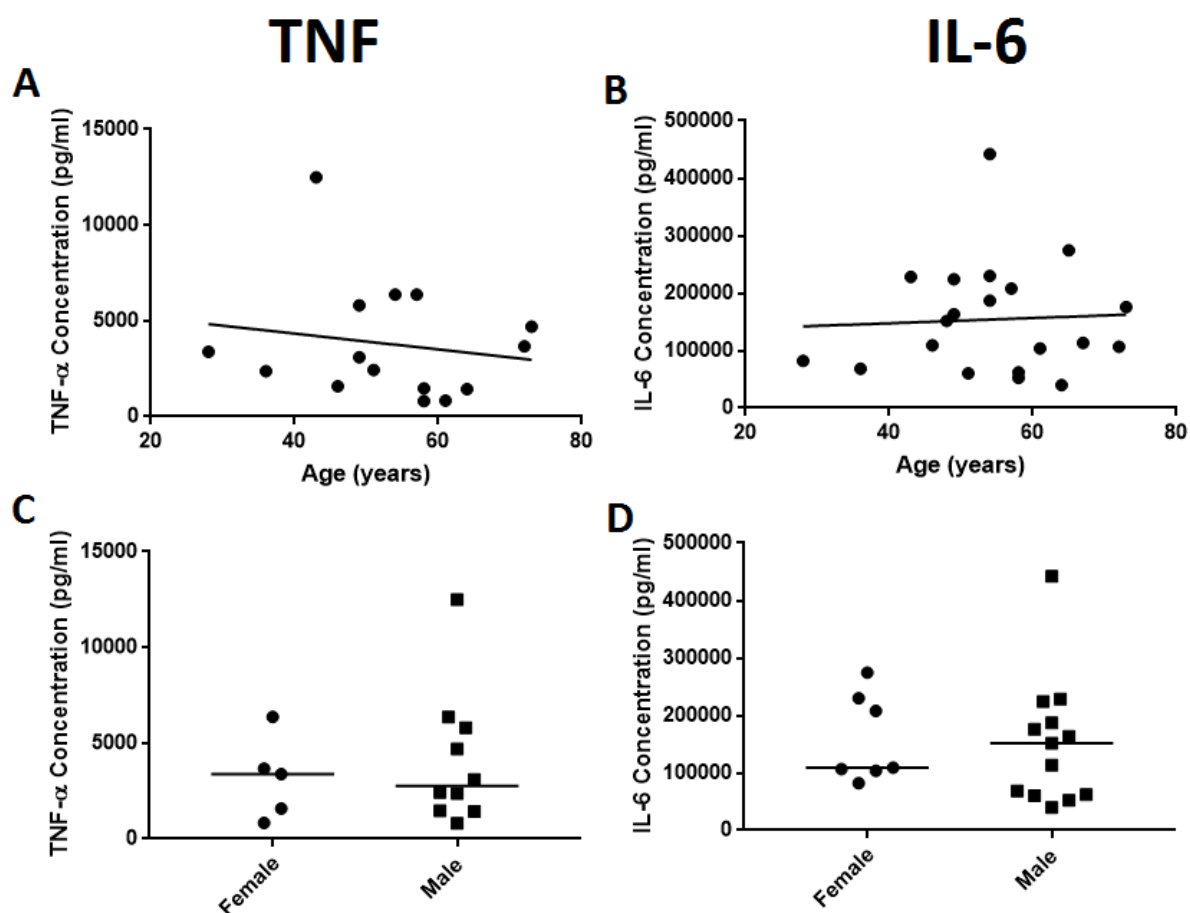
Figure A3.26 – Correlation between pro-inflammatory cytokine release from PHA stimulated whole blood and the age and gender of patients with sarcoidosis.

The age (**A, B**) and gender (**C, D**) of patients with sarcoidosis were correlated with TNF-α (**A** and **C**) and IL-6 (**B** and **D**) release. **A:** TNF-α correlated with age (p = 0.398, R^2 = 0.027). **B:** IL-6 correlated with age (p = 0.880, R^2 = 0.003). Black line = linear regression, statistical significance determined using Spearman's Rank Correlation Test. **C:** TNF-α correlated with gender (p = 0.953). **D:** IL-6 correlated with gender (p = 0.537). Black line = median, statistical significance determined using the Mann-Whitney U test.
