Monocyte profile and function in sarcoidosis

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Abstract: Monocyte profile and function in sarcoidosis

Sarcoidosis is a multisystem inflammatory disorder of unknown aetiology. The immune pathology is characterised by dysregulated T cell (T_H1) activity, macrophage activation and granuloma formation, resulting in systemic inflammation, and organ dysfunction. I hypothesised that, as the systemic precursor to the macrophage, altered monocyte activity in sarcoidosis may contribute to the early immune pathology of the disease. In this thesis, I examined their phenotype, four key monocytic functions: cytokine production, suppression of T cell proliferation, phagocytosis and fusion (as a precursor to granulomagenesis); and their gene expression profile compared to monocytes from healthy controls.

My data show that the expanded monocyte compartment comprise a greater proportion of the inflammatory ($CD14^{++}CD16^{+}$) and patrolling ($CD14^{+}CD16^{++}$) subsets, increased TNFα and IL-12 and decreased IL-10 and IL-4 production in sarcoidosis compared with healthy controls. The IL-10 deficit renders the monocytes less able to regulate T cell proliferation or their own fusion to multinucleate giant cells, potentially contributing to T cell expansion and granuloma formation respectively. Additionally, sarcoidosis monocytes are less able to phagocytose inert material. I also showed that previously reported deficiency in invariant NKT cells and low serum vitamin D levels in sarcoidosis may be linked to reduced IL-10 production by monocytes. Vitamin D treatment in vitro restored most of these deficiencies and provides a potential therapeutic method for manipulating monocyte function and disease genesis in sarcoidosis.

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List of Abbreviations

αGC	α-galactosylceramide, α-GalCer
ANOVA	Analysis of variance
APC(s)	Antigen presenting cell(s)
APC	Allophycocyanin
BAL(F)	Bronchoalveolar lavage (fluid)
BSA	Bovine serum albumin
CAS	Clinical activity score
CCL	Chemokine
CCR	Chemokine receptor
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinmidyl ester
CRP	C-reactive protein
CXR	Chest X-ray
Cy	Cyanine
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulphoxide
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescinisothiocyanate
FSC	Forward scatter
GM-CSF	Granulocyte macrophage colony-stimulating factor
GO	Gene ontology
GSEA	Gene set enrichment analysis
GWAS	Genome wide association studies
HC	Healthy control
HLA	Human leukocyte antigen
HS	Human serum
ICS	Intracellular staining
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILD	Interstitial lung disease
iNKT	Invariant natural killer T cells, Type 1 NKT cells
IPF	Idiopathic pulmonary fibrosis
LFA-1	lymphocyte function-associated antigen-1
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MDSC	Myeloid derived suppressor cell
MHC	Major histocompatibility complex
MS	Multiple sclerosis
NK	Natural killer cells
NKT	Natural killer T cell, in this document, type 1 or invariant NKT cell.
NOD	Non-obese diabetic
PAMP	Pathogen-associated molecular pattern
PBL	Peripheral blood leukocytes
PBMC	Peripheral blood mononuclear cells

PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein
PFTs	Pulmonary function tests
PHA	Phytohaemaglutinin
PMA	Phorbol-12-myristate-13-acetate
PPAR-γ	Peroxisome proliferator-activated receptor γ
RA	Rheumatoid arthritis
RPM	Revolutions per minute
RT	Room temperature
RT-qPCR	Real time quantitative PCR
SCAS	Sarcoidosis clinical activity score
SCID	Severe combined immunodeficiency
SHA	Sarcoidosis high activity
SLA	Sarcoidosis low activity
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper cells
TLR	Toll-like receptor
TNF	Tumour necrosis factor
Treg	Regulatory T cell

Chapter 1: Introduction

1.1. Sarcoidosis

1.1.1 Background

Sarcoidosis is a multisystem inflammatory condition characterised by noncaseating granulomata. The condition was first described in 1869 by Hutchinson, a British dermatologist, who reported a case of a 58-year-old coal wharf worker with non tender, purple skin lesions (1). The term “sarcoidosis” was not coined until 1897 when Boek, a Norwegian dermatologist, presented a patient with skin nodules, whose histology he felt was similar to sarcoma (2). The defining histopathogical finding of noncaseating granulomata is usually associated with mononuclear cell infiltration into the tissues but is nonspecific to sarcoidosis, leading many to argue that the condition remains a diagnosis of exclusion (3).

The true incidence of sarcoidois is difficult to ascertain, with many cases being clinically and radiologically silent (4). Even when clinically apparent disease is present, variable tenacity in securing the diagnosis by radiological and histological means, as well as differential reporting may mask the absolute incidence. Whilst reported in all racial groups, the reported incidence of sarcoidosis varies considerably; in the UK 5.1/100 000 is suggested (5) with the highest reported incidence worldwide seen in northern European countries (5-40/1000). A much lower incidence is reported in Japan (1.2/100 000) (6, 7). A peak incidence is seen between 20-29 years for both

men and women with a second peak in women, outside Japan, seen at 65-69 (8).

1.1.2. Clinical features

Whilst almost any organ can be affected, the lungs and intrathoracic lymph nodes (>90%), skin (20-30%) and eyes (20-25%) are most commonly affected (7, 9, 10). The clinical manifestations of disease result from the systemic burden of inflammation and the localised consequences of granuloma deposition. The clinical presentation is highly variable ranging from entirely asymptomatic to rapidly progressive pulmonary or cardiac failure, sight threatening uveitis and severe neurological impairment. Constitutional symptoms of fever, lethargy, malaise, anorexia and weight loss are reported by 20-50% of patients at presentation (11). The clinical manifestations of sarcoidosis are listed in Table 1.1 and illustrated in Figure 1.1.

As many as 50% of patients with sarcoidosis present with respiratory symptoms: most commonly dyspnoea, cough and chest discomfort. Pulmonary function tests are abnormal in many patients with sarcoidosis, but there is no diagnostic pattern (3); normal, restrictive or obstructive patterns are observed (12-14). It has been reported that up to 65% of patients have airflow limitation with spirometry usually indicative of restrictive ventilatory dysfunction, with reduced forced vital capacity (FVC) and reduced forced expiratory volume in 1 second (FEV1); additionally, concomitant obstructive disease is seen in up to 50% (9). Even where there are abnormal pulmonary function tests at presentation, as many as 80% of patients with sarcoidosis

are seen to recover to within the normal range at two years (15). Airway hyper-responsiveness can be seen in 5-83% of patients (16). Raised pulmonary artery pressure is a feature in 6-23% of patients at rest, increasing to 43% on exertion (17).

Affected organ	Frequency of occurrence	Common Findings
Lungs and thoracic lymph nodes	>90	Dyspnoea, cough, chest pain, pulmonary hypertension, mixed pulmonary function test abnormalities.
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, cardiomyopathies, sudden cardiac death
Central nervous system	10-25	Cranial neuropathy, mass lesions, aseptic meningitis and/or encephalitis, myelitis, spinal cord and peripheral neuropathy, pain, hypothalamic pituitary involvement
Sinuses and upper respiratory tract	5-10	Chronic sinusitis, laryngeal involvement, parotid gland involvement
Bones, joints and muscle	5-15	Chronic arthritis, dactylitis, lytic bone lesions, myopathy
Haematological system	>50	Peripheral lymphopenia, hypergammaglobulinaemia
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 1.1. Clinical manifestations of sarcoidosis
Adapted from *Chen, E. S. & Moller, D. R. Nat. Rev Rheumatol. 2011* (18).



Figure 1.1. Clinical features of sarcoidosis
From *Iannuzzi MC et al. N Engl J Med. 2007* (7). A. Waxy interscapular skin plaques; B. Lupus pernio (violaceous plaques); C. Anterior uveitis with synechiae; D. Enlarged, nodular lacrimal gland; E. Endobronchial cobblestoning; F. Ipsilateral peripheral facial- and cranial-nerve involvement with hearing loss; G. Spinal cord mass on a T_1-weighted MRI scan (arrow); H. Nasal, parotid, lung, liver, spleen, subcutaneous-nodule, and mediastinal and epitrochlear lymph-node involvement on a gallium scan; I. Hypermetabolism in the liver, spleen, and lymph nodes on a positron-emission tomographic scan with ^{18}F-fluorodeoxyglucose; J. Right-lung cavity with a gravity-dependent aspergilloma; K. Hypodense nodular splenic masses on an abdominal computed tomographic scan; L. Involvement of the optic chiasm on a gadolinium-enhanced MRI scan (arrow); and M. Granulomatous involvement of the humerus on a T_1-weighted MRI scan.

Löfgren's syndrome is an acute presentation of sarcoidosis characterised by bihilar lymphadenopathy, erythema nodosum, acute arthritis and/or uveitis. It

is more frequently seen in women and has a 70-80% chance of remission (19). The remaining patients with sarcoidosis can be divided into three groups, (i) acute self-limiting, (ii) chronic relapsing remitting and (iii) chronic progressive. Patients with fibrotic pulmonary disease, or the classically described skin lesion, lupus pernio, rarely achieve resolution. Organ involvement is typically defined early in the course of disease, with less than a quarter of patients having new organ system involvement during a two year follow-up period (15).

1.1.3. Aetiology

1.1.3.1. Environmental factors

The aetiology of sarcoidosis remains unclear and the disease is thought to occur as a result of diverse environmental triggers in a genetically susceptible host. Spatial clustering of unrelated cases of sarcoidosis tantalisingly suggests that exposure to common environmental agent(s) can trigger the development of sarcoidosis. The US-based multi-centre ACCESS (A case Control Etiologic Study of Sarcoidosis) study, failed to identify any statistically significant aetiological factor (pre-study goal of a two-fold risk [odds ratio]). The results did suggest that there is a modestly increased (approximately 1.5) risk of developing sarcoidosis after exposure to specific microbe-rich or mouldy environments and in agricultural workers (20). Smoking status (active, passive and former) were strongly associated with a reduced risk of sarcoidosis (0.2), but no association was found (positive or negative) between sarcoidosis and previously hypothesised risk factors such as non-

occupational wood dusts, occupational metals or silica. Some studies have suggested an increased incidence of sarcoidosis or sarcoidosis-like granulomatous pulmonary disease in firefighters and US military personnel exposed to the heavy dust burden from the destruction of the World Trade Centre in New York in 2001 (21, 22) but whether this presentation actually represents sarcoidosis remains contentious.

There is additional evidence to suggest that myco- and propionibacterial DNA and protein antigens are present in sarcoidosis tissue and that these are the target for T cell responses (23). This shall be discussed in greater detail later. Whilst no consistent antigen has been found, clonal expansion of T cells suggests that in an individual, a specific antigenic stimulus may be important (24).

1.1.3.2. Genetic factors

The assumption that there is an important genetic component is based on the observations that (i) the prevalence and incidence rates of sarcoidosis are different between ethnic groups and races and (ii) the disease tends to cluster in families (8, 25). The ACCESS study found a fivefold increase in the risk of sarcoidosis amongst sibling and all first- and second-degree relatives of affected individuals; spouses have no increased risk (26). Twin studies suggest that the co-twin has an 80-fold increased risk for monozygotic pairs compared with a 7-fold increase in dizygotic siblings (27).

The most consistent genetic study data suggest an association between sarcoidosis risk and class I and II HLA molecules (28), with different HLA alleles implicated in different ethnic groups. In the ACCESS study, *HLA-DRB**1101, *HLA-DRB**1201, *HLA-DRB**1501 and *HLA-DRB0402* alleles had the strongest association with the risk of sarcoidosis (29). Amino acid residues [Phenylalanine-47 (F47)] that form the polymorphic peptide binding domains of pocket 4 of the HLA-DR protein and pocket 9 of the HLA-DQ protein are associated with increased risk (29, 30). There is evidence to suggest that HLA genotypes are associated with clinical phenotype and progression, for example *HLA-DRB1*0301* is strongly associated with a Löfgren presentation and disease resolution (31-33); *HLA-DQB1*0201* is also associated with remitting disease in British and Scandinavian patients (34), although the allele responsible is difficult to ascertain because of strong linkage to HLA-DRB1. By contrast, *HLA-DQB1*0602-DRB1*150101* is associated with chronic unremitting disease (35). With the exception, perhaps, of variants in the *TNF* gene, associated with a 1.5 fold increased risk of sarcoidosis suggested by meta-analysis (36) no other candidate gene associations have consistently been reported.

Genome wide association studies also suggest a role for genes within the MHC locus and have implicated *BTNL2*, *ANXA11* and *FAM178A*; the former two are both involved in the regulation of T cell proliferation (37-39). BTNL2 has amino acid homology to the co-stimulatory B7.1 and signals to CTLA4 causing a second signal, which attenuates T cell activity. Mutation results in truncation of protein and decreased expression on T cells. However, the exact

role in sarcoidosis is uncertain and genes neighbouring *BTNL2*, such as the human leukocyte antigen *DRB1 (HLA-DRB1)*, have also been implicated; they show high linkage disequilibrium and therefore may be inherited together (40), perhaps explaining the association seen. ANXA11 (Annexin 11) has a number of regulatory functions in calcium signaling, cell division, vesicle trafficking and apoptosis although the consequence of Annexin 11 defects in sarcoidosis is unclear (41, 42). *FAM178A* is a gene of unknown function that is also associated with Crohn's disease and again, the mechanistic pathways relevant to sarcoidosis pathogenesis are unclear (37, 43).

1.1.4. Immune pathology

1.1.4.1. T_H1 polarisation

The immune pathology of sarcoidosis results from a hyperactive $CD4^+$ T cell inflammatory response with a T_H1 bias (7, 18, 44) resulting in excess T cell proliferation, macrophage activation and granuloma formation. The T_H1 cells produce interferon-γ (IFNγ) activating macrophages and together these cells produce a number of cytokines and chemokines including TNFα, TGFβ, IL-1β, GM-CSF, IL-1, IL-2, IL-15, IL-12, IL-18, CCL2 (45-49), which results in further monocyte and T cell recruitment. IFNγ enhances macrophage phagocytosis and oxidant production synergising with cytokines such as TNFα to cause microbial killing (18). TNFα is key in granuloma formation and propagation; treatment with anti-TNF agents, such as the monoclonal antibody Infliximab, is used with some success in severe disease. IL-2 and IL-15 have a proliferative and anti-apoptotic effect on T cells, contributing to the excess of T

cells (45). Both the transcription factor STAT1, which regulates the differentiation of T_H1 cells, and its phosphorylated form are upregulated in sarcoidosis.

The T_H2 response at sites of inflammation is attenuated in sarcoidosis with an observed reduction in the levels of IL-4 and IL-5, but interestingly not IL-13, released by T cells (47, 50, 51). The data on the role of T_H17 responses in sarcoidosis is conflicting with some studies suggesting a contributory role for T_H17 polarised T cells in granuloma formation (52) and others suggesting a decrease in IL-17A mRNA in the bronchoalveolar cells in sarcoidosis (53).

Another key feature is the oligoclonal expansion of T cells with a restricted TCR in the lungs, blood and skin (24, 54, 55). This phenomenon is particularly marked in individuals with *HLA-DRB1*0301* and *DRB3*0101* who have Va2.7+CD4+ T cells, the number of which correlates with prognosis and clinical course (56, 57). The antigen specificity of these oligoclonally expanded T cells in sarcoidosis remains unknown.

1.1.4.2. Immune regulatory cells

Regulatory T cells (T_{regs}) maintain immune homeostasis by inhibiting antigen-presenting cells and effector T cell function. The frequency, and function of $FoxP3^+$ T_{regs} to suppress cytokine expression and granuloma formation, is reduced in sarcoidosis patients (58-60), although the implication of this remains unclear.

Previous work from the group and others, have shown that there is a deficiency of the immune regulatory invariant natural killer T (iNKT) cells (discussed in detail later) in both acute and resolved sarcoidosis (61, 62). In these studies, there was no difference in iNKT cell number in the periphery, lungs or draining lymph nodes suggesting a true deficiency rather than accumulation at the site of disease. Again, the implication of this observation remains unclear.

Regulatory B cells (B_{reg}) are a subpopulation of IL-10 producing predominantly $CD24^{hi}CD27^{+}$ B cells with immunosuppressive function (63-65). $CD24^{hi}CD38^{hi}$ and $CD24^{int}CD38^{int}$ B-cell subpopulations have also been identified, with CD48 and CD148 also being reported to be useful in identification (66). It is thought that B_{regs} arise from autoreactive transitional 2 marginal zone precursor (T2-MZP) B cells (67). During inflammation, B_{regs} produce IL-10 upon activation by signals from CD40, B-cell receptors (BCR), CD80, and CD86 contributing to the differentiation of $FoxP3^{+}/CD4^{+}/CD25^{+}$ T_{regs} (67). The lack of B_{regs} is associated with a worsening of several autoimmune diseases such as collagen-induced arthritis, systemic lupus erythematosus, and experimental autoimmune encephalomyelitis in murine studies (63, 65). An increase in the number of B_{regs} has been reported in the peripheral blood in sarcoidosis, however the downstream consequences of this remains to be determined (68).

1.1.4.3. Granuloma formation

The granuloma in sarcoidosis is a dynamic, cytokine producing structure characterised by a core of macrophages and monocyte-derived epithelioid histiocytes attempting to engulf a nidus of poorly soluble material that provides a persistent inflammatory stimulus. Mature macrophages are characterised by increased cytoplasmic size, a larger number of organelles and by their ruffled cell membranes, which are thought to confer greater phagocytic and microbicidal potential (69-71). Within the granuloma, macrophages can undergo additional changes, they can fuse to form multinucleated giant cells (72) or differentiate into foam cells, which are characterised by lipid accumulation. The mature macrophages can undergo transformation into epithelioid histiocytes, which have tightly interdigitated cell membranes. $CD4^+$ T cells with a predominantly T_H1 bias, interspersed with a small number of $CD8^+$ T cells and B cells surround this core. $CD4^+CD25^{bright}FoxP3^+$ regulatory T cells, extracellular matrix-secreting fibroblasts and additional $CD8^+$ T cells accumulate at the periphery. The $CD4^+CD25^{bright}FoxP3^+$ regulatory T cells exhibit anti-proliferative effects but do not completely inhibit TNFα production and therefore are insufficient to control local inflammation (58). The local environment of the granulomata may later bias towards T_H2; where alveolar macrophages express an alternative M2 phenotype characterised by the production of IL-4 and IL-13 and up-regulated expression of chemokines, such as CCL-18 promoting extracellular matrix deposition and lung remodeling resulting in fibrosis (73).

1.1.4.4. Candidate antigens

It is widely believed that the sarcoidosis granulomata form around a nidus of poorly soluble material, however the precise antigen remains elusive. It is likely that any one of a number of potential antigens may form this nidus. The clinical and pathological similarities to tuberculosis continue to fuel speculation that mycobacteria cause sarcoidosis. However, microbial organisms are not detected on histological examination of sarcoidosis biopsy samples and there are no reproduced studies demonstrating the presence of viable mycobacteria or other organisms (18). PCR studies report mycobacterial nucleic acids in 0 to 80% of sarcoidosis samples. Interestingly the protein mycobacterial catalase-peroxidase (mKatG) has been found to be present in sarcoidosis tissue with the same physicochemical properties as the Kveim-Siltzbach reagent (74). mKatG has the ability to elicit a T cell response in sarcoidosis (but not other lung diseases), the strength of which correlates with disease severity (75-77).

A Japanese study found DNA from *propionibacterium* spp. in almost all tissue samples from patients with sarcoidosis, but also from many of the healthy controls (78). Studies attempting to replicate this finding of propionobacterial material vary from 40 to 78% positive (23, 79). *P. acnes* proteins produce a greater T cell proliferative response in sarcoidosis patients than in controls and also can induce granulomatous inflammation (80-82). They promote expression of T_H1-related cytokines through antigen-independent innate mechanisms via the stimulation of TLR9 (83).

The Kveim-Siltzbach reagent, historically used in the diagnosis of sarcoidosis, has no known observed viable organism and yet is able to induce granulomatous inflammation in individuals with sarcoidosis. It has been suggested by Chen and colleagues that the antigens from mycobacterium (or other organism) could be released on the death of the organism with a complex of host and (myco)bacterial proteins in response to the infection, leading to sarcoidosis (84).

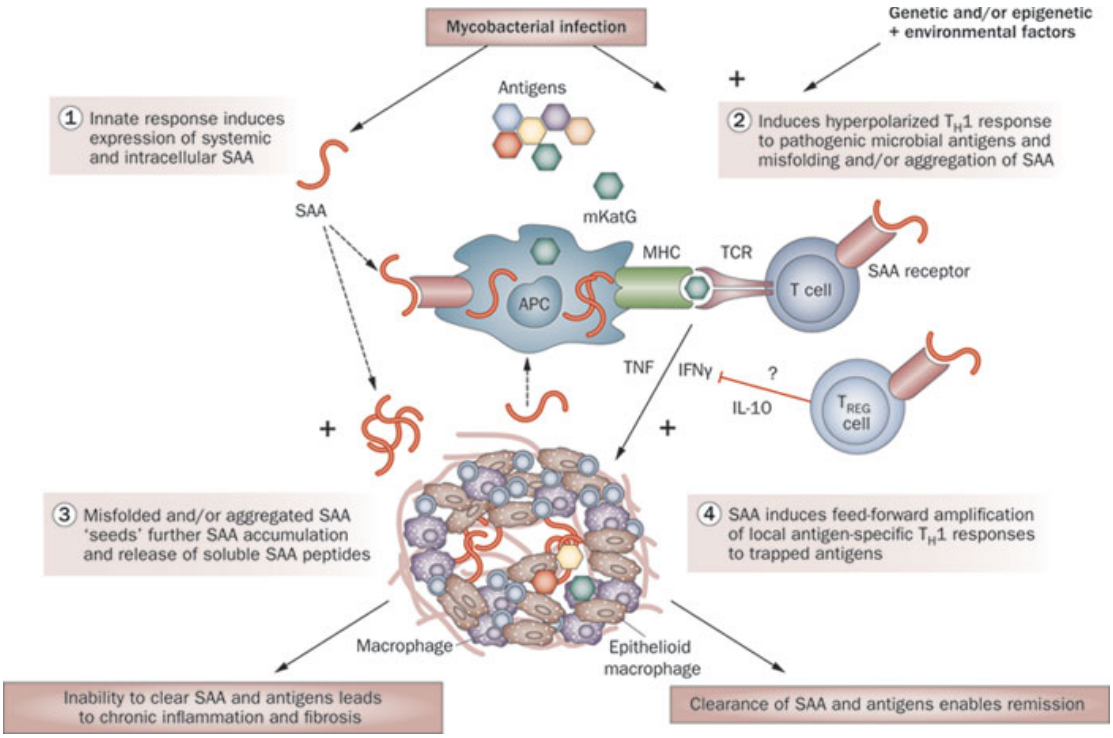


Figure 1.2. Serum amyloid A misfolding hypothesis of the pathobiology of sarcoidosis
From *Chen, E. S. & Moller, D. R. 2011* (18)*. Nat. Rev. Rheumatol.* Adapted from: *Chen, E. S. et. al. Am. J. Resp. Crit. Care Med. 2010* (85).

The same group has more recently demonstrated that granulomas in sarcoidosis are characterised by extensive deposition of serum amyloid A protein (SAA) (85) at greater levels than seen in other granulomatous disorders. SAA promoted granulomatous inflammation in animal models and

stimulated TNFα and a T_H1 response including the expression of IFNγ as well as the expression of the immune-regulatory cytokines IL-10 and IL-18 from human alveolar macrophages. The authors of this work hypothesise that an aberrant innate immune response in sarcoidosis results in the induction, misfolding and aggregation of SAA, which drives the persistent hyperactive TH1 response (Figure 1.2).

1.1.4.5. Role of TNFα

Alveolar macrophages from sarcoidosis patients with severe or progressive disease release more TNFα than those from individuals with inactive disease (86). TNFα is important in macrophage activation, promotion of cellular migration towards the site of inflammation and leukocyte adhesion (87, 88). In a mycobacterial-driven mouse model of granulomagenesis, TNFα is responsible for the early production of chemokines that attract mononuclear cells to the site of inflammation, such as RANTES, MIP-1α, MIP-1β, MIP-2 and CCL2 (MCP-1)(88). Increased levels of CCL2 are seen in the bronchoalveolar lavage fluid (BALF) of sarcoidosis patients (89-91). In active sarcoidosis, alveolar macrophages produce high levels of CCL20 when stimulated by TNFα and IL-1β, which drives DC, B and T cell homing to the lungs via CCL6(52, 92).

Traditionally, TNFα was thought to induce granuloma formation (87, 88, 93, 94), based on the finding that TNFα-deficient mice are unable to mount a satisfactory granulomatous response, with poorly formed granulomata containing multiple mycobacteria, rendering them hyper-susceptible to

tuberculosis (87). However temporal observation of granuloma formation has suggested that granulomata can be formed in the absence of TNFα and that this formation is, in fact, accelerated followed by early loss of the granuloma structure. This suggests that TNFα is more important in the maintenance of the granuloma, which perhaps explains the observation poorly formed granulomata (95). This observation is supported by computational modeling studies suggesting that TNFα deficiency does not impair granuloma formation (96). TNFα may have additional effects on granuloma formation by influencing T cell trafficking and activation, independently altering cell recruitment and the structural organisation of mature granulomata (97). Clinical trial data suggest that monoclonal antibody therapy directed against TNFα (Infliximab) improves lung function and extra-pulmonary manifestations in individuals with sarcoidosis (98). Other pro-inflammatory cytokines such as IL-1, IL-6 and osteopontin are upregulated in sarcoidosis, but the implication of this is unclear. Expression of the NFκB transcription factor family is upregulated whilst the inflammation suppressing transcription factor PPAR-γ is downregulated in individuals with sarcoidosis (99).

1.1.4.6. Innate immunity and sarcoidosis

There is some evidence to suggest the importance of innate immunity in the pathogenesis of sarcoidosis. TLRs are involved in regulating the innate response in patients with sarcoidosis; a polymorphism in *TLR2* (−16,934 A>T) is associated with enhanced induction of expression of the TNFα, IL-12 and IL-6 in PBMCs from patients with sarcoidosis (100). NOD2 stimulation has been implicated through a synergistic effect on TLR2-induced expression of

IL-1β in individuals with sarcoidosis (101). TLR2 stimulation by serum amyloid A (SAA) enhanced the production of TNFα, IL-18 and IL-10 in alveolar cells from patients with sarcoidosis (85).

1.1.4.7. Fibrosis

A proportion of patients with chronic active sarcoidosis develop fibrosis of the lungs, heart or liver, the mechanism for which is unclear. Both pro-fibrotic cytokines, such as TGFβ, IGF1 and matrix metalloproteinases, and IFNγ, which down-regulates fibroblast proliferation and extracellular matrix deposition, are found at sites of granulomatous inflammation (44, 102, 103). There is evidence to suggest that alveolar macrophages in fibrotic sarcoidosis are alternatively activated (M2 phenotype), with up-regulated expression of pro-inflammatory, pro-fibrotic CCL-18 with resultant lung remodeling (104-106). Given the lack of T_H2 cells expressing IL-4 in sarcoidosis, this raises the possibility that this is either being produced by other cells such as monocytes or that other cytokines such as IL-10 and/ or IL-13 are driving the pro-fibrotic phenotype.

1.1.5. Diagnosis

The diagnosis of sarcoidosis is made on the basis of clinical and radiological findings of granulomatous disease with supporting histology. Patients may have had symptoms, many of which may be non-specific, for more than three months and often require multiple consultations with health care providers before a specific diagnosis is made (107).

The chest radiograph is the most commonly used radiological imaging technique and radiographic disease staging is still based on the system proposed by Scadding in the 1960s (108). This has been modified to include stage 0: no adenopathy or infiltrates; stage 1: hilar and mediastinal adenopathy alone; stage 2: adenopathy and pulmonary infiltrates; stage 3 pulmonary infiltrates alone; and stage 4: pulmonary fibrosis. This system allows some prognostication, with patients with stage 1 radiographs at presentation having a greater than 90% chance of resolution by 2 years, compared with those with stage 3 who have less than a 30% chance of resolution. There is considerable inter-observer variability in staging, even amongst radiologists, which diminishes the clinical utility of this scale (109). In addition, there is little correlation between the chest radiographic findings and the level of dyspnoea, serum disease activity markers and physiological assessment (such as six-minute walk testing and pulmonary function testing).

Computed Tomography is not usually indicated if a confident diagnosis can be made from the clinical history and plain chest radiographic findings (110). The American Thoracic Society (ATS)/ European Respiratory Society (ERS)/ World association of Sarcoidosis and other granulomatous disorders (WASOG) consensus statement suggests that CT may be required in certain circumstances; if the clinical or radiographic findings are atypical, if there is a strong clinical suspicion of disease but normal radiography and to detect both pulmonary and extra-pulmonary complications (111). Additionally, CT may be useful in determining the degree of parenchymal involvement temporally, particularly prior to treatment and gives an indication of disease activity level.

The current British Thoracic Society (BTS) Guidelines reinforce this advice and stress the importance of consulting a dedicated chest radiologist, with expertise in High resolution CT (HRCT) and preferably an interest in interstitial lung disease (112). In cases where there is no apparent pulmonary involvement, F-18 fluorodeoxyglucose positron emission tomography (^{18}FDG PET) may prove useful in identifying areas with active granulomatous inflammation for biopsy (110, 113). The sensitivity and specificity is unknown and a positive result may be due to malignancy or an alternative inflammatory condition. Cardiac and neurological involvement may be better detected with ^{18}FDG PET and MRI with gadolinium than with CT (110, 114).

Biopsy must demonstrate non-caseating granulomata without evidence of infective organisms or focal particle and must be considered in conjunction with other clinical evidence for multisystem disease. In patients who present with Löfgren's syndrome (bilateral hilar lymphadenopathy, erythema nodosum and arthritis), a clinical diagnosis is usually sufficient and a biopsy may not be required. These patients generally have a good prognosis. Transbronchial biopsy has a diagnostic yield of 40-90% and it is suggested that 4-5 biopsies are required to maximise the diagnostic yield (115). The yield can be increased by performing endobronchial biopsy, in conjunction with transbronchial biopsy, giving a rate closer to 90% (16, 116). The yield can be increased yet further if endobronchial ultrasound is used to access smaller nodes and those not accessible by mediastinoscopy (116, 117). Examination of bronchoalveolar lavage fluid for lymphocytes (specifically the CD4/CD8 ratio) can be used as a complementary diagnostic test. If the fluid contains

more than 15% lymphocytes, the sensitivity for diagnosing sarcoidosis approaches 90%; the specificity is low (118).

Angiotensin converting enzyme (ACE) is produced in the epithelioid cells of the granuloma (119) and *may* reflect the systemic granuloma burden (120). Serum ACE is an insensitive test for the diagnosis of sarcoidosis (sensitivity 57%, specificity 90%, NPV 60%, PPV 90%) (3, 119). Whilst an elevated serum ACE is relatively specific to sarcoidosis, it is not sufficient to confirm the diagnosis and therefore is not useful as a screening diagnostic test. Serial ACE measurements have been proposed to monitor the course of the disease. The ACE level, however, does not overall correlate with disease activity levels, though may be useful in individual patients in whom the level is elevated at presentation and falls with remission. The Kveim-Siltzbach test similarly lacks specificity, and is no longer FDA-approved due to its use of human splenic suspension.

1.1.6. Treatment

There is reasonable consensus between the international thoracic societies on the indications for treatment in sarcoidosis (111, 112); this is summarised in Table 1.2. Treatment is usually limited to the symptomatic patient or those with evidence of organ dysfunction or the presence of hypercalcaemia. For most patients a conservative approach with close observation is indicated. The decision to treat has been complicated by a lack of understanding about the natural clinical course of the disease. Corticosteroids remain the mainstay of initial therapy.

1.	Treatment is not indicated for an asymptomatic patient with only lymphadenopathy.
2.	Treatment is not indicated in asymptomatic patients with pulmonary infiltrates and mildly abnormal lung function and stable disease.
3.	Oral corticosteroids are the first line therapy in patients with progressive disease as determined by radiology or lung function, presence of specific symptoms (including dyspnoea, cough or other chest symptoms) or extra-pulmonary disease.
4.	Treatment with prednisolone (or equivalent) at a dose of 20-40mg per day initially for 4 weeks then reduced to a maintenance dose, which will control symptoms and disease progression for a period of 6-24 months.
5.	Bisphosphonates are recommended to minimise steroid induced osteoporosis.
6.	Inhaled corticosteroids are not beneficial as initial treatment or for maintenance therapy. Inhaled corticosteroids could be considered for symptom control (cough) or bronchial hyper-reactivity in a subgroup of patients.
7.	Steroid-sparing immunosuppressive or anti-inflammatory treatments have an undefined role in sarcoidosis, but should be considered in patients when corticosteroids are not controlling the disease or side effects are intolerable. At present, methotrexate is the treatment of choice. Azathioprine is often used when methotrexate is contraindicated or not tolerated.
8.	Lung (and heart) transplantation should be considered in end-stage pulmonary and cardiac sarcoidosis.

Table 1.2. Consensus approach to treatment of sarcoidosis
Adapted from *Chen, E. S. & Moller, D. R. Nat. Rev Rheumatol. 2011* (18). Adapted from the joint guidelines from the British Thoracic Society, the Thoracic Society of Australia and New Zealand and the Irish Thoracic Society (112).

Meta-analysis of the available trial data on steroid therapy in sarcoidosis suggest an improvement in the chest radiograph appearances at 3-24 months, however, differences in methodology and radiological reporting may have impacted on the results seen. There were no other significant findings (121). It has been suggested that steroid treatment may actually have a deleterious effect on the natural course of sarcoidosis (121) which is

seemingly supported by study data suggesting that once steroids were commenced, treatment may be prolonged, with more frequent relapse and fewer steroid free periods. This is in contrast to those not initially started on treatment the majority of whom had still not required treatment by two years (122-124). It is likely that in all of these study cohorts, those with more severe disease were identified early and treated with steroids. Severe disease at outset is associated with a worse prognosis and more frequent relapses. Therefore it may just be that those with more severe disease are being treated and thereby being highlighted by steroid use. Alternatively it may be that the steroid treatment is somehow delaying resolution of the inflammatory cycle, prolonging the course of the disease.

Where steroid therapy alone is not sufficient or there are unacceptable side effects from the treatment, steroid-sparing agents such as Methotrexate, Azathioprine or Hydroxychloroquine may be added or substituted (112) although their precise role is unclear. Clinical study data (98, 125) is encouraging with respect to the use of anti-TNFα therapy in the treatment of sarcoidosis, which may be prescribed on an individual basis in the UK. Although the published clinical trial data, which includes only small numbers of patients, supports this (126, 127), there is not enough robust data available yet on clinical efficacy. As such, currently, the use of anti-TNFα therapy is not widely recommended but clinical trials are ongoing (http://www.clinicaltrials.gov/ct) (112).

1.2. Monocytes

1.2.1. Background

Monocytes are bone marrow derived leucocytes, which are important early in the immune response. Their functions include: sensing tissue damage (128, 129); phagocytosis; production of ROS, NO and type 1 interferons; scavenging viral components and immune complexes (130, 131). They are important in inflammation both through the production of inflammatory mediators such as IL-1α, TNFα, IL-6, IL-8 and CCL2 and through leucocyte recruitment and T cell activation (130).

1.2.2. Monocyte development

Haematopoietic stem cells (HSCs) in the bone marrow produce both myeloid (MP) and lymphoid (LP) committed precursors. MPs give rise to monocyte, macrophage, and DC precursors (MDPs), which subsequently differentiate into monocytes, macrophages, and common DC precursors (CDPs) (128). Monocyte progenitors express CD115 (M-CSF receptor) and develop in response to macrophage colony-stimulating factor (M-CSF). Monocytes migrate from the bone marrow in a CCR2 dependent manner (132, 133) and circulate in the blood. They can enter tissues under inflammatory conditions where they differentiate into macrophages and inflammatory DCs (TipDCs - TNFα, iNOS-producing DCs); however, it is now widely accepted that monocytes do not give rise to classical DCs or plasmacytoid DCs (134, 135).

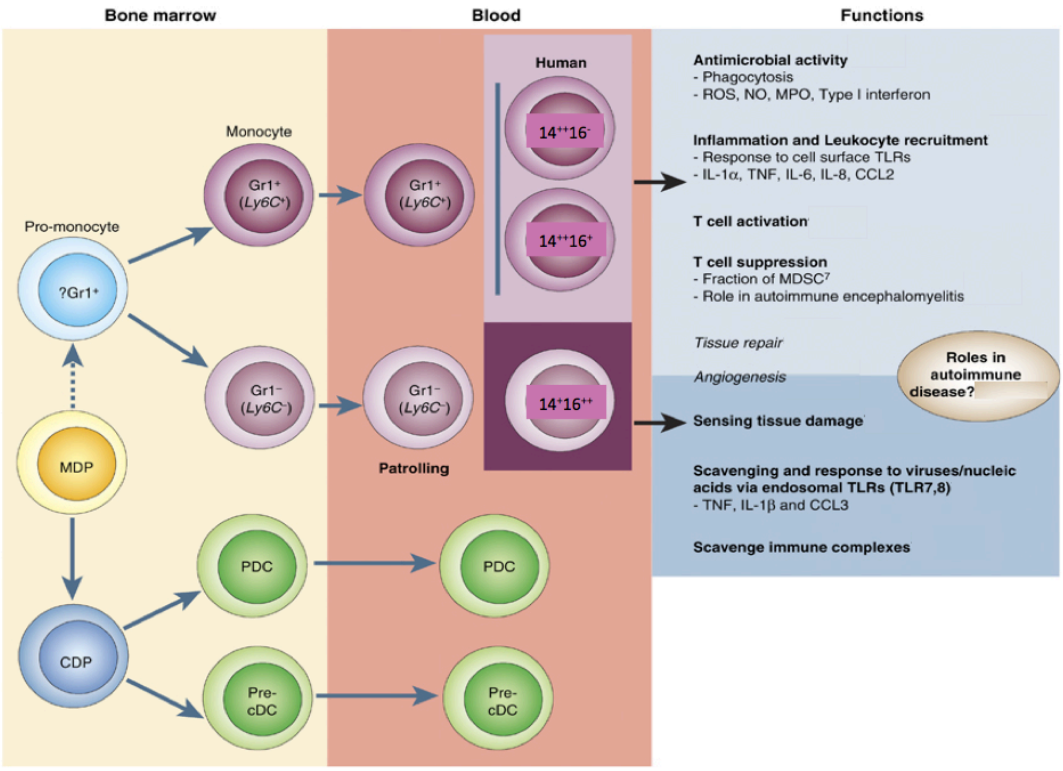


Figure 1.3. Monocyte development & effector functions.
Adapted from *Saha & Geissmann. Immunology and Cell Biology. 2011 (136).* Hematopoietic stem cells produce MDPs in the bone marrow via a myeloid committed precursor, these then give rise to monocytes, possibly through a Gr1+ pro-monocyte. In the mouse, two monocyte subsets Gr1+ (*Ly6C*+) and Gr1$^-$ (*Ly6C*$^-$) leave the bone marrow to enter the circulation; the corresponding human monocyte subsets are illustrated.

Differentiation of MDPs into monocytes and macrophages is less well understood than their differentiation into DCs. The developmental relationship between the 'mature' monocyte subsets is still unclear, in part because monocytes appear to be able to migrate both out of and back into the bone marrow (128, 137). Traditionally, monocytes have been considered as a developmental intermediate between bone marrow precursors and tissue macrophages. It is now known, firstly that monocytes carry out a number of specific effector functions during inflammation (137) and secondly, that many tissue macrophages and DCs do not originate from monocytes. Monocyte development is summarised in Figure 1.3.