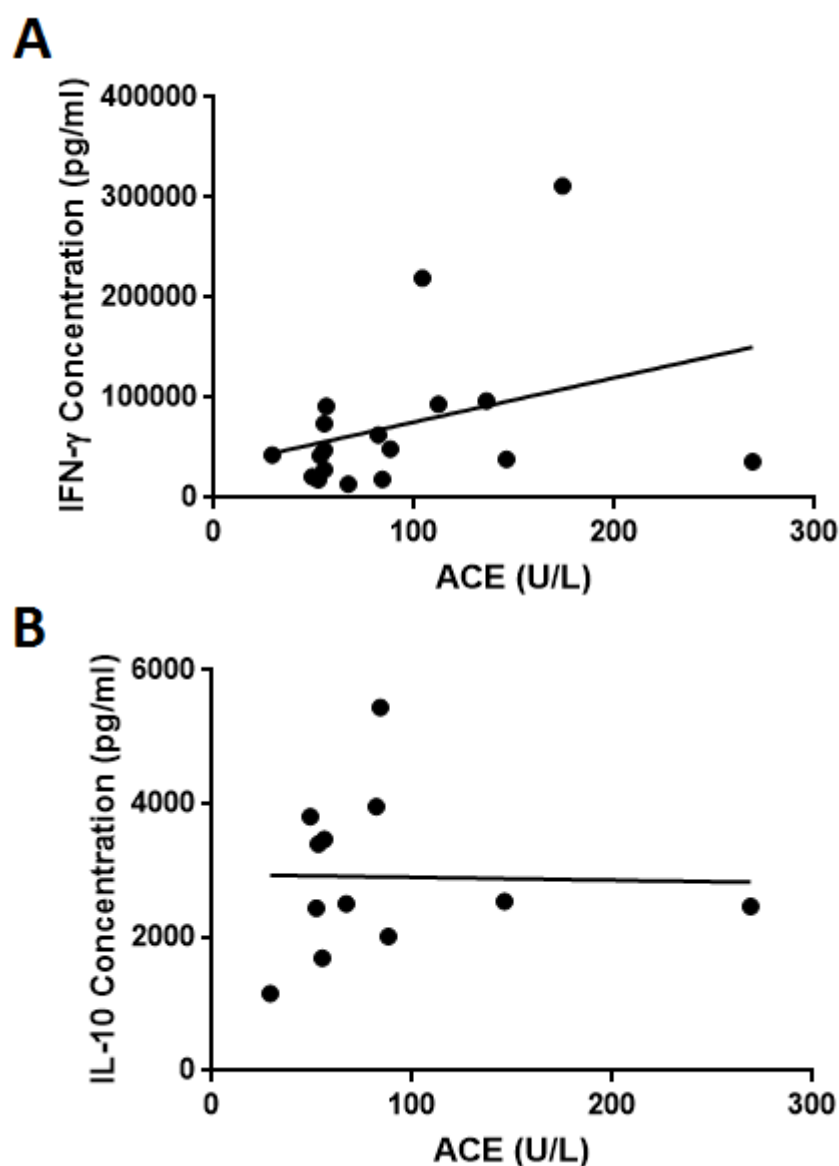
Figure A3.27 – Correlation between IFN-γ and IL-10 release from PHA stimulated whole blood and serum ACE levels from patients with sarcoidosis.

A: There was no statistically significant correlation between IFN-γ release and serum ACE from patients, p = 0.085, R^2 = 0.115. **B:** There was no statistically significant correlation between IL-10 release and serum ACE from patients, p = 0.558, R^2 < 0.001. Black line = linear regression, statistical significance determined using Spearman's Rank Correlation Test.

Cell Type	Phenotype	CD Marker	CD Marker Information
T-lymphocytes	CD3$^+$	CD3	T-lymphocyte marker only present on thymocytes. TCR co-receptor molecule.
Monocytes	CD14^{++}CD16$^-$ CD14^{++}CD16$^+$ CD14$^+$CD16^{++}	CD14	Pattern Recognition Receptor, also a co-receptor with TLR4. Can bind to LPS. Expressed on monocytes, macrophages, dendritic cells and lower expression on neutrophils.
		CD16	Fc receptor responsible for NK cell mediated lysis. Present on NK cells, monocytes/macrophages and neutrophils.
B-lymphocyte	CD19$^+$	CD19	B-cell antigen receptor component, present on B-lymphocytes and follicular dendritic cells.
Natural Killer Cells	CD3$^-$ CD56$^+$	CD3	
		CD56	Neural cell adhesion molecule (NCAM), multiple functions in adhesion and growth. Present on NK cells, neurons, glia and more.
T-helper lymphocyte	CD3$^+$CD4$^+$	CD3	
		CD4	TCR co-receptor specific for MHC class II. Present on T-helper cells, monocytes, macrophages and dendritic cells.
Cytoxic T-lymphocyte	CD3$^+$CD8a$^+$	CD3	
		CD8a	TCR co-receptor specific for MHC class I. Present on CTLs, NK cells and dendritic cells.
Activated T-lymphocyte	CD3$^+$CD25^{low}	CD3	
		CD25	Alpha chain of the IL-2 receptor. Expressed mainly by activated lymphocytes, but also present in myeloid cells. Highly expressed in T-regulatory cells.
T-regulatory lymphocyte	CD3$^+$ CD4$^+$ CD25^{high}	CD3	-
		CD4	-
		CD25	-
Natural Killer T-lymphocyte	CD3$^+$CD56$^+$	CD3	-
		CD56	-
Naïve/Memory T-lymphocyte	CD3$^+$ CD45RA$^+$ CD45RO$^-$ CD3$^+$ CD45RA$^-$ CD45RO$^+$	CD45RA/RO	Leukocyte common antigen, RA is on naïve, RO on memory.

**Table A3.1 – Leukocytes subsets matched with CD markers used in this study.**
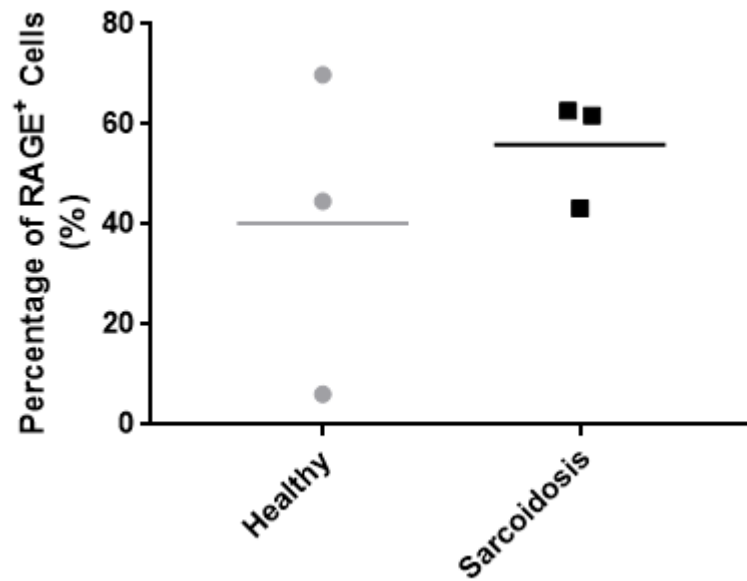
Figure A4.1 – Percentage of T-lymphocytes with intracellular RAGE in patients with sarcoidosis and healthy controls.