1.2.3. Monocyte subsets

1.2.3.1. Murine subsets

Murine monocyte subsets are divided on the basis of surface antigen and chemokine receptor expression (138). In mice, expression of Ly6C and CD11b identifies a subset that expresses high levels of CCR2, and low levels of CX_3CR1, these are termed inflammatory or $Ly6C^{hi}$ monocytes. These represent 2-5% of circulating leucocytes in an uninfected mouse and are rapidly recruited to sites of infection and inflammation (130, 139, 140). $Ly6C^{hi}$ monocyte trafficking is significantly impaired in the absence of CCR2, which is important in the release of these cells from the bone marrow (132). These monocytes are pro-inflammatory and key in early antimicrobial defence (130, 138). A second, less prevalent, subset of circulating murine monocytes, expresses high levels of CX_3CR1, low levels of CCR2 and Ly6C. They are referred to as $Ly6C^{lo}$ or patrolling monocytes because intravital microscopy has demonstrates that they crawl along the luminal surface of endothelial cells lining blood vessels (141), a process mediated by the integrin LFA-1. At this location they are ideally situated as early responders to infection and tissue damage (141, 142).

1.2.3.2. Human subsets

Human monocyte biology, until very recently, has been poorly characterised. Monocytes evade easy definition because they have diverse phenotype and function; the point at which a monocyte makes the transition from monocyte to either DC or macrophage (in terms of function and surface markers) is also

unclear. Monocytes are currently best identified using a combination of surface markers, although some groups use forward and side scatter on FACS sorting to isolate the cells, confirming surface expression after sorting.

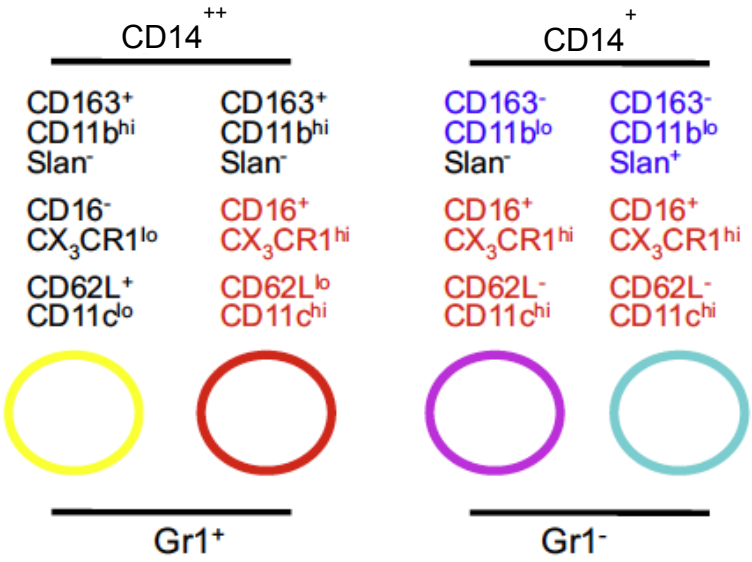


Figure 1.4. Proposed human monocyte surface antigen expression. Modified from *Cros et al. Immunity 2010.* (129). Monocyte populations are color-coded: $CD14^{++}CD16^{-}$ are yellow, $CD14^{++}CD16^{+}$ are red, and $CD14^{+}CD16^{-}$ are dark blue. Within $CD14^{+}$ cells, $Slan^{+}$ cells are light blue and $Slan^{-}$ cells are magenta.

Human monocyte subsets differentially express a number of surface proteins (Figure 1.4) but can be divided on the basis of their surface expression of CD14 (a pattern recognition receptor that acts as a co-receptor for TLR4 in the detection of bacterial LPS) and CD16 (the low affinity FcγRIII receptor that binds the FC portion of IgG antibodies) (143). The $CD14^{++}CD16^{-}$ subset, referred to as classical monocytes, accounts for 85% of circulating monocytes, and similarly, mouse $Ly6C^{hi}$ monocytes, express CCR2 (138). They are efficient phagocytes and reportedly produce IL-10, IL-8, IL-6 and CCL3 in vivo in response to LPS, but do not produce ROS. $CD16^{+}$ monocytes

can be further subdivided into $CD14^{++}CD16^{+}$ (intermediate) and $CD14^{+}CD16^{++}$ (non-classical) subsets.

The $CD14^{++}CD16^{+}$ account for 5% circulating monocytes, are also efficient phagocytes and produce ROS, IL-1β, TNFα and IL-6 in response to LPS, and reportedly, also IL10, but with different kinetics to $CD14^{++}CD16^{-}$ monocytes. They are increased in frequency in inflammatory conditions such as rheumatoid arthritis (RA), psoriatic arthritis and Crohn's disease(144-146).

The $CD14^{+}CD16^{++}$ population accounts for 7% of circulating monocytes. They are poor phagocytes and are thought to be the human homologue to murine "patrolling' $CD115^{+}GR1^{-}$ ($Ly6C^{-}$) monocytes which attach to and crawl along vascular endothelium to scavenge for foreign antigens (129). $CD14^{++}$ monocytes, regardless of CD16 expression, are thought to be the human homologue to the murine inflammatory $CD115^{+}GR1^{+}$($Ly6C^{+}$) monocytes. Although the different subsets identified in mice and humans are not entirely analogous, their development and role in immune defence are similar (147-149).

1.2.4. Monocyte trafficking

CCL2 (MCP1) is a CC-chemokine that binds to CCR2 to mediate inflammatory monocyte recruitment (150). CCL2 may be widely expressed in response to activation by pro-inflammatory cytokines or stimulation of innate immune receptors by a range of microbial molecules (151). The mechanism of action of the CCL2-CCR2 remains unclear and is likely to be multifarious;

(i) CCL2, primarily released by monocytes, macrophages and DCs, binds with glycosaminoglycans in specific tissues, establishing a gradient that is thought to guide monocytes towards sites of infection or inflammation (152) and (ii) CCL2 has been suggested to circulate in the lymph and bind to high endothelial venules in lymph nodes, thereby guiding monocytes into the lymph nodes (153). CCR2 has been shown to be important in the release of monocytes from the bone marrow into the circulation (132, 133). The Duffy antigen receptor for chemokines (DARC), a non-signaling receptor that binds CCL2 and transports it across endothelial cells to the luminal side, has been shown to be involved in the monocyte recruitment from the bloodstream into inflamed tissues (154). CCL7 (MCP3) also binds CCR2 and has been shown to contribute to inflammatory monocyte recruitment (150, 155). Deletion of either the *Ccl2* or *Ccl7* genes impairs monocyte recruitment during infection by approximately 40–50% (155).

Monocytes express CCR1 and CCR5, which bind multiple chemokines, including the shared ligands CCL3 (MIP1α) and CCL5 (156, 157). It has been shown that (i) CCR1 arrests monocyte migration in the presence of shear flow, (ii) CCR5 contributes to monocyte 'spreading', and (ii) both mediate transendothelial chemotaxis towards CCL5; suggesting a role in monocyte recruitment (158), the impairment of which has subsequently been implicated in the pathogenesis of conditions such as atherosclerosis and MS (151). It has also been suggested that CCR6, CCR7, CCR8 and CXCR2 have, as yet unclear, roles in monocyte recruitment.

Patrolling monocytes respond to CX_3CL1 (fractalkine), a membrane-bound chemokine, expressed in tissues and the marginal zone of the spleen. Deletion of the *CX3CR1* gene results in diminished crawling by the patrolling monocytes and impaired inflammatory monocyte recruitment to sites of infection (147, 159).

Monocyte (as with other leucocyte) migration is additionally dependent on multiple integrins and other adhesion molecules such as CD62L (L-selectin), PSGL1 (glycoprotein ligand 1), LFA1 (lymphocyte function-associated antigen 1, αLβ2 integrin), MAC1 (macrophage receptor 1, αMβ2 integrin), PECAM1 (platelet endothelial cell adhesion molecule) and VLA4 (very late antigen 4, α4β1 integrin) (151, 160).

1.2.5. Monocyte recruitment in infection and inflammation

Monocytes mediate direct antimicrobial activity in infected tissue and promote adaptive immune responses in draining lymph nodes. Patrolling monocytes, by virtue of their endothelial location can be recruited to sites of infection even earlier than the inflammatory monocytes, and participate in the initial inflammatory response by releasing TNFα and IL-1 (141). Pro-inflammatory cytokines and microbial PAMPs from the site of infection induce monocyte emigration from the bone marrow, and circulating inflammatory monocytes are targeted to inflammatory sites by the up-regulation of adhesion molecules on local endothelial cells. Much of the existing work has not compared the relative contributions of the patrolling and inflammatory subsets in the response to infection.

Monocyte recruitment contributes to the pathogenesis of various non-infective inflammatory conditions including atherosclerosis (and likely sarcoidosis). The role of monocytes in atherosclerosis has received considerable attention and so shall be considered here as a model for sterile inflammation. In atherosclerosis, monocytes initially undergo P-selectin- and E-selectin-dependent rolling and then use VCAM1 for firm adhesion. Inflammatory monocytes are recruited to the atherosclerotic lesions in a CCR2-, CCR5- and CX_3CR1-dependent manner where they differentiate into macrophages. Patrolling monocytes infiltrate lesions to a lesser extent. Their recruitment has been less well characterised but it is known that they require CCR5, but not CX_3CR1, for migration to lesions (151).

Monocytes may remain monocytic, or become macrophages or DCs. Understanding the types of signals that determine their fate, and potential reversibility of this commitment, could lead to the identification of modifiable targets in disease where monocytes dominate the immune-pathology.

1.3. Macrophages

1.3.1. Background

Macrophages may develop from bone marrow derived monocytes. Their function includes phagocytosis, antigen presentation and cytokine production. They are phenotypically heterogeneous which is likely to be the result of adaptation to their microenvironment (161). Specialised tissue-resident

macrophages include alveolar macrophages in the lung, osteoclasts in the bone, Langerhans cells in the skin, microglia in the brain and Kupffer cells in the liver. The spleen and lymph nodes also have distinct populations of macrophages that perform unique functions: splenic marginal zone macrophages suppress innate and adaptive immune responses to apoptotic cells (162); and lymph node subcapsular sinus macrophages clear viruses and initiate antiviral humoral immune responses (163, 164). These tissue-specific macrophage subpopulations ingest foreign materials and recruit additional macrophages from circulation during an infection or following injury. It is still not clear whether these tissue-specific subsets develop from distinct lineage-committed precursors or if they can be derived from circulating monocytes (161). Once cells, recruited to sites of inflammation have adopted tissue-specific characteristics, it is not known if their fate is sealed or if they are able to migrate and adapt their phenotype to their new location accordingly.

1.3.2. Macrophage subsets

Quantification of macrophage gene expression profiles after cytokine or microbial stimulation has proved useful in subset delineation where surface marker expression alone is not sufficient for subset identification (129, 165). A number of distinct macrophage subsets have been described. These include: classically activated macrophages (M1), which mediate host defence against bacteria, protozoa, viruses and tumourigenesis; alternatively activated macrophages (M2), which are anti-inflammatory producing large amounts of IL-10 (166); Tumour-associated macrophages (TAMs) and myeloid-derived

suppressor cells (MDSCs) which suppress antitumour immunity (166). It is generally believed that macrophages represent a spectrum of activated phenotypes rather than discrete stable subpopulations and studies have reported fluidity in macrophage functional phenotype in response to situation specific stimuli (167-171).

1.3.3. Macrophage activity

Macrophages have a multitude of functions including the (i) phagocytosis and tissue surveillance; (ii) pro-inflammatory anti-microbial defence, whereby they secrete mediators such as TNFα, nitric oxide, IL-1, IL-12 and IL-23; and (iii) regulation of tissue repair mediated through TGFβ1 and PGDF. In addition to these better-understood actions, macrophages have been shown to be important in (i) the regulation of metabolic function in adipocytes; (ii) the down-regulation of pro-inflammatory responses in allergy and asthma; (iii) the pathogenesis of atherosclerosis and (iv) the development of fibrosis in conditions such as idiopathic pulmonary fibrosis, hepatic fibrosis and systemic sclerosis. Macrophage subsets also regulate each other: M1 macrophages have been shown to have a protective role against the immunosuppressive tumourigenic activity of TAMs (166).

1.3.4. Macrophage reservoir renewal

It has been long understood that macrophages differentiate from recruited monocytes (166); this has been recently challenged by a study that demonstrated that both tissue resident and newly recruited macrophages

undergo massive IL-4-mediated proliferation in T_H2-mediated inflammation, resulting in increased numbers of M2 effector macrophages (172). After activation through inflammation or infection, tissue resident macrophages, including alveolar macrophages appear to 'disappear' which is felt to be due to a combination of factors including: increased tissue adherence, tissue emigration through draining lymphatics and possibly cell death (173, 174). Alveolar macrophages appear to traffick to the draining lymph nodes after bacterial infection (175) suggesting a potential role in transporting antigen. This migration from tissue to lymph node remains controversial; it is possible that phenotypically similar cells arise in the lymph nodes by alternate means. It has been argued that migration *does not* happen (176); analysis of whole tissue indicates that resident alveolar macrophages are relatively stable in number during inflammation (177). The counter-argument to this view is that *in situ* self-renewal of alveolar macrophages during inflammation could mask any net cellular loss. Self-renewal, bypassing the requirement for bone marrow-generated monocytes could potentially result in more effective local homeostasis. It is unclear whether this observed self-renewal occurs more generally or is context specific.

1.3.5. Pulmonary macrophage subsets

It is reportedly possible to identify four distinct populations of macrophages in lungs, according to anatomical location, each of which all have specific morphology and functional phenotypes: pleural, intravascular, interstitial and alveolar macrophages (178). Little is known about the pleural macrophages outside of the context of pleural disease such tuberculous, parapneumonic or

malignant effusions (179). They have lower CD14 expression than classical peripheral monocytes, low CD16 expression, the capacity to produce both IL-10 (constitutively) and TNFα (during inflammation) and phagocytic potential. Intravascular macrophages are abundant in ruminants and swine, but rare in humans and laboratory rodents; they are large cells (20–80 μm), form adhesive complexes with the endothelium and are highly phagocytic. Interstitial macrophages are usually referred only to as the immediate precursors of surface macrophages, in transit from the blood to the lung surface. This is probably an oversimplification, as they are functionally distinct from alveolar macrophages (180, 181). Interstitial macrophages are smaller, $CD11b^{+}HLADR^{+}ICAM\text{-}1^{+}$ 'monocyte-like' smaller cells with a high proliferative capacity. They are ideally situated in the connective tissue, by virtue of direct contact, for interaction with T cells, DCs and extracellular matrix (ECM). AMs are larger, more mature, exhibit increased phagocytosis, tumour cell killing, and superoxide anion release, and participate primarily in innate immune responses. AMs can easily be obtained by BAL; in humans BAL cells are more than 80% macrophages with about 18% lymphocytes, a few neutrophils and eosinophils (182). Rodent BAL cells comprise more than 95% macrophages with few lymphocytes and neutrophils. Work from the group has shown that there is an influx of monocytes during lung inflammation (139). The recovery of macrophages by BAL is incomplete; from 3 to 50% of the macrophages have been reported to remain in lungs (183). It may be that these remaining macrophages comprise a distinct phagocyte population with the more activated cells being the most adhesive.

1.3.6. Monocyte-macrophage axis in sarcoidosis

The origin of the macrophages that comprise the sarcoidosis granuloma is unclear. The lack of a satisfactory animal model and the difficulty of obtaining biopsy samples from locations other than lymph nodes, lung and skin mean that the precise comparative phenotype of these granuloma macrophages in different locations is unknown. Are they the specialised AM, the interstitial macrophages, both or neither? Do they all behave in the same way in sarcoidosis, regardless of location? Are the granuloma macrophages derived from the circulating monocytes, which seems more likely given the increased number of monocytes seen peripherally in the disease and inflammatory burden of the disease? In the lung, for example, the granuloma form in the interstitial compartment (Figure 1.5) rather than on the luminal surface of the alveolus where the AMs reside.

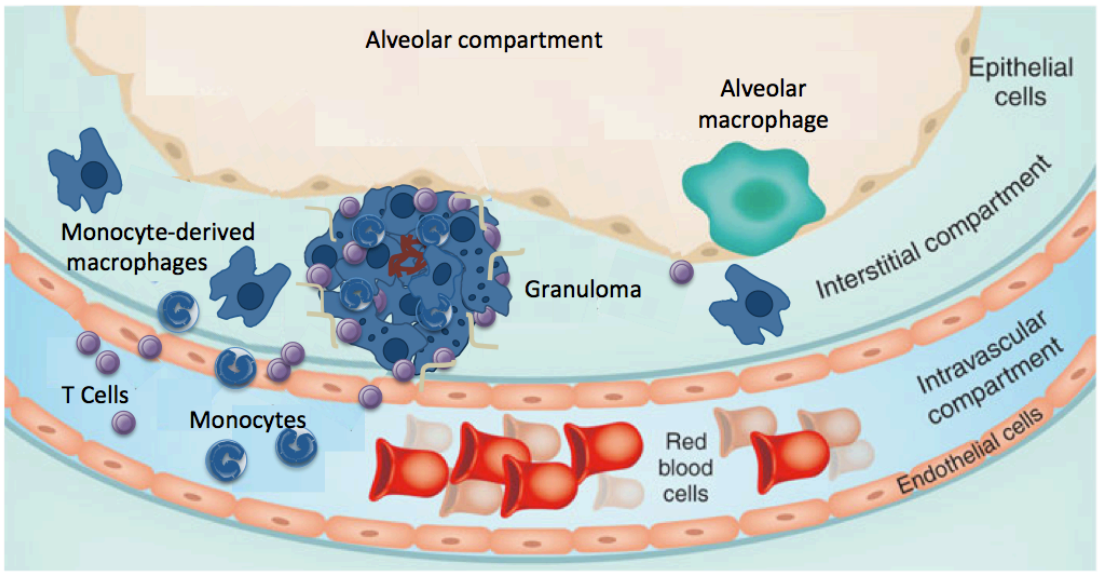


Figure 1.5. Interstitial compartment location of the sarcoidosis granuloma.
Adapted from *Ley & Zarbock. Nat Med. 2008* (184). BALF, used in many of the existing studies in sarcoidosis, harvests cells from the alveolar space, collecting the [green] alveolar macrophages, rather than the [blue] monocyte-derived interstitial macrophages.

The relative longevity of the AM, reportedly more than 80 days in humans (185), also seems to argue against their being solely responsible for the granuloma macrophages, are there enough to account for the granuloma burden, could they replicate quickly enough over a sustained period? It is known that monocytes home rapidly to the inflammatory lung (139). Additionally, it has previously been shown that monocytes, including the patrolling subset, are likely to undergo initial differentiation to macrophages in the interstitial, or parenchymal space; here they form an interstitial macrophage intermediary before undertaking the final commitment to alveolar macrophage (186, 187). Altered monocyte activity in sarcoidosis has been described (188-190), but reports are sparse with focus having been predominantly on T cell and AM activity. It may be, that in focusing on the tissue specific alveolar macrophage in previous sarcoidosis studies, that a potentially important contributor with distinct phenotypic characteristics, the interstitial macrophage, has been overlooked.

1.4. Phagocytosis

Monocytes, macrophages, DCs, neutrophils and mast cells are all ‘professional phagocytes’ and may be distinguished from ‘non-professional phagocytes’ by their phagocytic proficiency (191). Professional phagocytes express such as scavenger and pattern recognition receptors (PRRs) such as the toll like receptors (TLRs) that detect signals not normally found in healthy tissues. Scavenger receptors bind apoptotic and necrotic cells, opsonized pathogens and cell debris; TLRs detect 'non-self' or 'damage', but the way in

which they interact to result in phagocytosis is still largely unknown (161, 192).

The mechanisms through which phagocytosis is controlled is varied and depends on both the location and stimulus. It is an actin dependent process whereby particulate targets, typically greater than 0.5μm in diameter, are actively internalised. This may be through FC receptor- or dectin-1-mediated 'reaching' phagocytosis, by complement mediated 'sinking' phagocytosis or by macropinocytosis or 'triggered/ cell drinking/ non-specific' phagocytosis (193). The internalised particles are degraded as the compartment acidifies and fills with proteolytic enzymes. Although a number of receptors have been reported as having a role in phagocytic uptake, only a few have been reproducibly demonstrated to induce phagocytosis when expressed on otherwise non-phagocytes. These include Fc receptors for antibody-opsonised particles and dectin1 for fungal β-1,3-glucans (194, 195). The identification of candidate receptors is made more difficult by the fact that bacterial cell walls, and other target particles, engage many receptors on the phagocyte such that it may not be clear which is triggering internalisation. Target particle size, shape and rigidity all affect phagocytosis efficiency and subsequent phagocyte cytokine production. In sarcoidosis, the lack of a consistently identifiable antigen suggests that any one of a number of candidate antigens may be responsible for triggering phagocytosis and thence granulomagenesis. The identification of such an antigen would be invaluable, albeit seemingly unlikely, in delineating the early events triggering the sarcoidosis granuloma.

1.5. Invariant natural killer T (iNKT) cells

1.5.1. Background

iNKT cells are a unique subpopulation of T lymphocytes with immune regulatory properties that bridge innate and acquired immunity. They recognise glycolipid antigens presented by CD1d on a wide range of antigen presenting cells (APCs), including dendritic cells (DCs), monocytes, macrophages, B cells, thymocytes, adipocytes and hepatocytes. Upon activation they are able to produce a wide range of cytokines and chemokines, notably IL-4 and IFNγ. The term NKT cell was initially used to refer to any cell co-expressing natural killer (NK) cell antigens (NK1.1 in mice, CD56 and CD161 in humans) and a T cell receptor (TCR) derived from randomly rearranged TCR-α and TCR-β chains. This original definition of NKT cells is problematic because it does not define a T cell linage with unique phenotypical or functional attributes. Heterogeneity in NKT cell definition previously may, in part, explain some of the apparently conflicting data on NKT cell activity and function in both human and murine disease.

1.5.2. NKT subtypes

There are two undisputed classes of NKT cells, both of which are CD1d restricted (types I and II) (Figure 1.6). They both can play an important modulatory role in the induction and/or prevention of autoimmune disease, inflammation and cancer, which has been the focus of much recent work (196-201). Despite this, the precise location of exactly how and where the

NKT-APC interactions occur *in vivo*, and how they regulate the host immune response remains unclear (202).

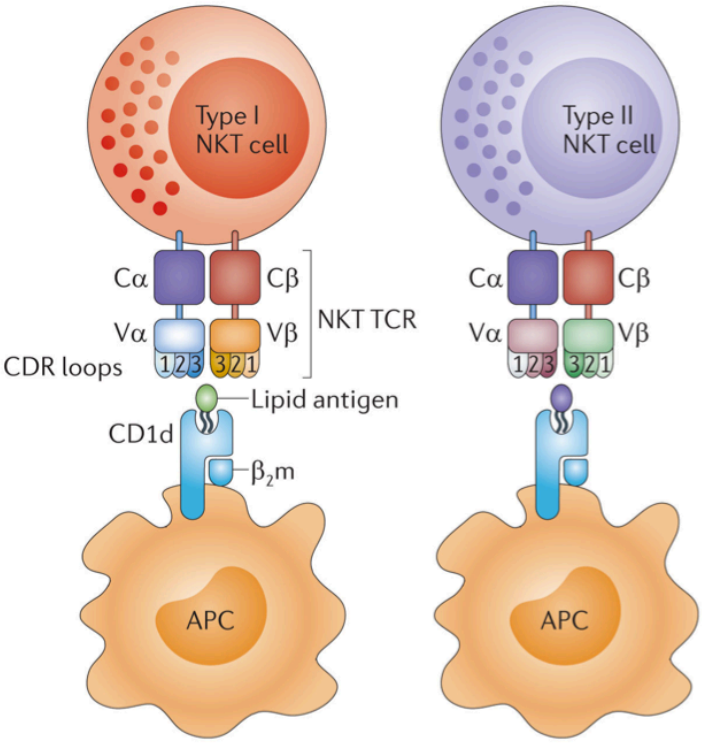


Figure 1.6. Type I (iNKT) and Type II NKT cells
From *Rossjohn et al. Nat Rev Immunol. 2012 (203).* The two subsets use different variable (V) region gene segments in the α- and β-chains of their T cell receptors (TCRs), and recognise different CD1d-restricted antigens.

Type I (iNKT), are defined by their invariant TCR-α chain (Vα14-Jα18 in mice, Vα24-Jα18 in humans), limited TCR-β repertoire (Vβ8.2, Vβ7 and Vβ2 in mice, Vβ11 in humans) and their reactivity with the exogenous marine-derived glycosphingolipid antigen α-galactosylceramide (αGC) (204). They are identified and isolated through their ability to recognise αGC-loaded CD1d multimers (204-206). The 6B11 monoclonal antibody, which recognises the invariant CDR3 region of the Vα24-Jα18 human NKT TCR also identifies iNKT cells with excellent specificity (207, 208). It has been hypothesised that, while

iNKT cells can promote both pathogenic and regulatory immune responses, they are more frequently pathogenic (202).

Type II or non-classical NKT cells, whilst being CD1d restricted, do not react with αGC and do not express the invariant TCR that characterises type I NKT cells; instead they have a semi diverse TCR with some bias (in mice Va3.2-Ja9,Va8/Vb9). Type II NKT cells are hypothesised to be predominantly inhibitory and protective (202). Type II NKT cells may recognise sulfatide, a naturally occurring self-antigen, enriched in membranes found in myelin in the CNS, in the pancreas, kidney and liver. Sulfatide-reactive type II NKT cells express oligoclonal TCRs by utilisation of a limited number of Vα- and Vβ-chains. They have been shown to be protective against the inflammatory response induced by iNKT cells in autoimmune diseases (209, 210) although it has been suggested that non-sulfatide reactive type II NKT cells may play a pathogenic role in other diseases, such as ulcerative colitis (211). In comparison to iNKT, where the TCR is encoded predominantly by a germline Vα gene (75-88%), in type II NKT cells, only about 14% of the TCR Vα are encoded by germline gene segments (212). The TCR Vβ chains of both iNKT and type II NKT are more diverse and predominantly non-germline encoded. Type II NKT TCRs engage their ligands via their β chain rather than α chain, suggesting that the TCR Vβ chain contributes significantly to antigen fine specificity (213).

One (possibly two) further group(s) of NKT-like cells possessing both a TCR and NK1.1 have been described in mice (204, 214), but they are not CD1d

restricted and have a diverse TCR which interacts with MHC molecules. The identity of these cells, which have been described as type 3 (and type 4) NKT cells is uncertain and their function and role in immunity are poorly understood.

Since the identification of NKT cells, a further group of T cells with a restricted TCR has been described: mucosal associated invariant T cells (MAITs) (215). MAITs account for 1-10% of peripheral T cells (216) and preferentially reside in the gut. They possess a semi-invariant TCR (Vα7.2-Jα33 in humans and Vα19-Jα33 in mice) with a limited TCR-β repertoire (Vβ2 and Vβ13 chains in humans and Vβ6 and Vβ8 chains in mice) (204), express NK lineage markers such as NK1.1 (CD161), produce a broad range of cytokines (including IFNγ, IL-4, IL-5 and IL-10) upon activation they are evolutionarily conserved between mice and humans to the extent that both cell types show cross-species reactivity. These similarities and the suggestion that they respond to a limited repertoire of glycolipid antigen (216) has led to them being dubbed by some as mucosal NKT cells (mNKT), although it is currently accepted that they are a distinct, non NKT population.

In humans, the frequency of iNKT cells is variable, though stable within individuals over time, accounting for between 0.1% and 0.2% of T cells. This proportion is highly variable and can range from < 0.1% to > 2% (207, 217, 218). Twin studies suggest that the number of iNKT cells in peripheral blood monocytes (PBMCs) is genetically regulated (219). Similar frequencies are seen in the bone marrow and spleen (61, 218, 220). The frequency of iNKT

cells is lower in the thymus (0.001–0.01% of lymphocytes), whilst being enriched in the omentum (about 10% of T cells) but not in the liver (221-224). In mice, iNKT cells constitute 0.2–0.5% of lymphocytes (and 0.5–2% of total T cells) in the thymus, spleen, blood and bone marrow, and 15–35% of liver lymphocytes (30–50% of liver T cells) (208). Humans have higher frequencies than mice of other innate and innate-like T cells, such as type II NKT cells, MAITs, CD1a-, CD1b- and CD1c-restricted T cells and it is possible that these cell types might compensate functionally for the comparatively smaller number of NKT cells in humans.

Human iNKT cells can be divided into functionally distinct subsets: $CD4^+CD8^-$, $CD4^-CD8^-$ and a third, $CD4^-CD8^+$ that is not found in mice. Human $CD4^+CD8^-$ iNKT cells are broadly associated with T_H0-type immune responses, whereas $CD4^-$ NKT cells have a T_H1 cell-type phenotype (217, 218, 225, 226). There is increasing evidence for the existence of additional subsets, with distinct functions and cytokine profiles.

1.5.3. iNKT cell activation

iNKT cells can be activated both directly, via exogenous microbial or endogenous lipid antigen presented by CD1d, and indirectly without the need for TCR binding. The direct pathway is CD1d-dependent and involves engagement of the NKT TCR. αCG, the prototypic NKT cell ligand, derived from a marine sponge, binds with very high avidity to CD1d and is an extremely strong agonist and therefore studies using αCG may not reflect the true physiological responses to iNKT activation. Most microbial lipids and

other self-antigens, including isoglobotrihexosylceramide, or isogloboside 3 (iGB3), do not effectively stimulate iNKT cells. More physiological and perhaps, more pathologically relevant glycolipid antigens have been isolated from a number of infective agents including *Sphingomonas* spp, *Borellia burgdoferi* (αGalDAG), *Erlichia* spp, and *Streptococcus pneumoniae* (αGlcDAG) (227-229).

Three distinct pathways may drive indirect activation. The first requires both cytokine presence (IL-12, IL-18) and an as yet undetermined endogenous glycolipid antigens; although β-D-glucopyranosylceramide (β-GlcCer) has been recently shown to be an example (230). During *Salmonella typhimurium* infection, lipopolysaccharide (LPS) stimulates Toll-like receptors (TLRs) on dendritic cells (DCs) and induces IL-12 release. iNKT cells are then activated by the combination of IL-12 (produced by LPS-stimulated DCs) and endogenous antigen presented by CD1d. Other self-lipids recognised to activate iNKT cells include lysophosphatidylethanolamine (LPE) and lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC) and lysosphingomyelin (LSM) (231). A second indirect pathway does not require TLR-mediated DC activation, but does rely on DC recognition of an endogenous antigen. Thirdly, it has also been shown that iNKT cells can be activated without TCR engagement through IL-12 and IL-18, or IL-12 and IFNγ production by APCs (232-235). The activation of iNKT cells is summarised in Figure 1.7.

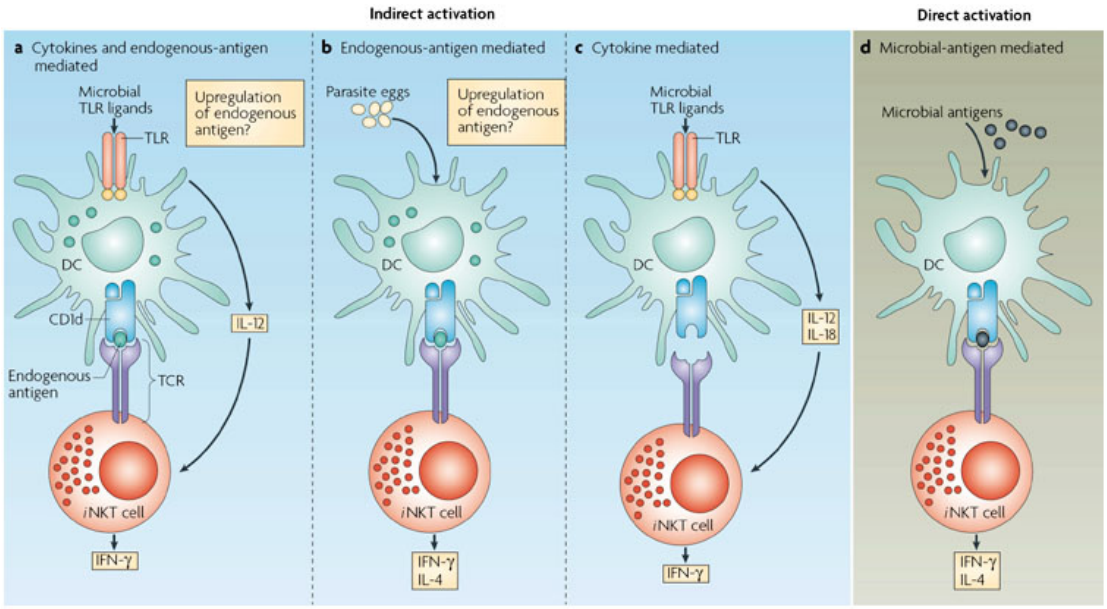


Figure 1.7. Direct and indirect pathways can activate iNKT cells. From *Tupin et al. Nat Rev Microbiol. 2007* (232).

Upon activation iNKT cells become potent secretors of a wide range of cytokines, depending on iNKT cell type, polarisation, mode and strength of stimulation. These include: IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21 and TNFα, IFNγ and GMCSF. iNKT cells also participate in perforin-, CD95-CD95L- and TNF-induced cytotoxicity and may also express granulysin. Peripheral blood iNKT cells have a far greater capacity for cytokine production than the more immature thymic iNKT cells (208).

1.5.4. iNKT cells and human disease

iNKT cell defects have been implicated in the pathogenesis of certain cancers, as well as number of inflammatory, autoimmune and infectious conditions (208). The currently known associations between iNKT cells and disease are summarised in Figure 1.8.

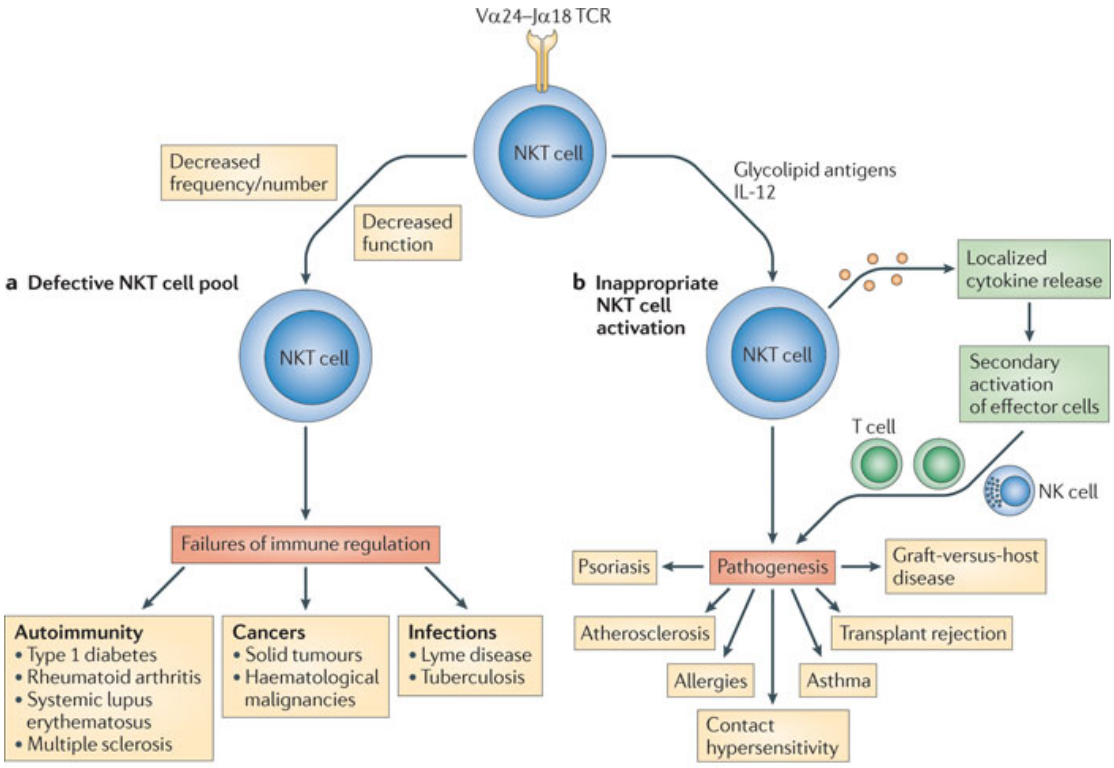


Figure 1.8. iNKT cell defects in number and activation are associated with disease.
From *Berzins et. al. Nat Rev Immunol. 2011 (208).*

iNKT defects are thought to cause failures of immune regulation and deficiencies in numbers and function have been shown in type 1 diabetes (T1DM), multiple sclerosis (MS), rheumatoid arthritis (RA), systemic lupus erythematosus (208, 236-239) and Sjogren's syndrome. Work from my supervisor's group and others, have shown that iNKT cells are deficient in number in sarcoidosis but the functional consequence of this remains unclear (61, 62). Interestingly, individuals with myasthenia gravis patients have increased iNKT cells frequency, in contrast to those with MS, RA and T1DM. The reason for these differences is unclear, but it has been shown that in MG, iNKT cell levels return to normal after treatment (240). In sarcoidosis, iNKT cells levels do not appear to return to normal, despite treatment, over a two

year follow up period (61). The most frequently cited association between iNKT number and disease is that between T1DM and iNKT cell deficiency. Diabetes-prone non-obese diabetic (NOD) mice have defects in iNKT cell frequency and cytokine production; the development of diabetes in these mice can be prevented by adoptive transfer of iNKT cells, overexpression of the iNKT cell TCR or stimulation of iNKT cells with αGC. In addition, the rate of diabetes is higher in iNKT cell-deficient (*Cd1d*$^{-/-}$ or *Ja18*$^{-/-}$) NOD mice compared with wild-type NOD mice (241-244). It is important to consider however, that the frequency of iNKT cells in the peripheral blood of NOD mice is actually normal, despite there being significant deficiencies in other tissues. Human studies of iNKT cells in type 1 diabetes are conflicting (208). iNKT cells have a key role in host defence against infection mediated by a wide range of microorganisms including influenza A, streptococcus pneumonia, *pseudomonas aeruginosa* and *Borrelia burgdorferri* (139, 245-249). The evidence to suggest that the activation of iNKT cells is protective far outweighs the evidence that their absence is detrimental. iNKT cells also modulate the immune response in both haematological and solid tumours (250, 251). Differences in iNKT cell definition between studies may account for conflicting findings in iNKT study and further characterisation of human iNKT cells is required.

1.5.5. iNKT and APC cross-talk

Surprisingly little is known about the dynamics of iNKT-APC interaction *in vivo*, although recent advances in intravital imaging techniques now enable these interactions to be studied (252, 253). Whilst DCs are the major APC to

interact with iNKT after lipid antigen stimulation, other APCs are also involved. The DC-iNKT interaction often, but not exclusively, occurs in the regional lymph nodes and is bidirectional. For example, during infection, pathogen-derived signals stimulate DCs to produce IL-12 and enhance their production of lipid antigens that stimulate iNKT cells. These activated iNKT cells then recognise self or foreign lipid antigens presented by CD1d on DCs. Upon ligation of CD40 to CD40 ligand, the iNKT cells in turn induce further IL-12 production by DCs, which binds to IL-12R on the iNKT cells and further activates them. Given that the location of most iNKT-APC interaction is unknown, it would be interesting to compare the location, time and strength of interactions between APCs and iNKT cells. The technique could also be used to answer other answered questions such as whether iNKT cells (i) resume circulation after interaction, (ii) how they home to the site of pathology or (iii) if undergo proliferation following APC encounters.