T-lymphocytes were gated as CD3^{+} lymphocytes. RAGE^{+} cells established using an isotype control. Healthy controls (grey circles, n = 3) and patients with sarcoidosis (black squares, n = 3) displayed with mean (grey and black horizontal lines respectively). Mean values = 40.21 and 55.9 for controls and patients respectively. No statistical significance displayed (p > 0.05) using the Student's T-test.
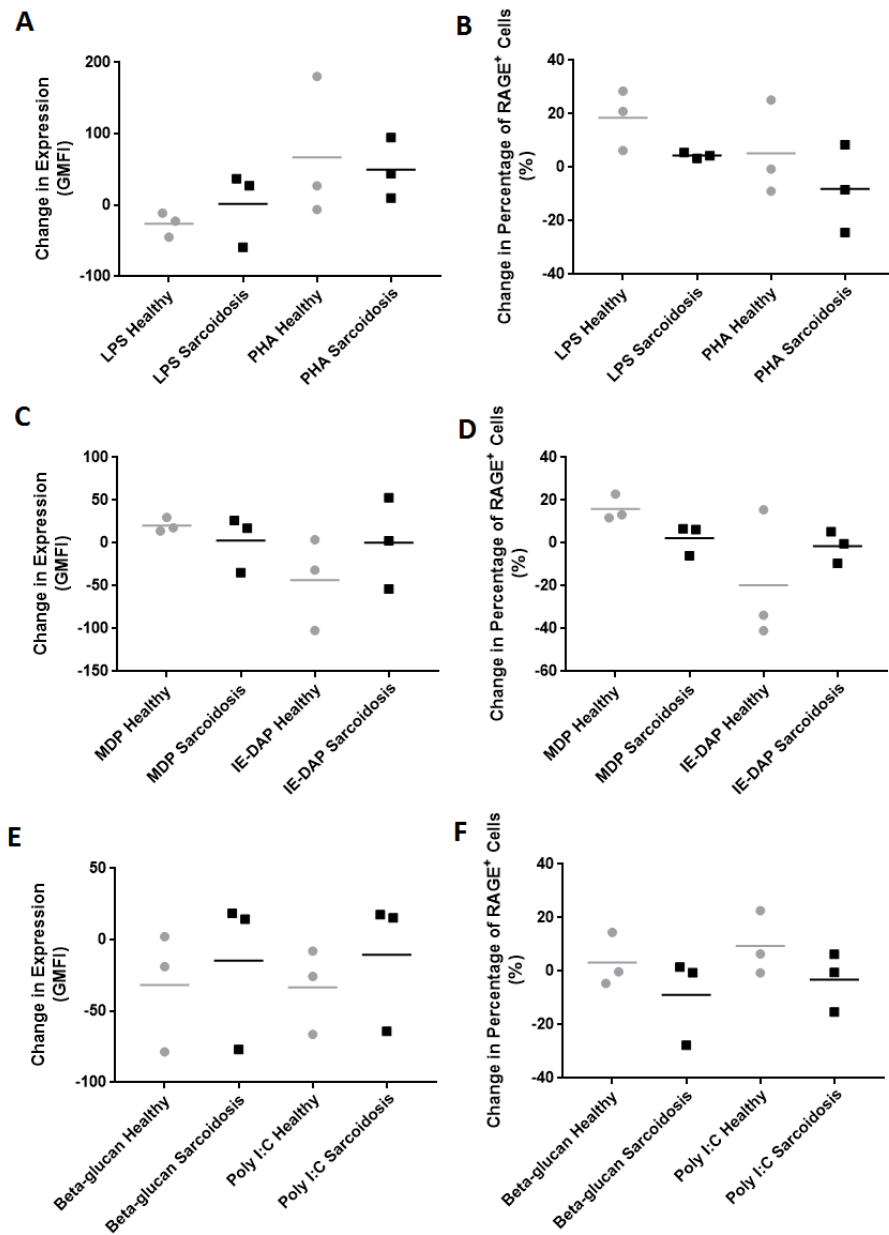
T-lymphocytes

Figure A4.2 – Change in T-lymphocyte RAGE expression and change in the percentage of RAGE+ T-lymphocytes in response to a range of PAMPs and stimuli.

T-lymphocytes were gated as CD3+ lymphocytes. RAGE+ cells established using an isotype control. Expression of RAGE determined by flow cytometry using geometric mean fluorescence intensity (GMFI). Healthy controls (grey circles, n = 3) and patients with sarcoidosis (black squares, n = 3) displayed with mean (grey and black horizontal lines respectively). PBMCs were stimulated for 6 hours with 10 µg/ml of LPS, MDP, IE-DAP, Beta-glucan, Poly I:C and 3 µg/ml of PHA. **A, C** and **E:** Change in expression. **B, D** and **F:** Change in the percentage of RAGE+ T-cells. No statistical significance displayed (p > 0.05) using the Student's T-test.