Monocytes constitutively express CD1d and have a similar chemokine receptor expression pattern as iNKT cells, suggesting that they may co-localise in vivo (138, 226, 254) and there is growing evidence in murine and in vitro models to support iNKT-monocyte cross-talk. Work from the group has previously shown that monocytes accumulate at the site of pathology in iNKT-deficient murine models of both influenza A (139) and multiple sclerosis (EAE, experimental autoimmune encephalomyelitis) (140). iNKT cells have been shown to demonstrate CD1d-dependent killing of $CD68^+$ tumor-associated monocytes/macrophages (TAMs) in NOD/SCID mice (255). iNKT cells have also been shown to instruct human peripheral blood monocytes, via GM-CSF

and IL-13, to differentiate into myeloid APCs (immature DCs) (256). DCs that resulted from iNKT cell interactions with monocytes appear to have a non-inflammatory phenotype regardless of whether the iNKT cells were activated by self-antigens or by α-GalCer. This suggests that iNKT cells can also expand the tolerogenic DC population by recruiting monocytic progenitors into the DC lineage.

1.6. Project aims

Our understanding of the immune pathology driving sarcoidosis remains heavily biased towards the hyperactive T_H1 response and the activity of the highly specialised, long-lived alveolar macrophages. Remarkably little is known about the activity of monocytes in sarcoidosis, potentially key upstream players in the immune pathology of the disease. Monocyte number is increased in sarcoidosis but the specific implication of this is unclear. Is their function altered in the disease? In this thesis, I focus on monocyte cytokine production, suppressive activity and granulomagenesis. Additionally, the group has previously demonstrated a deficiency of iNKT cell number in both acute and resolved sarcoidosis (61, 62), the loss of which may contribute to immune pathology and I therefore explore the interaction between iNKT cells and monocytes in sarcoidosis.

Gene expression and quantitative proteomic profiling work in sarcoidosis has thus far focussed primarily on whole blood, PBMCs and AMs (257-260). Gene expression profiling in healthy monocytes has helped illuminate inflammatory

and polarisation signatures (261-263) and monocyte genetic profiling in sarcoidosis has the potential to suggest abnormal function.

The specific objectives of this work are detailed at the start of each chapter, however, the overall aim of the project was to characterise the phenotype and functional profile of monocytes in sarcoidosis and to explore the implications of any anomalies found. My objectives were therefore to:

1. Characterise monocyte phenotype and functional profile in sarcoidosis monocytes compared with healthy controls
2. Explore the relationship between abnormalities in monocyte function and iNKT cell loss in sarcoidosis
3. Investigate how any anomalous function determined may be modulated
4. Interrogate monocyte gene expression profiling in sarcoidosis.

A more complete understanding of monocyte biology in sarcoidosis may help identify pathways that contribute to early immune pathology of the disease. The identification of abnormal monocyte activity prior to localised inflammation and granulomagenesis could illuminate new potential disease targets, the peripheral accessibility of the monocyte facilitating potential therapeutic modulation.

Chapter 2: Materials and methods

2.1. Patient and control and recruitment

Patients were recruited from the Sarcoidosis-ILD service at the Oxford department for respiratory medicine. Controls were recruited through local advertising in accordance with local R&D policy and national ethics approval: REC 10/ H0505/ 70 (Berkshire research and ethics committee). All participants were non-smokers and not receiving treatment for sarcoidosis (corticosteroids or other immune modulatory therapy) at the time of sampling. Patient demographics are supplied in the methods summary at the start of each chapter.

2.2. Laboratory methods

2.2.1. Media

Name	Composition
R0	RPMI-1640 (Sigma) + 2mM 1-glutamine, 50µg/ml penicillin (PAA) + 50µg/ml.
R10	R0 + 10% sterile filtered heat inactivated FCS (Sigma).
H10	R0 + 10% HS (First Link UK [HUT5726/ HUT5141]).
X5/ 10	X-VIVO 10 (Lonza) + 5% / 10% HS (pooled de-complemented serum from recruited donors)

Table 2.1. Media used in experiments

2.2.2. Isolation of human peripheral blood mononuclear cells (PBMCs)

To avoid activation of monocytes by density gradient preparations (264), peripheral blood mononuclear cells were prepared from fresh whole blood through erythrocyte lysis. Blood was collected into tubes containing sodium heparin (≥1U/ml) as an anticoagulant (Greiner Bio-One) or into 50ml polypropylene tubes (BD Falcon) prepared with 100μl (1000U/ml) of sodium heparin (Wockhardt). This was then mixed with an equal volume of RBC lysis solution (Qiagen) and incubated at room temperature (RT) for 15 minutes. The cells were then washed with R0 and centrifuged at 1200 rpm for 10 minutes. The cells were then resuspended in half the volume of RBC lysis buffer and incubated at RT for 5 minutes. The cells were then washed twice more in R0 and re-suspended in R10 ready for use.

Where monocyte activation was not a concern, mononuclear cells were separated from whole blood using standard density gradient centrifugation. Whole blood, obtained as above, was diluted 1:1 with R0 and a maximum of 25mls of the diluted blood was layered onto 15mls of Lymphoprep™ solution (AXIS-SHIELD). This was centrifuged at 2000 rpm for 20 minutes with no brake and the interface containing PBMCs harvested with a Pasteur pipette. The cells were washed in R10 and centrifuged at 1600 rpm for 15 minutes, with two further washes undertaken with subsequent centrifugation occurring at 1200 RPM.

To count the cells, they were resuspended in R10 and diluted 1:1 in 0.4% Trypan blue solution (Sigma), loaded onto a FastRead disposable cell counting slide (Immune Systems, BVS100) and counted under a microscope (Nikon). Alternatively an automated cell counting machine (ABX Pentra 60) was used where cell subset differential counts were required.

2.2.3. Isolation of Monocytes

For the isolation of purified, untouched monocytes cells were negatively selected using magnetic MACS MicroBeads (Pan Monocyte isolation kit, Miltenyi Biotec) according to manufacturer's protocol. Single-cell suspensions were obtained. Up to 10^7 cells were incubated with 30µl MACS buffer (PBS pH7.2, 0.5% BSA and 2mM EDTA), 10µl of FcR Blocking Reagent and 10µl of Biotin-Antibody Cocktail at 4°C for 10 minutes. A further 30µl of MACS buffer and 20µl of Anti-Biotin MicroBeads were added prior to further incubation for 15 minutes at 4°C. The cells were washed once, re-suspended in 500µl MACS buffer, passed through a 30µm filter for use with an LS column. Where monocytes were used to generate DCs, MACS CD14 positive selection was used (Miltenyi Biotec) from density gradient separated blood. For positive selection, up to 10^7 cells were incubated with 80µl of MACS buffer and 20µl of CD14 MicroBeads 4°C for 15 minutes. The cells were washed once, re-suspended in 500µl MACS buffer, passed through a 30µm filter for use with an LS column.

Pan monocyte kit:
(http://www.miltenyibiotec.com/~/media/Images/Products/Import/0001900/IM0001975.ashx).
CD14 isolation kit:
https://www.miltenyibiotec.com/~/media/Images/Products/Import/0001200/IM0001260.ashx

2.2.4. Isolation of $CD3^+$ T cells

$CD3^+$ cells were isolated magnetically using $CD3^+$ microbead positive selection ($CD3^+$ T cell isolation kit, Miltenyi Biotec) according to manufacturer's protocol. Single-cell suspensions were obtained. Up to 10^7 cells were incubated with 80µl of MACS buffer and 20µl of CD3 MicroBeads 4°C for 15 minutes. The cells were washed once, re-suspended in 500µl MACS buffer, passed through a 30µm filter for use with an LS column.

CD3 isolation kit:
http://www.miltenyibiotec.com/~/media/Images/Products/Import/0001200/IM0001261.ashx

2.2.5. Generation of iNKT cell clones and lines

The iNKT22 cell clone was generated as previously described (265). In brief, $V\alpha24^+$ cells from the PBMCs of a healthy donor were isolated by MACS separation, seeded onto 96-well plates, stimulated with PHA in RPMI-1640 supplemented with 10% human serum (HS; First Link). IL-2 (Peprotech) was

added to the medium on day 3. After 15-20 days, $V\alpha24^+ V\beta11^+$ and CD1d/α-GalCer tetramer$^+$ T cells were further expanded by PHA and allogenic irradiated feeders. Feeders were irradiated with 3300 rads using the irradiator in the Nuffield Department of Surgery in the John Radcliffe Hospital.

An additional line was generated from iNKT cells isolated directly into 5ml polypropylene tube (BD Falcon, lines, 1000 cells per tube) by FACS sorting (see below), using 6B11 and CD3 to identify cells. These were then cultured and expanded and phenotyped as previously described 2004 (265).

2.2.6. Cryopreservation of PBMCs and iNKT clones and lines

PBMCs and iNKT cells were pelleted by centrifugation at 1500 rpm for 5 minutes, the supernatant removed and cells resuspended at 5-10x10^6 cells/ml in freezing media (10% dimethyl sulphoxide [Sigma] in FCS). Cells were frozen in 1ml aliquots in labelled cryovials (Alpha laboratories) and transferred to storage at -80°C in a freezing container (Nalgene). For long-term storage cells were transferred to liquid nitrogen.

2.2.7. Thawing of PBMCs and iNKT cells

PBMCs and iNKT cells were thawed by placing cryovials in a water bath at 37°C for rapid thawing. They were then transferred to a 15ml Falcon tube (BD) and washed twice in R10.

2.2.8. Flow cytometric studies

2.2.8.1. Surface antigen staining

Table 2.2 lists the antibodies and Table 2.3 lists the isotype controls used during the project. Cells were incubated for 20 minutes at 4°C in the dark with fluorochrome conjugated monoclonal antibodies (mAb) in 96 well plates at $0.25x10^6$ - $0.5x10^6$ cells in FACS buffer (10% foetal calf serum, 1% bovine serum albumin in PBS). All mAbs were optimised for flow cytometry, appropriate concentrations of antibodies were determined by staining $0.5x10^6$ cells with different concentrations of mAb. Cells were washed twice and resuspended in FACS buffer. To exclude dead cells, cells were also stained with 30µl of a 1 in 1000 dilution of 10mg/ml Hoechst (Sigma) or (ViVid violet live/dead stain; Invitrogen) for 15min at RT before washing in FACS buffer. Samples were resuspended in 400µl FACS buffer + 1% formaldehye (Sigma), unless Hoechst was used in which case the formaldehyde was omitted. Samples were analysed on a 3 laser, 9 colour, CyAn™ ADP Flow Cytometers using Summit 4.3™ (both Beckman Coulter, DAKO, Cambridgeshire, UK).

2.2.8.2. Intracellular cytokine staining (ICS)

Brefeldin A (Sigma) was added to each well after 14 hours of an 18-hour stimulation period. Surface antigens were stained as described above, after which the cells were fixed in 1% formaldehyde (Sigma) for 15 minutes at RT. Cells were then washed and resuspended in 50µl of 0.5% Saponin/PBS (Sigma) with conjugated mAbs. Cells were then washed twice, fixed as described, resuspended in FACS buffer and analysed by flow cytometry.

Antibody	Fluorochrome	Clone	Isotype	Supplier	Catalogue number
Anti-6B11	APC	6B11	Mouse IgG1κ	eBioscience	17-5806-42
Anti-CCR2	Alexa Fluor® 647	48607	Mouse IgG2b	BD Pharmingen	558406
Anti-CCL2	APC	5D3-F7	Mouse IgG1κ	eBioscience	17-7099-81
Anti-CCL2	PE	2H5	Armenian Hamster IgG1	eBioscience	12-7096-41
Anti-CD1d	PE	CD1d42	Mouse IgG1κ	BD Pharmingen	550255
Anti-CD3	APC-e780	UCHT1	Mouse IgG1κ	eBioscience	47-0038-42
Anti-CD3	eFluor® 450	OKT3	Mouse IgG2a	eBioscience	48-0037-42
Anti-CD4	PECy7	SK3	Mouse IgG1κ	eBioscience	25-0047-42
Anti-CD8	eFluor® 450	RPA-T8	Mouse IgG1κ	eBioscience	48-0088-42
Anti-CD8a	FITC	RPA-T8	Mouse IgG1κ	eBioscience	11-0088-42
Anti-CD11b	PE	ICRF44	Mouse IgG1κ	eBioscience	12-0118-42
Anti-CD14	APC	61D3	Mouse IgG1κ	eBioscience	17-0149-42
Anti-CD14	eFluor® 450	61D3	Mouse IgG1κ	eBioscience	48-0149-41
Anti-CD14	PECy7	61D3	Mouse IgG1κ	eBioscience	25-0149-42
Anti-CD15	eFluor® 450	HI98	Mouse IgGM1κ	eBioscience	48-0159-42
Anti-CD16	eFluor® 450	CB16	Mouse IgG1κ	eBioscience	48-0168-42
Anti-CD16	FITC	CB16	Mouse IgG1κ	eBioscience	11-0168-42
Anti-CD32	APC	6C4	Mouse IgG1κ	eBioscience	17-0329-42
Anti-CD33	eFluor® 450	P67.6	Mouse IgG1κ	eBioscience	48-0337-42
Anti-CD36	eFluor® 660	NL07	Mouse IgM	eBioscience	
Anti-CD62l	APC	DREG-56	Mouse IgG1κ	eBioscience	17-0629-41
Anti-CD62l	eFluor® 450	DREG-56	Mouse IgG1κ	eBioscience	48-0629-41
Anti-CD62l	PE	DREG-56	Mouse IgG1κ	eBioscience	12-0629-42
Anti-CD69	PE	FN50	Mouse IgG1κ	eBioscience	12-0699-42
Anti-CD80	PE	2D10.4	Mouse IgG1κ	eBioscience	12-0809-41
Anti-CD86	PE	IT2.2	Mouse IgG2b	eBioscience	12-0869-42
Anti-CD115	FITC	61708	Mouse IgG1κ	R&D	FAB329F
Anti-CD115	PE	61708	Mouse IgG1κ	R&D	FAB329P
Anti-CD163	PE	GHI/61	Mouse IgG1κ	eBioscience	12-1639-42
Anti-CD206	eFluor® 450	19.2	Mouse IgG1κ	eBioscience	48-2069-42
Anti-CX3CR1	FITC	2A9-1	Rat IgG2b	Biolegend	341606
Anti-Dectin-1	PerCP-eFluor® 710	15E2	Mouse IgG2a	eBioscience	46-9856-41
Anti-eCadherin	488	DECMA-1	Rat IgG1κ	eBioscience	53-3249-80
Anti-FoxP3	eFluor® 450	236A/E7	Mouse IgG1κ	eBioscience	48-4777-41
Anti-HLADR	APC	LN3	Mouse IgG2b	eBioscience	17-9956-42
Anti-HLADR	PE	LN3	Mouse IgG2b	eBioscience	17-9956-42
Anti-IFNγ	APC	4S.3B	Mouse IgG1κ	eBioscience	17-7319-82
Anti-IFNγ	eFluor® 450	4S.3B	Mouse IgG1κ	eBioscience	48-7319-42
Anti-IL-4	PE	8D4-8	Mouse IgG1κ	eBioscience	12-7049-42
Anti-IL-6	Alexa Fluor® 700	MQ2-13A5	Rat IgG1κ	eBioscience	56-7069-41
Anti-IL-10	Alexa Fluor® 647	JES3-9D7	Rat IgG1κ	eBioscience	50-7108-42
Anti-IL-10	eFluor® 660	JES3-9D7	Rat IgG1κ	eBioscience	50-7108-42
Anti-IL-10	eFluor® 450	JES3-9D7	Rat IgG1κ	eBioscience	48-7108-42

Anti-IL-10	PE	JES3-9D7	Rat IgG1κ	eBioscience	12-7108-82
Anti-IL12 p35	Alexa Fluor® 647	C8.6/HP 40	Mouse IgG1κ	eBioscience	51-7359-42
Anti-IL-17A	PE	eBio64DEC17	Mouse IgG1κ	eBioscience	12-7178-42
Anti-IL-17F	PE	SHLR17	Mouse IgG1κ	eBioscience	12-7169-41
Anti-IL17F	PECy7	SHLR17	Mouse IgG1κ	eBioscience	25-7169-42
Anti-PD1	PE	J105	Mouse IgG1κ	eBioscience	12-2799-42
Anti-TLR2	eFluor® 450	T2.5	Mouse IgG1κ	eBioscience	48-9024-82
Anti-TLR4	Alexa Fluor® 488	HTA125	Mouse IgG2a	eBioscience	53-9917-42
Anti-TNFα	eFluor® 450	MAb11	Mouse IgG1κ	eBioscience	48-7349-42
Hoescht	λ_{ex} ~355 nm, λ_{em} ~ 465 nm	N/A	N/A	Sigma	B 2261
Live/Dead fixable stain	Violet	L34955	N/A	Invitrogen	N/A

Table 2.2. Flow cytometry antibodies used

Antibody	Fluorochrome	Clone	Supplier	Catalogue number
Armenian Hamster IgG1	PE	eBio299Arm	eBioscience	12-4888-81
Mouse IgG1κ	APC	P3.6.2.8.1	eBioscience	17-4714-41
Mouse IgG1κ	APCe780	P3.6.2.8.1	eBioscience	47-4714-82
Mouse IgG1κ	eFluor® 450	P3.6.2.8.1	eBioscience	48-4714-80
Mouse IgG1κ	FITC	P3.6.2.8.1	eBioscience	11-4714-81
Mouse IgG1κ	FITC	11711	R&D	IC002F
Mouse IgG1κ	PE	MOPC-21	BD Pharmingen	554680
Mouse IgG1κ	PE	P3.6.2.8.1	eBioscience	12-4714-41
Mouse IgG1κ	PE	11711	R&D	IC002P
Mouse IgG1κ	PECy7	P3.6.2.8.1	eBioscience	25-4714-41
Mouse IgM1κ	eFluor® 450	11E10	eBioscience	48-4752-80
Mouse IgGM κ	eFluor® 660	11E10	eBioscience	50-4752-80
Mouse IgG2a	Alexa Fluor® 488	eBM2a	eBioscience	53-4724-80
Mouse IgG2a	eFluor® 450	eBM2a	eBioscience	48-4724-80
Mouse IgG2a	PerCP-eFluor® 710	eBM2a	eBioscience	46-4724-8
Mouse IgG2b	Alexa Fluor® 647	27-35	BD Pharmingen	557903
Mouse IgG2b	APC	eBMG2b	eBioscience	17-4732-41
Mouse IgG2b	PE	eBMG2b	eBioscience	12-4732-41
Rat IgG1κ	Alexa Fluor® 488	eBRG1	eBioscience	53-4301-80
Rat IgG1κ	Alexa Fluor® 647	eBRG1	eBioscience	50-4301-80
Rat IgG1κ	Alexa Fluor® 700	eBRG1	eBioscience	56-4301-80
Rat IgG1κ	eFluor® 660	eBRG1	eBioscience	50-4301-80
Rat IgG2b	FITC	RTK4530	Biolegend	400633

Table 2.3. Isotype controls used for flow cytometry

2.2.8.3. Imagestream™

To determine monocyte and NKT cell co-localisation, monocytes were incubated with NKT clone at a ratio of 1:1 in 96 well round bottomed plates for 18 hours; for monocyte phagocytosis studies, monocytes were treated as described below. The cells were then stained as described for surface staining above and data for morphology and co-localisation acquired on an Amnis Imagestream flow cytometer and analysed using IDEAS® analytical software (https://www.amnis.com/ideas.html).

2.2.9. Enzyme-linked immunosorbent assay (ELISA)

Cytokine concentration in the culture supernatant was measured by ELISA. Each wash step included 5 washes in PBS+ 0.05% Tween-20 (Sigma-Aldrich) using the Wellwash plate washer (Sigma-Aldrich).

For IL-10 ELISA, 96 well plates (Maxisorp immunoplate, Nunc-Thermofischer) were coated overnight with antibody coating buffer (2µg/ml of capture antibody in 0.1M $NaHCO_3$; BD Pharmigen Purified Rat anti-human and viral IL-10). The plates were washed before blocking with 10% FCS for 1 hour at 37°C. Samples were then added at serial dilutions and incubated overnight at 4°C. Further wash steps were performed prior to 1-hour incubation at RT with 2µg/ml of detection antibody (BD Pharmingen Biotin anti-human and viral IL-10).

IL-4, IL-12, TNFα and CCL2 were measured using Human ELISA Ready-SET-Go! (eBioscience) kits. 96 well flat bottom plates (Maxisorp immunoplate,

Nunc-Thermofischer) were coated with 100μl/ well of capture antibody and incubated overnight at 4°C. Plates were washed as above and blocked for one hour with assay diluent. Standards were prepared with the top standard at a concentration of 500pg/ml. The top standard was double diluted to give a 7-point curve, ranging from 500pg/ml to 7.81pg/ml. A further wash step was performed before the addition of undiluted samples which were incubated overnight at 4°C. 100μl/ well of detection antibody was added after a further wash step and incubated for one hour at RT.

In all cases, at this stage, a further wash step was performed before 30-minute incubation at RT with 0.001% avidin-HRP. A further wash step was then performed before addition of 3,3',5,5' tetramethylbenzidine. The reaction was quenched with 2M sulphuric acid (Fisher Scientific) and the plates read at 450nm on a microplate reader (Bio-Rad, Model 6800) using the microplate manager 5.2.1 software. Files were exported to Microsoft Excel for analysis.

IL-4: http://www.ebioscience.com/media/pdf/tds/88/88-7046.pdf

IL-12: http://www.ebioscience.com/media/pdf/tds/88/88-7126.pdf

TNFα: http://www.ebioscience.com/media/pdf/tds/88/88-7346.pdf

CCL2: http://www.ebioscience.com/media/pdf/tds/88/88-7399.pdf

2.2.10. Multiplex bead analysis

Multiplex-cytokine detection kits for human IL-4, IL-12, IL-13, IL-17A, CCL2 (MCP-1) and TNFα were obtained from BioRad. The assay was performed according to the manufacturer's instructions:

http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10014905.pdf

Briefly, wells were pre-wet and 50μl of the cytokine bead mix (preprepared by manufacturer, thoroughly mixed at bench prior to use) were added to each well and washed twice. Standards were prepared by four fold dilutions of a premixed standard. 50μl of standards, samples or blanks of media were added to appropriate wells and incubated on a plate shaker at room temperature in the dark for two hours. The plate was then washed three times and incubated for another thirty minutes with 50μl of detection antibody. After washing, plates were incubated for 10 minutes with 50μl of streptavidin-HRP. A final wash step was performed and the plates read immediately on the Bio-Plex™ 200 System (Bio-Rad) using Bio-Plex manager software version 5.0. Files were exported to Microsoft Excel for analysis.

2.2.11. Monocyte-iNKT co-culture assays

Untouched monocytes were incubated with iNKT clone at a ratio of 1:1 (250 000 cells total) in 96 well round bottomed plates for 18 hours. Where ICS was to be performed Brefeldin A was added as above. Cell ratios, concentrations and incubation times determined and optimised by preliminary studies.

2.2.12. Generation of monocyte-derived DCs

Monocytes were isolated using MACS CD14 positive MACS selection as described above. They were plated at a concentration of $4x10^5$/ml in 6 well plates with 100ng/ml Human recombinant IL-4 and 50ng/ml Human GM-CSF

(both Peprotech) and incubated at 37°C at 5% CO_2. DCs were used on day 5-8 after checking morphology and surface antigen expression (absence of CD3, CD14, presence of CD11c, CD80, CD86).

2.2.13. T cell proliferation assays

T cell proliferation was measured using a standard Carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen) fluorescence assay. Peripheral blood leucocytes (PBL) were prepared by MACS CD14 positive sorting as described above. The CD14 negative fraction, predominantly comprising T cells, were then stained with CFSE as per manufacturers protocol. Cells were washed in R0 and resuspended in warm PBS supplemented with 1% bovine serum albumin at a concentration of $1x10^6$/ml. 10mM of CFSE was added to the cell suspension, mixed thoroughly and incubated for 10min at 37°C in the dark. After incubation, 10mls of ice cold R10 was added to the cells prior to a 10 minute incubation on ice. The cells were then washed four times with R10 and a small aliquot of cells removed to check by flow cytometry that CFSE labeling has been successful before use.

To induce proliferation, $2x10^5$ CFSE labeled cells were incubated with 5×10^4 monocyte-derived DCs were plated in round-bottomed 96 well plates. After 120 hours of co-culture at 37°C in 5% CO_2, CFSE fluorescence was measured using a flow cytometer. Reduction in CFSE staining intensity indicates that cells have divided.

Alternatively, $2x10^5$ PBLs were plated in triplicate without CFSE and were incubated with 5×10^4 monocyte derived irradiated DCs as above. Triplicates per condition, leave space between each condition. After 120 hours of co-culture at 37°C in 5% CO_2, 1uCi of tritiated thymidine [3H-Tdr] and 25µl media were added (total tritiated Thymidine (3H-TdR) = 30µlCi/1.1MBq per plate) and incubated for a further 15-18h in designated radiation incubator. The cells were then harvested cells on a filter mat and the amount of [3H]-Thymidine incorporated into the DNA measured with a scintillation counter.

IL-10 receptor blockade was titrated and 4µg of LEAF™ purified anti-human IL-10R (CD210) antibody (Biolegend) used ($2x10^5$ T cells). CD1d blockage was titrated and 10µg/ml added at the start of co-culture (anti-human CD1d, EBioscience).

2.2.14. IL-10 capture assay

IL-10 producing monocytes were enriched/ depleted using MACS IL-10 secretion assay, enrichment and detection kit (Miltenyi Biotec) according to manufacturer's instructions:
https://www.miltenyibiotec.com/~/media/Images/Products/Import/0001200/IM0001207.ashx

Briefly, isolated monocytes were stimulated overnight with 1µg/ml LPS. They were then labelled for 5 minutes on ice with IL-10 catch reagent [anti-IL-10 monoclonal antibody (mouse IgG1) conjugated to cell surface specific monoclonal antibody (mouse IgG2a)]. The cells were then incubated to allow

for IL-10 secretion at 37°C under slow continuous rotation. The cells were then labeled for 10 minutes on ice with IL-10 detection antibody (anti-IL-10 monoclonal antibody (mouse IgG1) conjugated to PE). The cells were then labeled with anti-PE MicroBeads and separated as described above.

2.2.15. Monocyte phagocytosis assay

Monocytes were CD14 negatively selected from Ficoll isolated PBMCs. These were then incubated for two hours with yellow-green carboxylate-modified polystyrene latex beads (Sigma) at a monocyte: to bead ratio of 1:20. The beads have a mean particle size of 1.0μm and fluorescence range of λ_{ex} ~470nm; λ_{em} ~505nm. The monocytes were then stained with anti-CD14 surface marker in APC. Dose and time-point finding optimisation were conducted using a Cyan flow cytometer for data acquisition and Summit for analysis as previously detailed. Final assays were acquired on the Amnis Imagestream flow cytometer and analysed using IDEAS® analytical software. Cells included in analysis were in focus, $CD14^+$ single cells.

2.2.16. Monocyte Fusion assay

Monocytes were CD14 negatively selected from Ficoll isolated PBMCs. These were then resuspended in X-10 (X-Vivo Medium + 10% decomplemented mixed human serum from 10 different control donors). These were then plated on Permanox chamber slides at a density of 50 000 or 10 000 monocytes per chamber in 400µl final volume. The media was partially changed (200µl) on day 4 and 20ng/ ml IL-4 added to ¾ chambers.

Cells were fixed and stained with hematoxylin and methylene blue (24-72h after IL-4). Where IL-10 or IL-10 blocking antibody used (both Peptrotech), these were titrated and added to the chambers (40ng/ml and 4ng/ml).

2.2.17. Fluorescence microscopy

The monocyte-fusion chamber slides were washed twice with pre-warmed PBS. The samples were then fixed in 3.75% methanol-free formaldehyde solution and then washed two further times. Extraction was performed in glass petri dishes with 0.1% Triton X-100 in PBS for 3-5 minutes and washed twice. The cells were then stained for 20 minutes with Alexa 488 conjugated phallotoxin (Invitrogen) (5µl of methanolic stock in 200ul PBS containing 1% BSA per chamber) at room temperature in a covered staining chamber. The cells were then washed twice with PBS and air dried after which the chambers were removed. Cells were then covered in VECTASHIELD ® Mounting Medium with DAPI and visualised by Zeiss Axiovert S100 microscope.

2.2.18. Microarray

2.2.18.1. Cell preparation

Monocytes were isolated from Ficoll isolated PBMCs using CD14 negative selection within two hours of venesection. Purity was determined by FACs to be ≥98% They were then washed, treated and stored immediately in 350µl of RLT buffer (Qiagen) at -80°C. RNA extraction was performed within two weeks of storage.

2.2.18.2. RNA extraction

RNA extraction was performed using the RNeasy mini kit (Qiagen) according to manufacturer's instructions:

http://www.qiagen.com/knowledge-and-support/resource-center/resource-download.aspx?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en

All buffers used were supplied with the kit. Briefly, samples in RLT were thawed on the bench and transferred onto a QIAshredder spin column and centrifuged at 13000 rpm for two minutes. Following centrifugation, 350μl of 70% ethanol was added to the sample before transfer to an RNeasy spin column. This was centrifuged for fifteen seconds at 10000 rpm and the flow-through discarded. The column membrane was then washed with 350μl RW1 wash buffer during a fifteen second centrifugation at 1000 rpm. DNAse incubation mix was prepared by adding 10μl DNase solution, prepared with RNA free water according to manufacturer's instructions) to 70ul RDD buffer. 80μl DNase I incubation mix was then transferred directly onto the RNeasy spin column membrane and incubated at room temperature for fifteen minutes. One further was with 350μl RW1 buffer with fifteen seconds centrifugation at 10000 rpm was conducted and immediately followed by two further washes with RPE buffer, each with 500μl RPE buffer. The first centrifugation was for fifteen seconds, the second for two minutes, both at 10000 rpm. The column was then dried by centrifugation at 13000 rpm for two minutes. 30μl of RNAse free water was transferred directly onto the column membrane, incubated for one minute and centrifuged for one minute at 10000rpm. This final wash step wash repeated and the RNA then divided into

separate aliquots for storage, nanodrop and high-resolution electrophoresis bioanalyser (Agilent) readings to minimise freeze-thaw of the samples. Nanodrop and Agilent readings were undertaken in order to assure sufficient quality and quantity of RNA for microarray analysis. Samples were then stored at -80°C prior to batch hybridisation to Illumina HT12 V4 Beadarrays by Karene Argoud at the Wellcome Trust Centre for Human Genetics, Oxford.

2.2.18.3. Microarray data analysis

A full description of the data preprocessing and analysis is given in chapter 7 (section 7.3.3-4). Briefly, after initial quality control steps, data was VSN normalised and a mean intensity was computed for each probeset. A Bayesian moderated t-test was applied to identify differentially expressed genes between sarcoidosis monocytes control monocytes, using the limma (linear models for microarray analysis) package from BioConductor (266). The Benjamini–Hochberg false discovery rate (FDR) procedure was then applied to correct for multiple testing (267). The inverse log of the absolute log2 fold change (FC) generated by limma was used to determine the FC between groups, with the direction of change indicated by the sign

Fold change in gene expression and adjusted p values the array were entered into the web based programme MetaCore™ (Thomson Reuters) in order to identify biological networks and Gene Ontology (GO) processes differentially represented in the dataset: (https://portal.gengo.com/cgi/data_manager.cgi#).

Gene-set analysis methods (GSEA) is a powerful method of testing the biological meaning of differentially expressed genes (268) and was performed using web-based software (www.broad.mit.edu/gsea/).

2.2.19. Quantitative PCR (qPCR)

2.2.19.1. Complementary DNA (cDNA) synthesis

1μl (100ng/μl) of random hexamers (Qiagen) were added to 1μg of RNA, RNase-free water was added to give a final volume of 12.5μl. This was incubated for 10 minutes at 70°C before being placed on ice. 7.5μl of mastermix (4μl 5x1st strand buffer, 2μl 0.1M DTT, 1μl 10mM dNTPs, 0.5μl Superscript II RT (Invitrogen) was then added. An additional sample was prepared without RT as a control. Samples were then incubated at 42°C for 60 minutes and the reaction then terminated by raising the temperature to 70°C for 15 minutes. cDNA was then stored at -20°C prior to RT-qPCR the following day.

2.2.19.2. RT-qPCR

The TaqMan® Gene expression assay (Applied Biosystems) was used. A mastermix was prepared on ice using: 5μl 2X TaqMan master mix (Applied Biosystems), 0.5μl of required primer/probe (listed in Table 2.4), 1.5μl RNase-free water. 3μl of cDNA template and 7μl master mix were plated in triplicate per sample (plus -RT and non template controls) on a 96 well plate. The plate was sealed and centrifuged at 1500rpm for 4 minutes. The amplification reactions were performed in a 7500 Fast Real-Time PCR system (Applied

biosystems) and consisted of one cycle of 95°C for 20 seconds then 40 cycles of 95°C for 15 seconds followed by 60°C for 60 seconds. The ΔΔCT method was used to determine the relative expression of the genes with GAPDH as the endogenous control.

Gene	Applied Biosytems ID	Entrez Gene ID
IL-1B	Hs01555410_m1	3553
IL-6	Hs00985639_m1	3569
IL-8	Hs00174103_m1	3576
GAPDH	Hs02758991_g1	2597

Table 2.4. Primers/ probes used for RT-qPCR

2.2.20. Analytical methods

All statistical analysis was performed using GraphPad Prism 5.01 (GraphPad, La Jolla, USA). Normality of data distribution was established using the D'Agostino & Pearson omnibus normality test. Where data was normally distributed, Student t test was used to compare data and presented as mean ± SEM. Where data were not normally distributed, the Mann-Whitney test was used to compare data sets and presented as median [inter quartile range]. To compare multiple, normally-distributed paired data sets simultaneously, a one-way ANOVA with Tukey multiple comparisons post-test analysis was used. Where multiple data sets were not normally distributed, Kruskal-Wallis with Dunn's multiple comparison post-test analysis was performed. A p value of less than 0.05 was considered significant. Error bars on graphs represent SEM unless otherwise stated.

Chapter 3: Monocyte phenotype in sarcoidosis

3.1. Introduction

Human monocytes can be divided on the basis of expression levels of the lipopolysaccharide (LPS) co-receptor CD14 and the Fcγ receptor III CD16, into three subsets: classical ($CD14^{++}CD16^{-}$), inflammatory (or intermediate, $CD14^{++}CD16^{+}$) and patrolling (or non classical, $CD14^{+}CD16^{++}$) (136, 269). In addition to CD14 and CD16, other markers, such as HLA-DR, CCR2, CD163, CD11b, CX_3CR1 and CD62L can be used in combination to distinguish the three human monocyte sub-populations (129, 270, 271).

Previous studies of immune pathology in sarcoidosis have predominantly focussed on the T cell compartment or the alveolar macrophage; the monocyte, by comparison has received little attention. Alveolar macrophages are a distinct population of lung-resident macrophages and may not entirely originate from the peripheral monocyte compartment (172); by focusing on them and assuming that their activity directly relates to monocyte activity, key insights into the early pathogenesis of the disease may have been overlooked. The specific activity of the peripheral monocyte in terms of cytokine production, phagocytosis and granulomagenesis remains to be determined.

Increased numbers of inflammatory monocytes are seen in sarcoidosis (188, 190) as well as in other immune mediated conditions such as rheumatoid

arthritis, type 1 diabetes mellitus and atherosclerosis (143, 272). The frequency of other monocyte markers in sarcoidosis has not been widely studied and little is known about the monocyte surface phenotype and functional anomalies, if any, in sarcoidosis.

The cytokine profile of both T cells and alveolar macrophages in sarcoidosis has been widely studied and TNFα in particular has been identified as being an important driver of immune activity in sarcoidosis (18, 273-275). The cytokine profile of peripheral monocytes in sarcoidosis is, less well understood.

3.2. Aim

The aim of this chapter was to investigate the surface marker and cytokine expression of monocytes from individuals with sarcoidosis compared with healthy controls in order to more fully determine their phenotype in the disease.

3.3. Methods

Individuals with sarcoidosis were recruited via the Sarcoidosis-ILD service at the Oxford Centre for Respiratory Medicine and controls through local advertising (REC 10/ H0505/ 70). All participants were non-smokers (with exception of a subset of the H1N1 influenza cohort), with no history of atopic or autoimmune disease and were not receiving corticosteroids or other immune modulatory therapy at the time of sampling. All participants were

Caucasian or of South Asian origin (with the exception of the H1N1 cohort, which additionally included persons of African and South East Asian origin). Study subject demographics for this chapter are given in Table 3.1.

	Sarcoidosis				Control			H1N1			
	# (#Ex Pulm/SA)	Age (SD)	Male	CAS (0/1/2/3/4)	# (SA)	Age (SD)	Male	# (SA/SEA/AF)	Age (SD)	Male	NS/ES/ CS
Fig 3.1b, Fig 3.3a, Fig 3.4a-b	18 (4/0)	45 (10.7)	8	(0/3/11/3/1)	18 (2)	32 (5.0)	11				
Fig 3.3b	23 (5/1)	46 (9.8)	10	(0/3/14/5/1)	23 (2)	31 (5.0)	12	23 (5/1/4)	40 (12.4)	10	(10/6/6)
Fig 3.4c	27 (6/1)	45 (10.1)	12	(1/3/16/5/2)	27 (3)	31 (5.0)	15				
Fig 3.4d	21 (5/0)	44 (10.7)	10	(0/3/12/3/2)	20 (1)	31 (5.0)	11				
Fig 3.5a-d & Fig 3.6a	18 (1/0)	38 (8.5)	8	(0/8/7/2/1)	18 (3)	31 (4.4)	7				
Fig 3.6b	9 (0/0)	42 (8.4)	4	(0/2/3/2/0)	9 (1)	30 (4.8)	7				
Fig 3.6c	12 (2/1)	45 (8.4)	5	(0/4/4/5/0)	12 (1)	32 (5.6)	8				
Fig 3.6d	6 (1/1)	42 (7.7)	3	(0/2/2/2/0)	6 (1)	31 (4.3)	4				
Fig 3.7a	11 (2/0)	43 (8.0)	6	(0/4/5/2/0)	11 (2)	32 (5.4)	5				
Fig 3.8b-c	10 (1/0)	45 (7.3)	4	(0/3/6/1/0)	10 (1)	32 (5.6)	6				
Fig 3.9b	25 (2/0)	46 (8.6)	12	(0/8/14/3/0)	25 (2)	31 (4.8)	8				
Fig 3.9a & Fig 3.10a	6 (0/0)	53 (5.8)	4	(0/3/2/1/0)	6 (1)	34 (5.1)	4				

Table 3.1. Donor demographics
Number of participants per figure indicated in table, number with extra-pulmonary involvement (Ex Pulm) or South Asian (SA) origin indicated in parenthesis. CAS indicates serum biomarker clinical activity score: Lymphocyte count $<1.0x10^9$/l = 1; $>1.0x10^9$/l = 0. Serum ACE: >100 U/l = 2; 55-100U/l = 1; <55 U/l = 0. IgG >13g/l = 1; ≤ 13g/l = 0. Maximum score = 4. The H1N1 influenza cohort additionally included (i) persons of South East Asian (SEA) and African (AF) origin (i) current smokers (CS) [never smokers (NS) and ex smokers (ES) in this cohort indicated].

Blood was collected directly into heparinised tube (LiHep), erythrocyte lysis was performed and monocytes were isolated by magnetic bead CD14 negative selection within four hours of venesection. Monocytes are extremely adherent to tissue culture plastic, a property previously widely used in order to isolate them from PBMCs. This inadvertent loss of monocytes may artificially reduce their number, especially when activated and expressing greater numbers of adhesion molecules, and may be an explanation for the under reporting of this subset in sarcoidosis. Therefore, in order to avoid monocyte loss to tissue culture plastic, whole blood was separated at room temperature

and, once isolated the monocytes were used immediately and maintained at 4°C. Surface antigen staining for flow cytometry was then undertaken directly for the ex vivo studies, or after 18 hours of *E. coli* LPS stimulation when in conjunction with intracellular cytokine staining. I examined the frequency of circulating monocytes, their surface antigen, cytokine and chemokine profile, comparing sarcoidosis monocytes with healthy control monocytes.