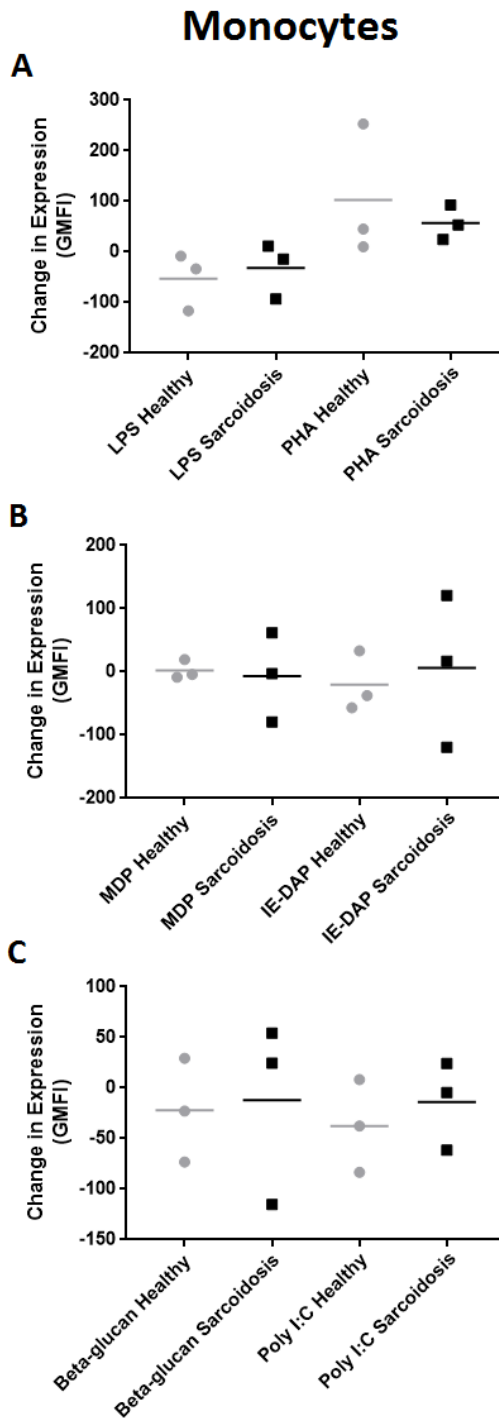
Figure A4.3 – Change in monocyte RAGE expression in response to a range of PAMPs and stimuli.

Expression of RAGE determined by flow cytometry using geometric mean fluorescence intensity (GMFI). Healthy controls (grey circles, n = 3) and patients with sarcoidosis (black squares, n = 3) displayed with mean (grey and black horizontal lines respectively). PBMCs were stimulated for 6 hours with 10 µg/ml of LPS, MDP, IE-DAP, Beta-glucan, Poly I:C and 3 µg/ml of PHA. **A:** Change in expression after LPS or PHA stimulation. **B:** Change in expression after MDP or IE-DAP stimulation. **C:** Change in expression after Beta-glucan and Poly I:C stimulation. No statistical significance displayed (p > 0.05) using the Student's T-test.
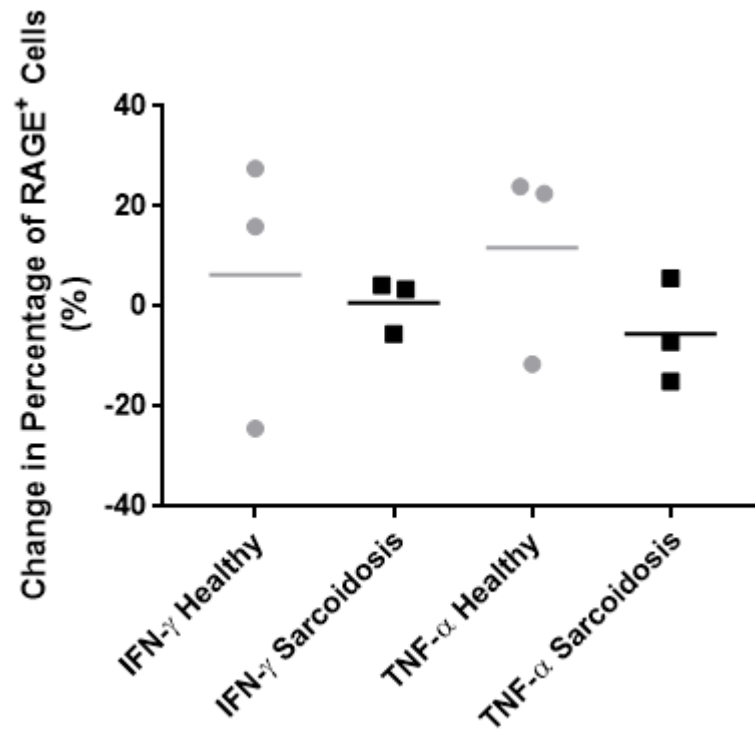
Figure A4.4 – Change in the percentage of RAGE+ T-lymphocytes in response cytokines.

Expression of RAGE determined by flow cytometry using geometric mean fluorescence intensity (GMFI). Healthy controls (grey circles, n = 3) and patients with sarcoidosis (black squares, n = 3) displayed with mean (grey and black horizontal lines respectively). PBMCs were stimulated for 6 hours with 5 ng/ml of TNF-α or 5ng/ml of IFN-γ. Mean values for IFN-γ stimulations = 6.38 and 0.73 % for controls and patients respectively. Mean values for TNF-α stimulations = 11.69 and -5.47 % for controls and patients respectively. No statistical significance displayed (p > 0.05) using the Student's T-test.