3.4. Results

3.4.1. Monocytes were increased in relative and absolute number in sarcoidosis.

Individuals with sarcoidois had a greater proportion of circulating monocytes than healthy controls (Figure 3.1a-b). Because sarcoidosis patients are known to have peripheral lymphopenia, the absolute cell number per ml of whole blood was also determined in a subset of these individuals to ensure that the finding of monocytosis was not artefactual. This confirmed the observation by demonstrating that monocyte absolute number per ml of blood was also increased.

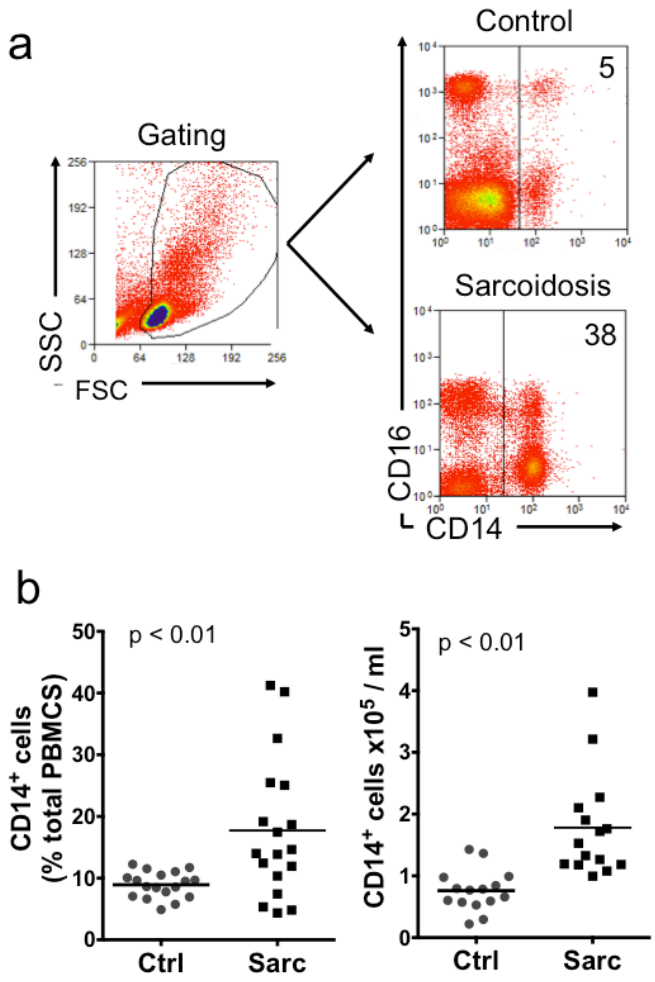


Figure 3.1. Individuals with Sarcoidosis had more monocytes than healthy control donors.
"$CD14^{+}$" and "monocytes" refer to all $CD14^{+}$ cells regardless of CD16 expression (i.e. both $CD14^{+}$ and $CD14^{++}$) throughout the report unless otherwise stated. (a) Representative plots illustrating monocytes ($CD14^{+}$ cells ± CD16) as a percentage of total PBMCs. Number indicates percentage of total cells in the illustrated gate. (b) CD14+ cells comprise a greater proportion of total PBMCs in sarcoidosis (17.73 ± 2.56) than controls 8.93 ± 0.5 (n=18 per group), confirmed by an increased absolute $CD14^{+}$ cell count (sarcoidosis 0.76 ± 0.22, controls 1.78 ±0.09) (n=15 per group). Mean ± SEM illustrated. D'Agostino and Pearson omnibus normality test and unpaired Student t test were used for statistical analysis.

3.4.2. Monocytes were activated by standard isolation methods.

Since density gradient media such as Ficoll and Percoll have been reported to increase monocyte CD16 and CD11b expression (129, 264, 276) whilst

decreasing CD62L expression (276), I next tested whether this method of monocyte separation did indeed activate monocytes. PBMCs were extracted from half a blood sample, cells were extracted from the other half after erythrocyte lysis (Chapter 2.2.1). I found that the percentage of activated, inflammatory ($CD14^{++}CD16^{+}$) monocytes was increased after Ficoll separation compared with blood in which the erythrocytes had been lysed (Figure 3.2a and b). In addition, CD16 expression on monocytes was increased when Ficoll was used, reflected by an additional population of $CD14^{++}CD16^{++}$ cells. Since CD16 expression is used to indicate the inflammatory monocyte subset, Ficoll separation of blood was therefore avoided in the study of monocyte phenotype and function.

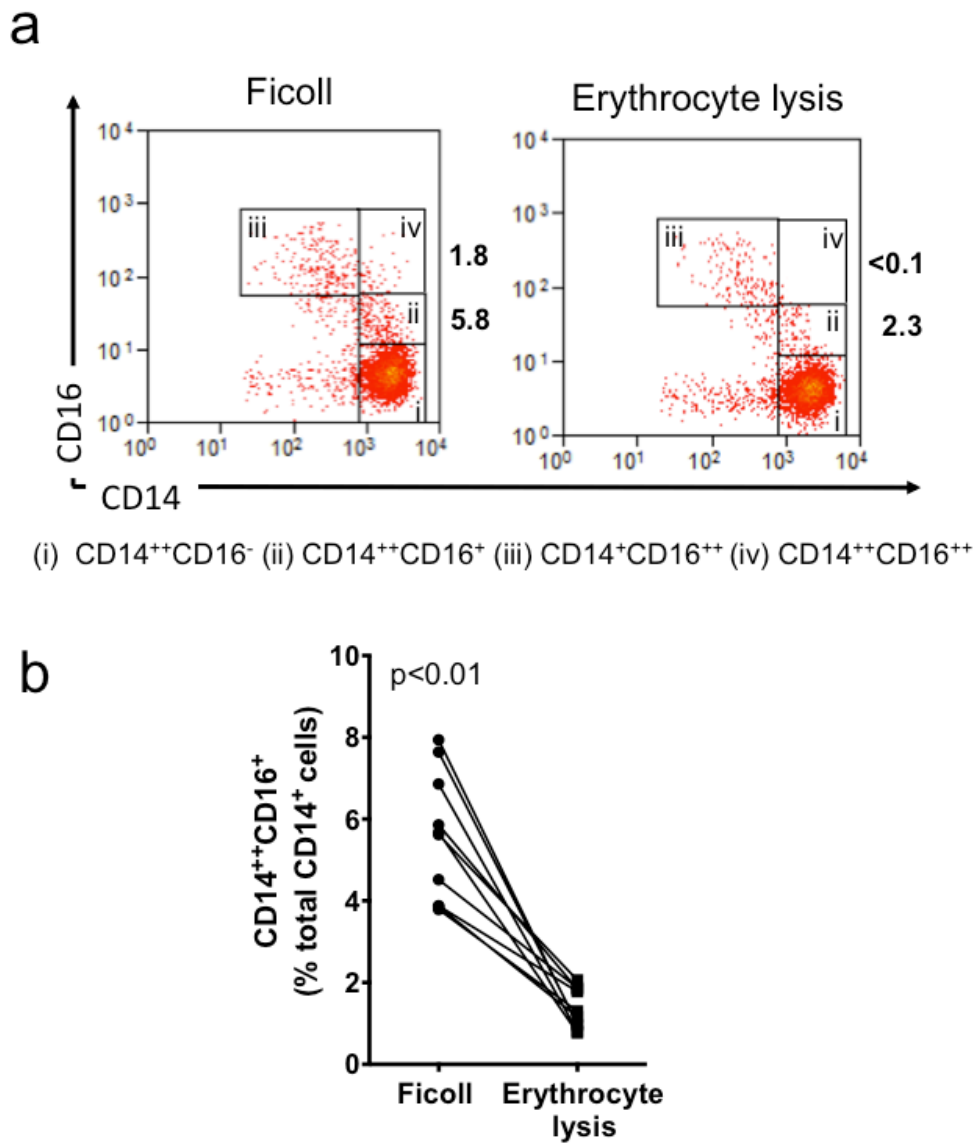


Figure 3.2. Monocytes were activated by standard blood separation techniques.

(a) The proportion of inflammatory $CD14^{++}CD16^{+}$ monocytes (% total $CD14^{+}$ cells) was increased when density gradient media (Ficoll) was used (iii). In addition, CD16 expression on monocytes was increased when Ficoll was used, reflected by an additional population of $CD14^{++}CD16^{++}$ cells (iv). Representative plots, gated on all live cells, illustrating the monocyte percentage in each gate. (b) Percentage of $CD14^{++}CD16^{+}$ monocytes after concurrent separation of control blood (n=10). D'Agostino and Pearson omnibus normality test and paired Student t test were used for statistical analysis.

3.4.3. Inflammatory monocytes were expanded in sarcoidosis.

The relative proportion of the three main monocyte subsets was then assessed. I found that the proportion of total monocytes that comprise both the inflammatory ($CD14^{++}CD16^{+}$) and patrolling ($CD14^{+}CD16^{++}$) subsets more than doubles in sarcoidosis compared with healthy controls (Figure 3.3a). A corresponding reduction in the relative proportion of classical ($CD14^{++}CD16^{-}$) subset was observed.

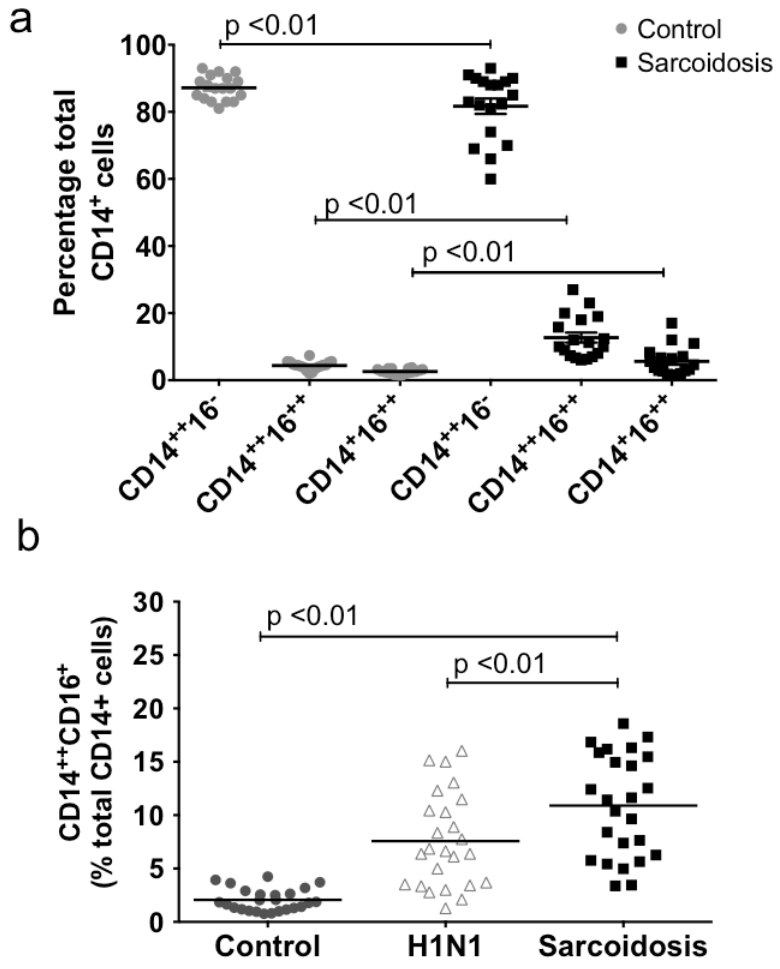


Figure 3.3. Monocyte subset profile was altered in sarcoidosis. (a) The proportion of both inflammatory ($CD14^{++}CD16^{+}$) and patrolling ($CD14^{+}CD16^{++}$) is increased in sarcoidosis (n =18 per group). (b) The proportion of inflammatory monocytes is increased in sarcoidosis 10.90 ± 0.96 compared with both healthy control donors 2.06 ± 0.21 and H1N1 disease controls 7.57 ± 0.88. (n=23 per group). Mean ± SEM. One-way ANOVA and Tukey multiple comparisons tests were used for statistical analysis.

I then compared these findings with another acute inflammatory condition affecting the lung, Influenza A (H1N1). All persons in this cohort had disease severe enough to require admission to the intensive care unit. The H1N1 data (unpublished) was collected and analysed by Suzanne Cole (Ho laboratory, Weatherall Institute of Molecular Medicine) as part of the 'Mechanisms of Severe Acute Influenza Consortium (MOSAIC)'. The proportional increase in inflammatory monocytes was greater in sarcoidosis than the individuals with influenza (Figure 3.3b).

3.4.4. Monocyte surface antigen expression was altered in sarcoidosis

I then examined surface antigen expression on sarcoidosis monocytes compared with healthy controls. PBMCs were first isolated by erythrocyte lysis and then monocytes were derived using CD14 negative magnetic bead separation in order to avoid activation. These were then stained immediately (ex vivo) with: CD11b, CD32, CD115, CX3CR1, CD62L, CCR2, CD163, CD15, CD43, PD-1, CD1d and CD206, a panel of surface antigens that collectively represent monocyte activity including inflammatory activity, activation, crawling and phagocytosis (Figures 3.4-3.6).

The percentage of monocytes expressing the activation markers CD11b and CD115 was significantly higher in individuals with sarcoidosis than in healthy controls (Figure 3.4a, c), whilst there was no statistically significant difference in the expression of CD32 or CD62L (Figure 3.4b). These findings were reflected in the MFI for each antigen.

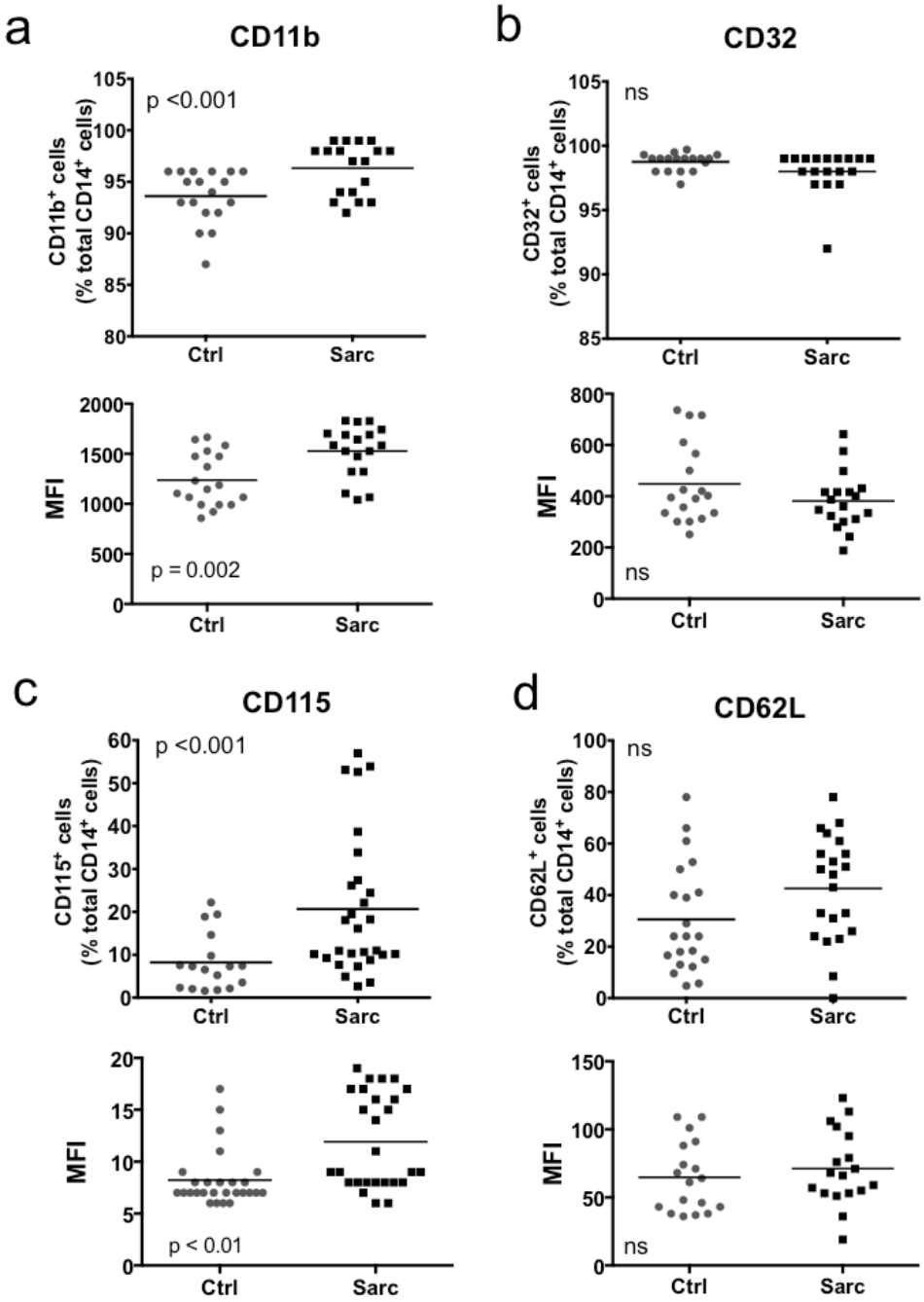


Figure 3.4. CD11b and CD115 expression was greater on sarcoidosis monocytes.

Surface antigen expression on monocytes (% total $CD14^+$ cells and mean fluorescence intensity [MFI]): (a) CD11b % mono: sarcoidosis 97.50 [93.73-98.25], control 94.5[92.0-96.0]; MFI: sarcoidosis 1528 [258], control 1238 [265] (n=18 each group), (b) CD32 % mono: sarcoidosis 98.5 [97.75-99.0], control 99.0 [98.0-99.08]; MFI: sarcoidosis 382 [112], control 448 [156] (n=18 each group), (c) CD115 % mono: sarcoidosis 13.56 [9.45-27.09], control 7.29 [2.24-12.20]; MFI: sarcoidosis 9 [8-17], control 7 [7-8] (n=27 each group), (d) CD62L % mono: sarcoidosis 24.00 [14.00-45.50] (n=21), control 30.58 [4.58]; MFI: sarcoidosis 71 [28], control 65 [26] (n=20). ns = not significant to p value < 0.05. D'Agostino and Pearson omnibus normality test and, where data was normally distributed, an unpaired Student's T test was used and mean [SD] indicated. Where data did not conform to a Gaussian distribution, a Mann-Whitney test were used for statistical analysis and Median [IQR] indicated. Plots illustrate representative staining for each surface antigen with isotype control (i.c) given in Appendix 1.

The percentage of monocytes expressing both CX_3CR1 and CCR2, involved in monocyte migration and adhesion to inflammatory sites, were significantly increased in sarcoidosis as was the MFI (Figure 3.5a-b), although it is worth noting that overall, monocytes are either high or low CCR2 expressing.