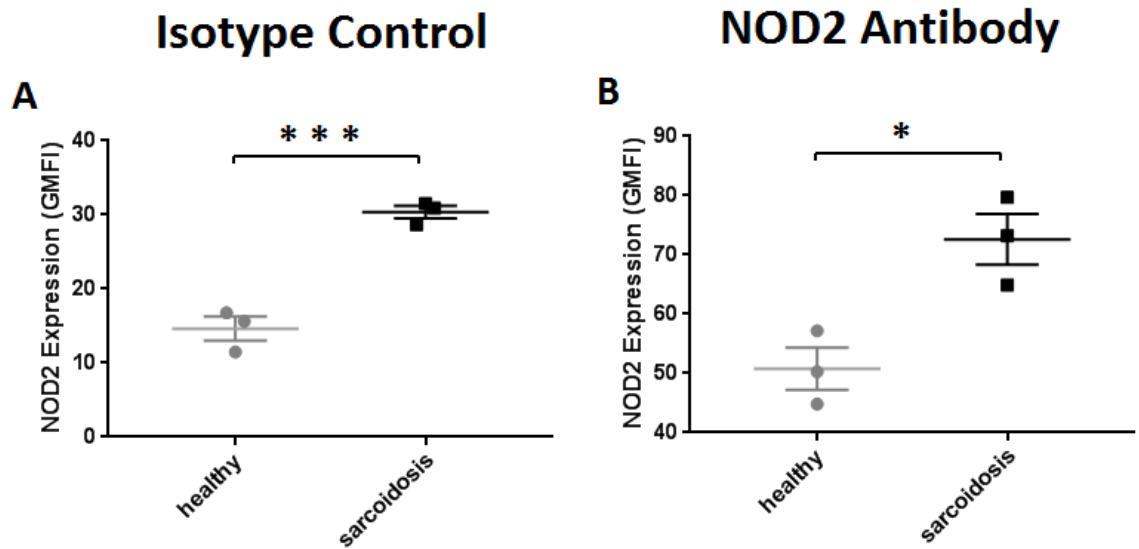
Figure A4.5 – Isotype control and NOD2 fluorescence in monocytes of patients with sarcoidosis and healthy controls.

Expression of NOD2 determined by flow cytometry using geometric mean fluorescence intensity (GMFI). Healthy controls (grey circles, n = 3) and patients with sarcoidosis (black squares, n = 3) displayed with mean (grey and black horizontal lines respectively). **A:** Isotype control antibody staining in monocytes. *** = 0.001, as determined by the Student's T-test. **A:** NOD2 antibody staining in monocytes, GMFI is not corrected with the geometric mean of the isotype control. * = 0.017, as determined by the Student's T-test.
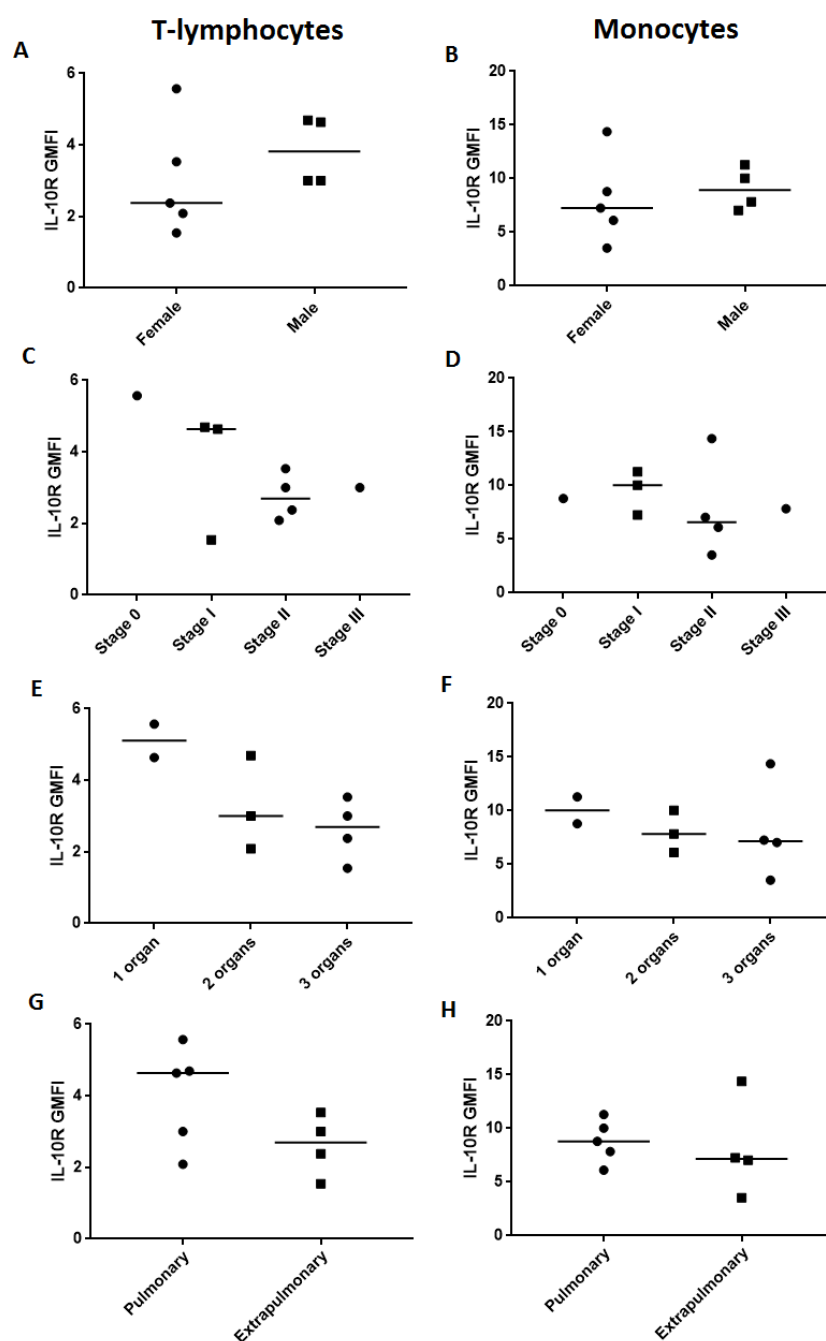
Figure A5.1 – IL-10R expression correlated with clinical features of patients with sarcoidosis. T-lymphocyte IL-10R expression (**A**, **C, E** and **G**) and monocyte IL-10R expression (**B, D, F** and **H**) were correlated with gender (**A** and **B**), Scadding CXR stages (**C** and **D**), the number of organs involved (**E** and **F**) and pulmonary restricted verses extrapulmonary organ involvement (**E** and **F**). There was no statistically significant differences in any of the correlations (p > 0.05) as determined by Mann-Whitney U tests (**A, B, G** and **H**) and Kruskal-Wallace Tests (**C, D, E** and **F**).
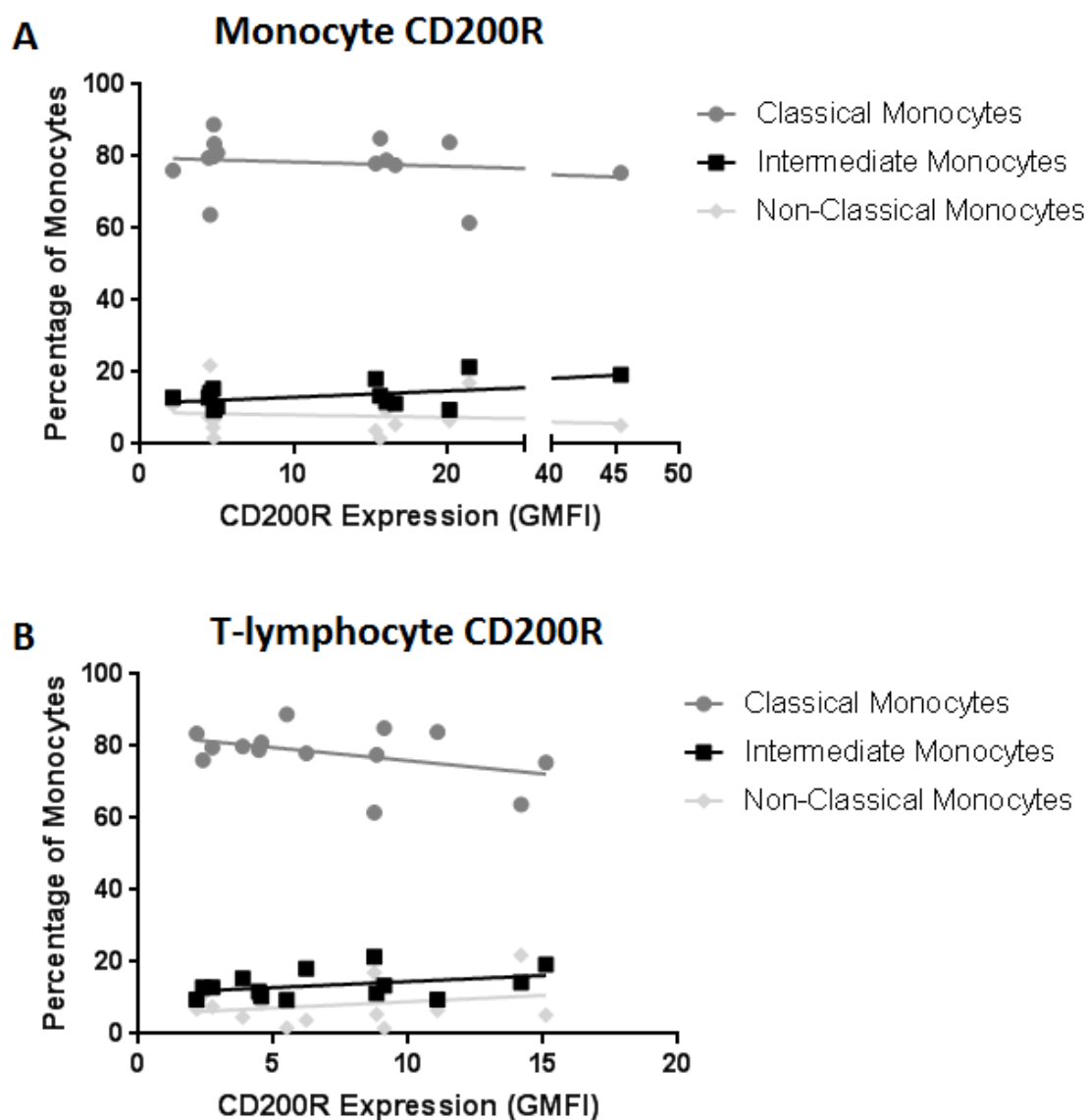
Figure A5.2 – Correlation between CD200R expression and the percentage of monocytes in classical, non-classical and intermediate subsets in patients with sarcoidosis. Classical monocytes (CD14^{++} CD16$^-$, dark grey circles), intermediate monocytes (CD14^{++} CD16$^+$, black squares) and non-classical monocytes (CD14$^+$CD16^{++}, light grey diamonds) were correlated with CD200R expression on monocytes (**A**) and T-lymphocytes (**B**). **A:** Classical (p = 0.418, R^2 = 0.170), intermediate (p = 0.325, R^2 = 0.141) and non-classical (p = 0.594, R^2 = 0.070). **B:** Classical (p = 0.649, R^2 = 0.034), intermediate (p = 0.553, R^2 = 0.280) and non-classical (p = 0.553, R^2 = 0.0187).
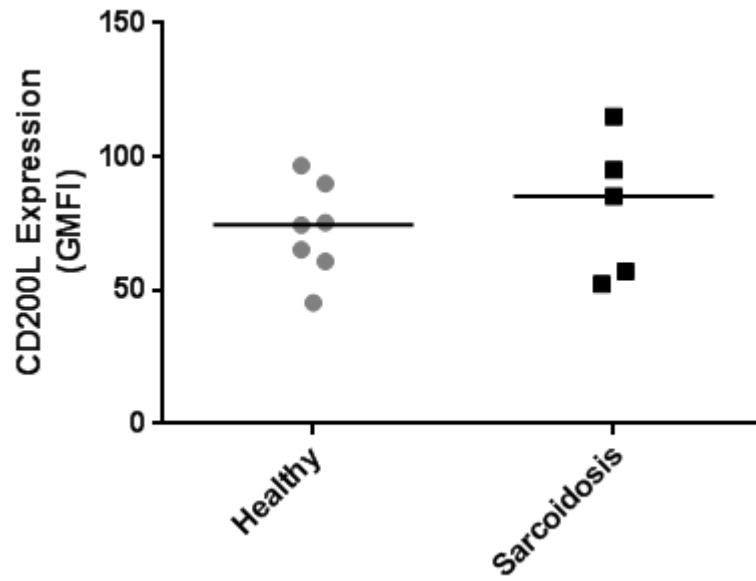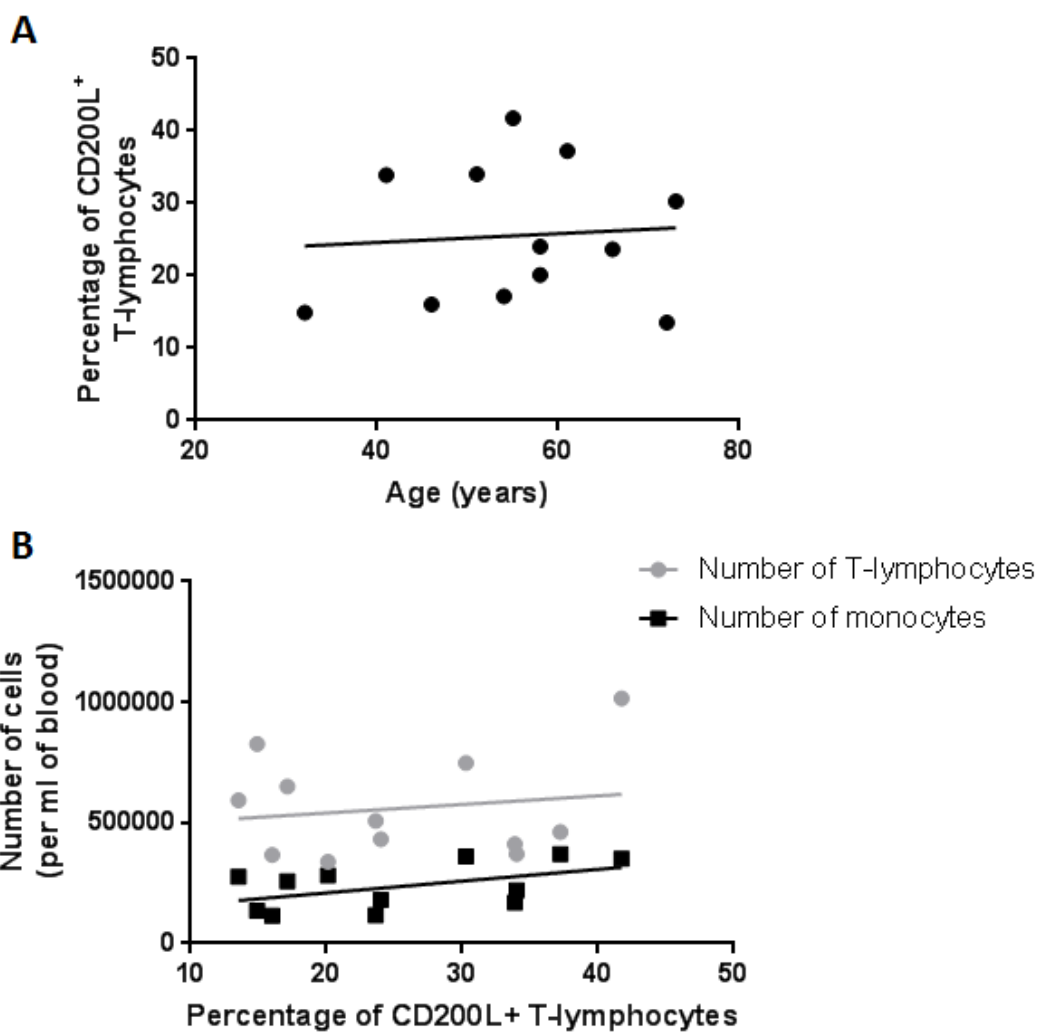
Figure A5.3 – CD200L expression on B-lymphocytes (CD19^{+} lymphocytes) in patients with sarcoidosis and healthy controls. The expression (GMFI) of healthy controls (n = 7) and patients with sarcoidosis (n = 5), p = 0.755 as determined by the Mann-Whitney U test.

Figure A5.4 – Correlation between the percentage of CD200L^{+} T-lymphocytes and either the age or number of leukocytes in patients with sarcoidosis. **A:** Correlation between age and the percentage of CD200L^{+} T-cells (n = 12, p = 0.829, R^2 = 0.006). **B:** Correlation between the percentage of CD200L^{+} T-lymphocytes and the number of T-cells (grey circles, n = 12, p = 0.956, R^2 = 0.026) or the number of monocytes (black squares, n = 12, p = 0.134, R^2 = 0.246).
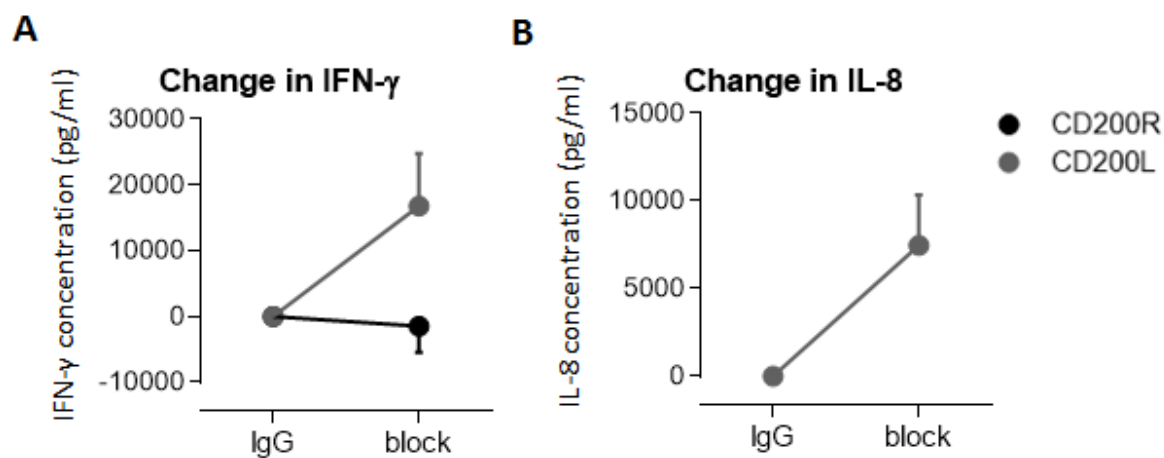
Figure A5.5 – Blocking CD200R or CD200L in a PHA stimulated PBMC or whole blood assay in healthy controls detecting IFN-γ or IL-8. CD200R blocking with an antibody took place in isolated PBMCs, CD200L blocking took place in a whole blood system. **A:** Change in IFN-γ release (pg/ml) in PBMCs and whole blood after incubation with CD200R (n=4) or CD200L (n=5) antibodies compared with isotype control IgG. Cells were stimulated with 100μg/ml PHA for 16 hours. **B:** Change in IL-8 (pg/ml) in PBMCs CD200L (n=4) antibodies compared with isotype control IgG. Cells were stimulated with 100μg/ml PHA for 16 hours. Results are presented as differences from isotype control IgG, with SEM error bars; $p > 0.05$ for all data using paired Student T tests.
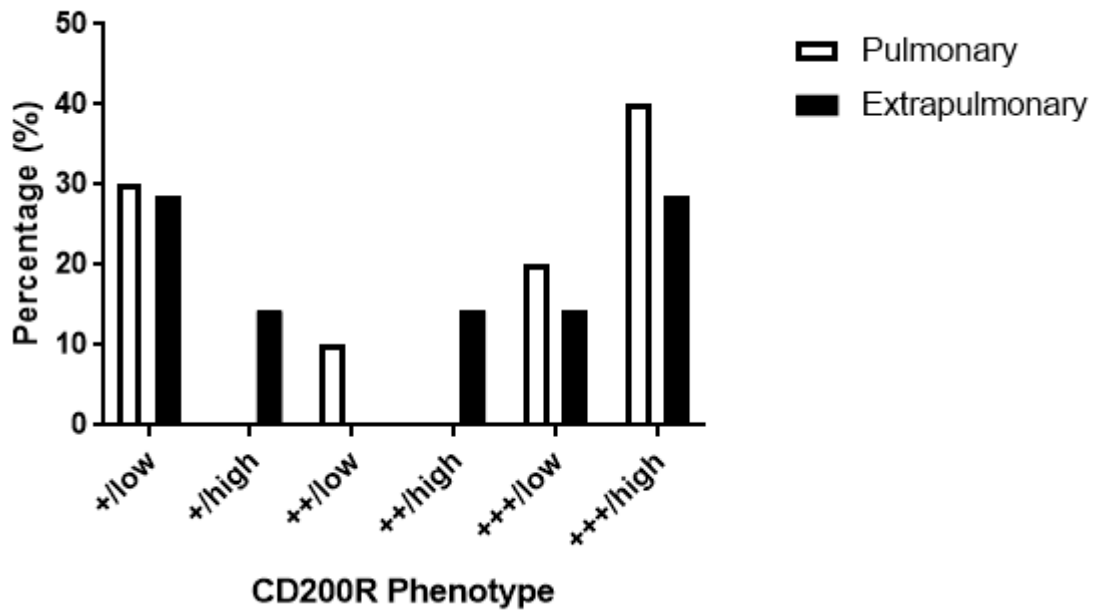
Figure A5.6 – The number of patients with sarcoidosis displaying pulmonary restricted or extrapulmonary organ involvement with specific phenotypes of CD200R expression on their T-lymphocytes and monocytes. Patients with extrapulmonary organ involvement (n = 7, black bars) and pulmonary restricted organ involvement (n = 10, white bars) are displayed in each category of CD200R expression. Monocytes were classified as 'low' or 'high'. T-lymphocyte CD200R expression was separated into 3 profiles labelled '+', '++' or '+++' representing low, medium and high expression respectively. Data represents the percentage of each group, pulmonary or extrapulmonary, with a particular CD200R expression phenotype.