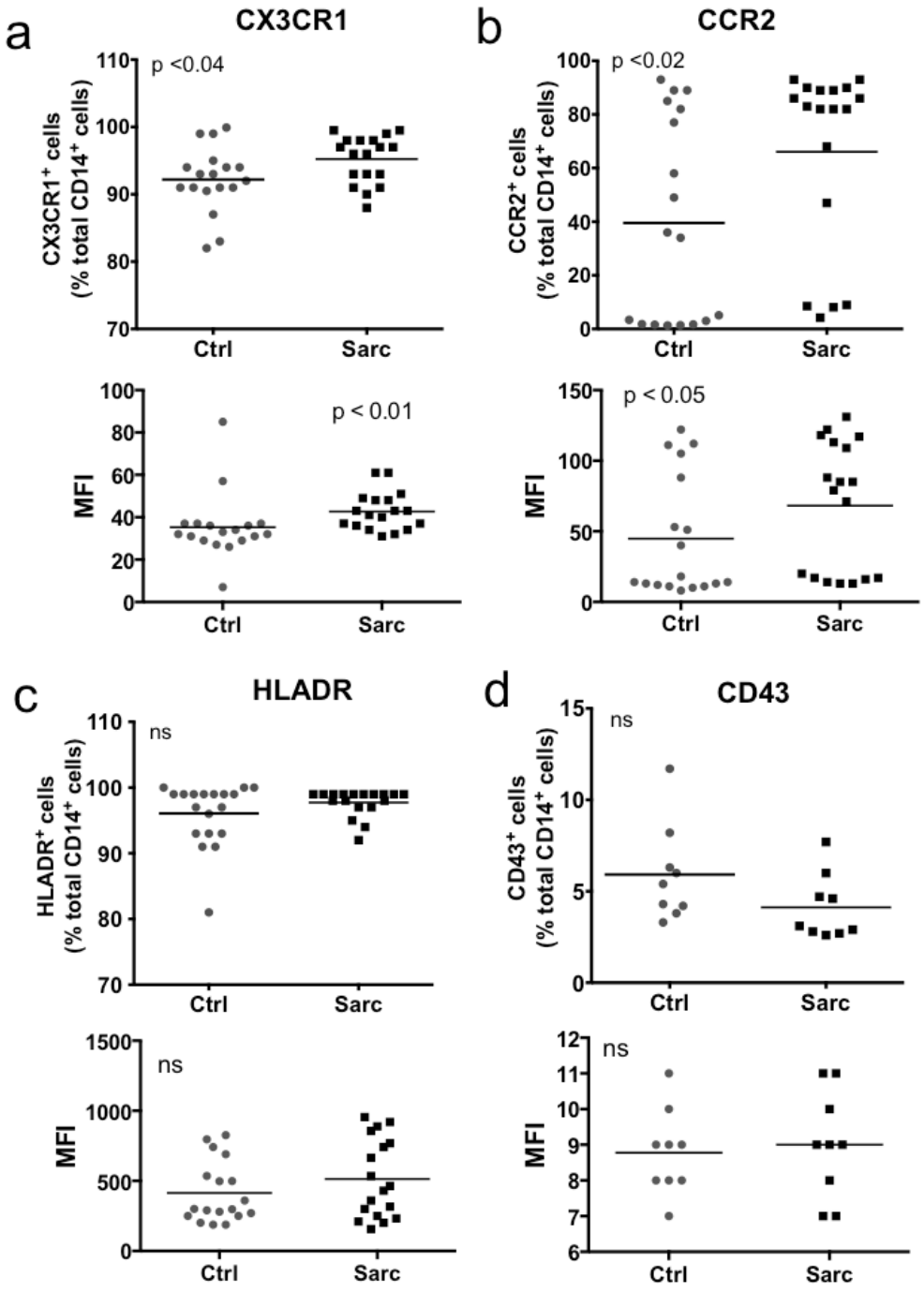


Figure 3.5. CX3CR1, CCR2 and CD43 expression was greater on sarcoidosis monocytes.

Surface antigen expression on monocytes (% total $CD14^+$ cells and MFI): (a) CX3CR1 (% mono): sarcoidosis 96.50 [92.50-98.00], control 92.50 [90.88-94.25]; MFI: sarcoidosis 42.0 [35.5-48.3], control 32.5 [29.0-37.0], (b) CCR2 (% mono): sarcoidosis 82.50 [37.50-89.25], control 35.00 [1.78-82.75]; MFI: sarcoidosis 82.0 [16.75-114.0], control 16.0 [11.75-92.3], (c) HLADR (% mono): sarcoidosis 99.00 [97.00-99.00], control 99.00 [93.0-99.0]; MFI: sarcoidosis 448 [247-791], control 299 [251-575], (d) CD43 (% mono): sarcoidosis 3.10 [2.75-5.35] control 5.40 [4.00-7.25], MFI: sarcoidosis 9.0 [1.5], control 8.8 [1.2], (n=9 each group). D'Agostino and Pearson omnibus normality test and, where data was normally distributed, an unpaired Student's T test was used and mean [SD] indicated. Where data did not conform to a Gaussian distribution, a Mann-Whitney test were used for statistical analysis and Median [IQR] indicated. Plots illustrate representative staining for each surface antigen with isotype control (i.c) given in Appendix 1.

A greater proportion of monocytes expressed HLA-DR, reflected by the MFI. Although this did not reach statistical significance, there was a suggestion that expression may be greater in sarcoidosis (Figure 3.5c). There was no significant difference in sarcoidosis monocyte expression of CD43, thought to be involved in the maintenance of cell-cell interactions (Figure 3.5d).

The scavenger receptor CD163, and the carbohydrate adhesion molecule CD15, both implicated in phagocytic activity, were significantly increased in sarcoidosis monocytes compared with healthy controls (Figure 3.6a-b). There was reduced expression of the scavenger receptor and maturation marker CD206 (Figure 3.6c). Both CD163 and CD206 expression suggest an M2 bias in cells of the monocyte-macrophage lineage.

Expression of PD-1 (programmed cell death protein 1) was significantly reduced in sarcoidosis monocytes suggesting decreased ability to interact with T cells (Figure 3.7a). Interestingly however, there was no difference in the almost universal expression of the CD1d on monocytes in sarcoidosis compared with controls (Figure 3.7b).

Overall, this suggests an activated phenotype with increased expression of activation markers such as CD11b, CD115. The increased expression of antigens associated with migration and phagocytosis suggest a possible alteration in these monocytic functions is sarcoidosis.

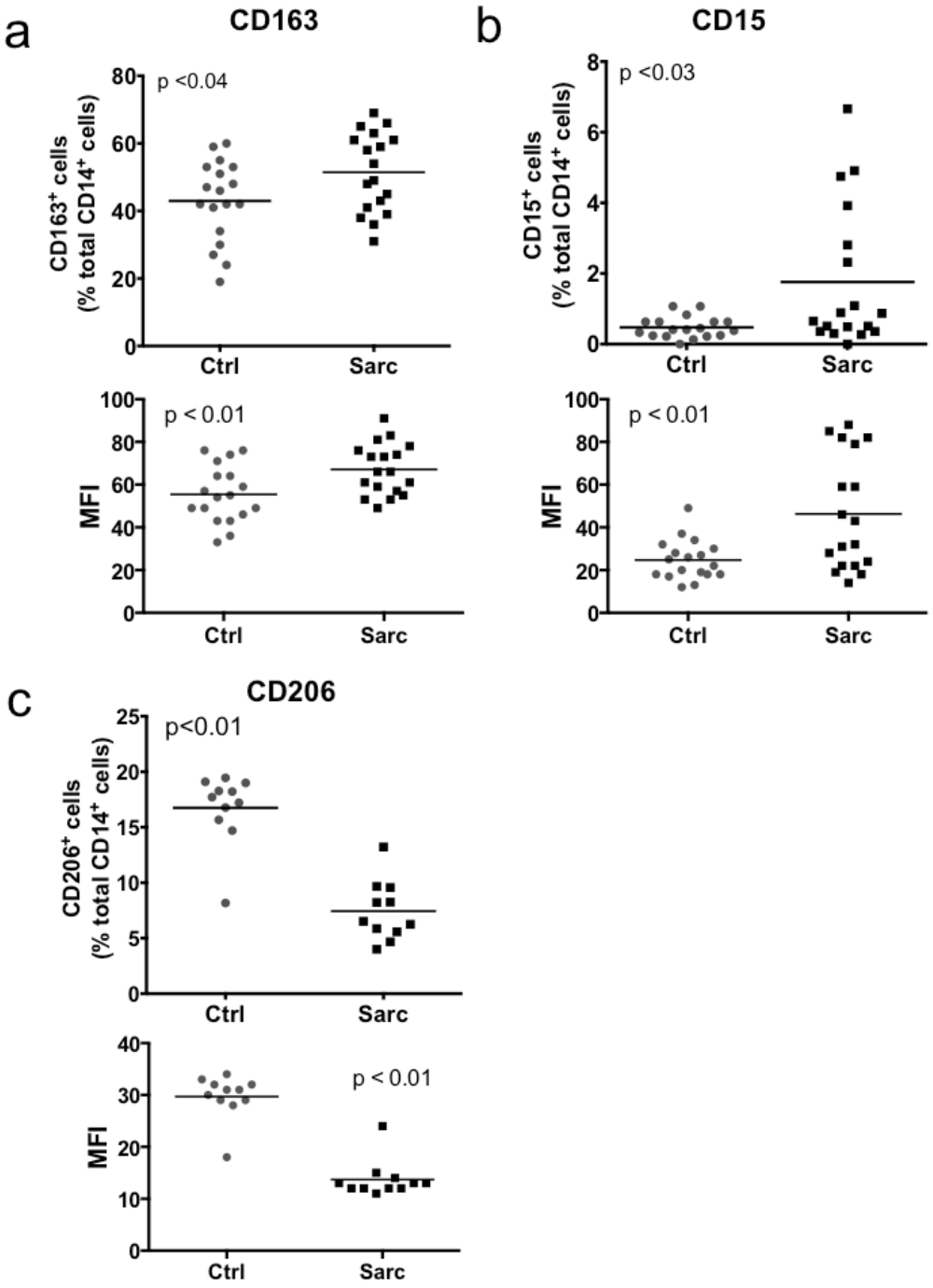


Figure 3.6. CD163 and CD15 expression was greater and CD206 less on sarcoidosis monocytes.

Surface antigen expression on monocytes (% total CD14+ cells and MFI): (a) CD163 (% mono): sarcoidosis 99.00 [97.00-99.00], control 99.00 [93.00-99.00]; sarcoidosis 67.17 [12.00], control 55.44 [13.29], (b) CD15 (% mono): sarcoidosis 0.76 [0.36-3.09], control 0.41 [0.24-0.63]; MFI: sarcoidosis 46.28 [26.82], control 24.72 [9.34] (n=18 each group), (c) CD206 (% mono): sarcoidosis 6.52 [5.57-9.57], control 17.71 [15.67-19.00]; MFI: sarcoidosis 13.00 [12.00-14.00], control 31.00 [29.00-32.00] (n=11 each group). D'Agostino and Pearson omnibus normality test and, where data was normally distributed, an unpaired Student's T test was used and mean [SD] indicated. Where data did not conform to a Gaussian distribution, a Mann-Whitney test were used for statistical analysis and Median [IQR] indicated. Plots illustrate representative staining for each surface antigen with isotype control (i.c) given in Appendix 1.

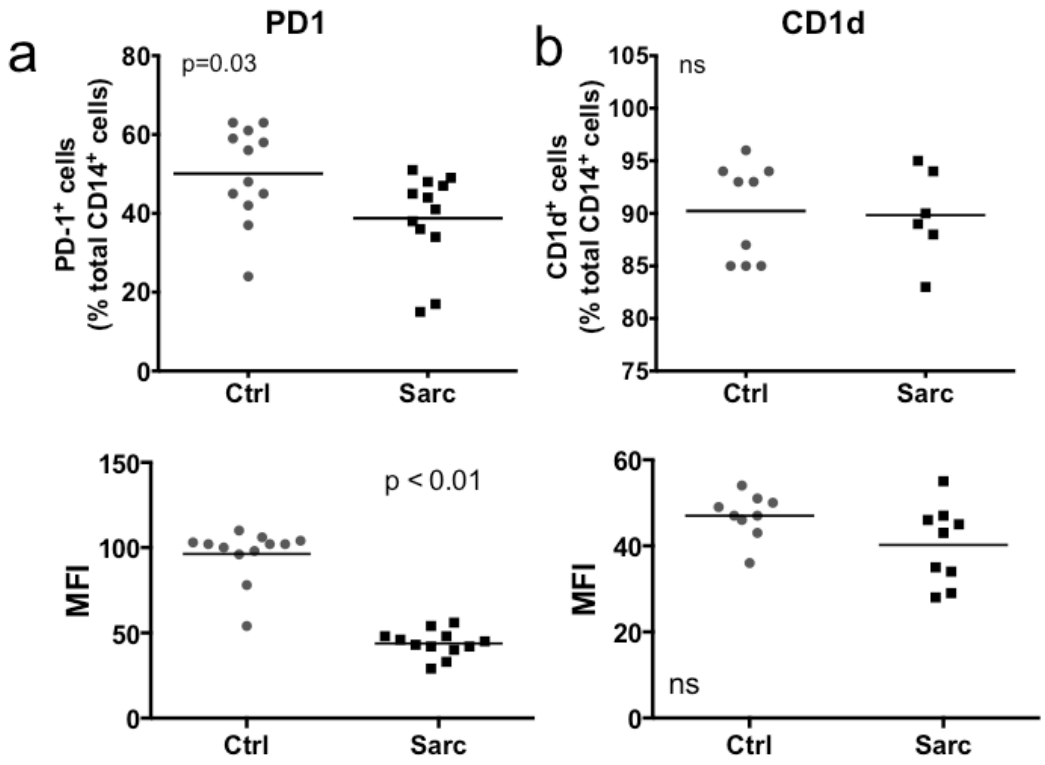


Figure 3.7. PD-1 expression was reduced on sarcoidosis.
Surface antigen expression on monocytes (% total $CD14^+$ cells): (a) PD-1 (% mono): sarcoidosis 38.75 [11.86], control 50.08 [3.49]; MFI: sarcoidosis 44.0 [40.5-48.0], control 102.0 [96.5-103.8] (n=12 each group) and (d) CD1d (% mono): sarcoidosis 89.50 [86.75-94.25], control 93.00 [85.00-94.00]; MFI: sarcoidosis 40.2 [9.15], control 47.0 [5.20] (n=9 each group). D'Agostino and Pearson omnibus normality test and, where data was normally distributed, an unpaired Student's T test was used and mean [SD] indicated. Where data did not conform to a Gaussian distribution, a Mann-Whitney test were used for statistical analysis and Median [IQR] indicated. Plots illustrate representative staining for each surface antigen with isotype control (i.c) given in Appendix 1.

3.4.5. Monocyte cytokine profiles are altered in sarcoidosis.

Monocytes, isolated as described, were stained both ex vivo and after stimulation in order to examine cytokine profiles. I used TNFα expression to determine the LPS concentration and time point for cytokine analysis (Figure 3.8a) as monocyte TNFα production is widely reported. Accepting that different cytokines may be produced with different kinetics, 1μg/ml LPS over an 18-hour stimulation was chosen because, for TNFα at least, these conditions provided the optimal stimulation for cytokine study.

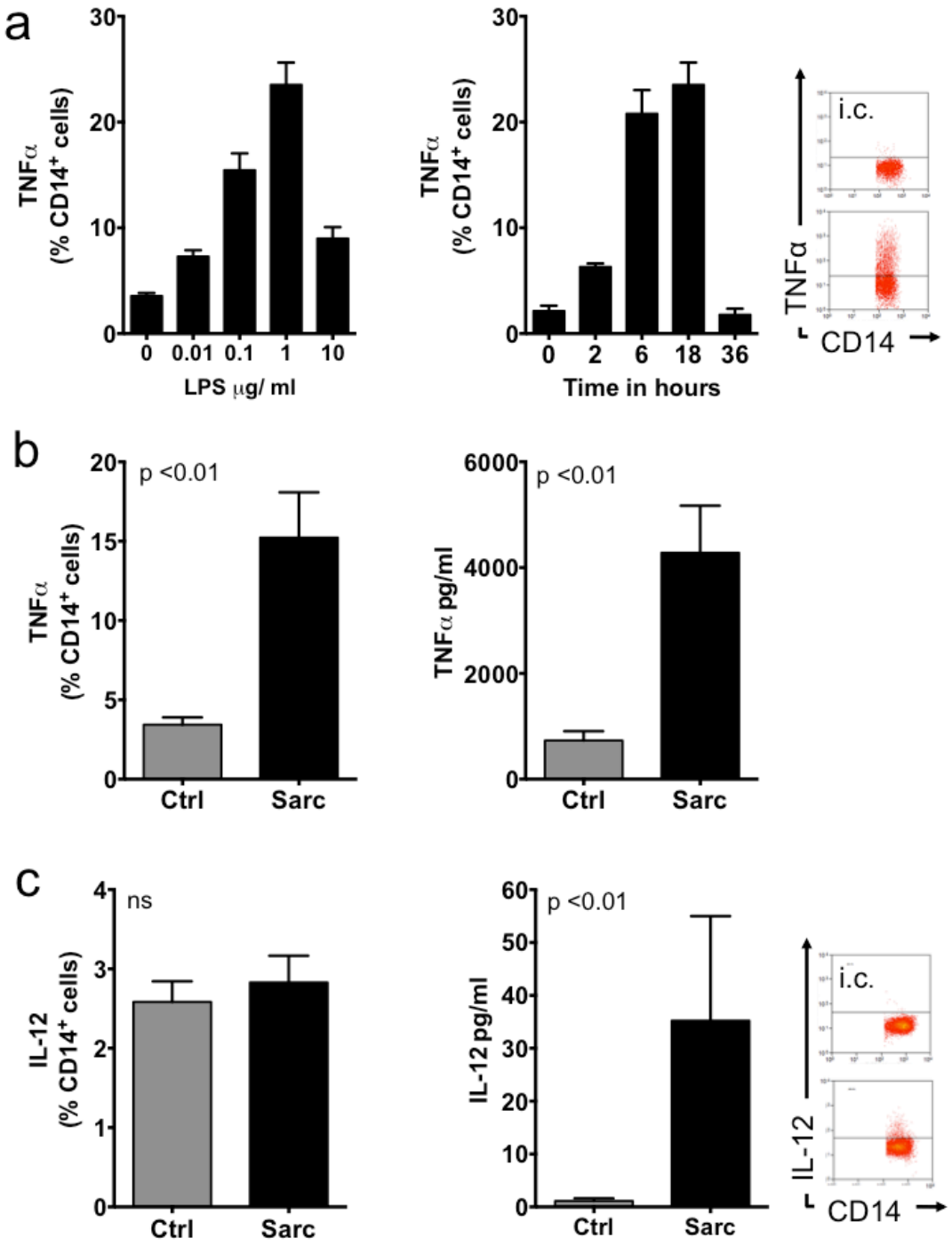


Figure 3.8. Sarcoidosis monocytes produced more TNFα and IL-12 than healthy monocytes.

(a) LPS concentration and stimulation duration was determined using TNFα (n=3 controls). Sarcoidosis monocyte cytokine production by ICS (% total $CD14^+$ cells) and ELISA (pg/ml in culture supernatant (125 000 monocytes) at 18 hours): (b) TNFα ICS: sarcoidosis 15.23 ± 2.85, control 3.44 ± 0.45 (n=10 per group), ELISA: sarcoidosis 4280 ± 890.8, control 731.6 ± 174.4 (n=6 per group), (c) IL-12: sarcoidosis 2.83 ± 0.33, control 2.59 ± 0.25 (n=12 per group), ELISA: sarcoidosis 35.23 ± 19.75, control 1.13 ± 0.47 (n= 6 per group) Mean ± SEM illustrated. D'Agostino and Pearson omnibus normality test and a Student T test was used for statistical analysis.

At 36 hours, there were very few monocytes left in culture, most cells were either adherent (suggesting transition to macrophages) or dying. Brefeldin A was added for the final four hours of stimulation (after 14 hours); additional samples were retained without Brefeldin A for the assessment of protein in the supernatant. With the exception of TNFα, the tested cytokine expression levels were undetectable by FACS ex vivo and as such, cytokine phenotype illustrated is after stimulation.

Increased TNFα expression by sarcoidosis monocytes was confirmed with a greater than four-fold increase in the total protein concentration in the supernatant, as measured by ELISA (Figure 3.8b). There was no significant difference in the percentage of monocytes that produce IL-12 in sarcoidosis compared with healthy controls but the quantity of IL-12 produced by sarcoidosis monocytes was greater (Figure 3.8c).

There was no difference in the proportion of monocytes in sarcoidosis that secrete the regulatory cytokine IL-4, but strikingly, there was an almost thousand fold decrease in the quantity of IL-4 produced by sarcoidosis monocytes [mean (SEM) 0.786 90.05) pg/ml sarcoidosis, 732 (174.4) pg/ml controls] (Figure 3.9a).

Interestingly, both the proportion of monocytes capable of producing IL-10 and the total quantity produced was reduced in sarcoidosis (Figure 3.9b). IL-10 expression was not found to be subset specific (Figure 3.9c).

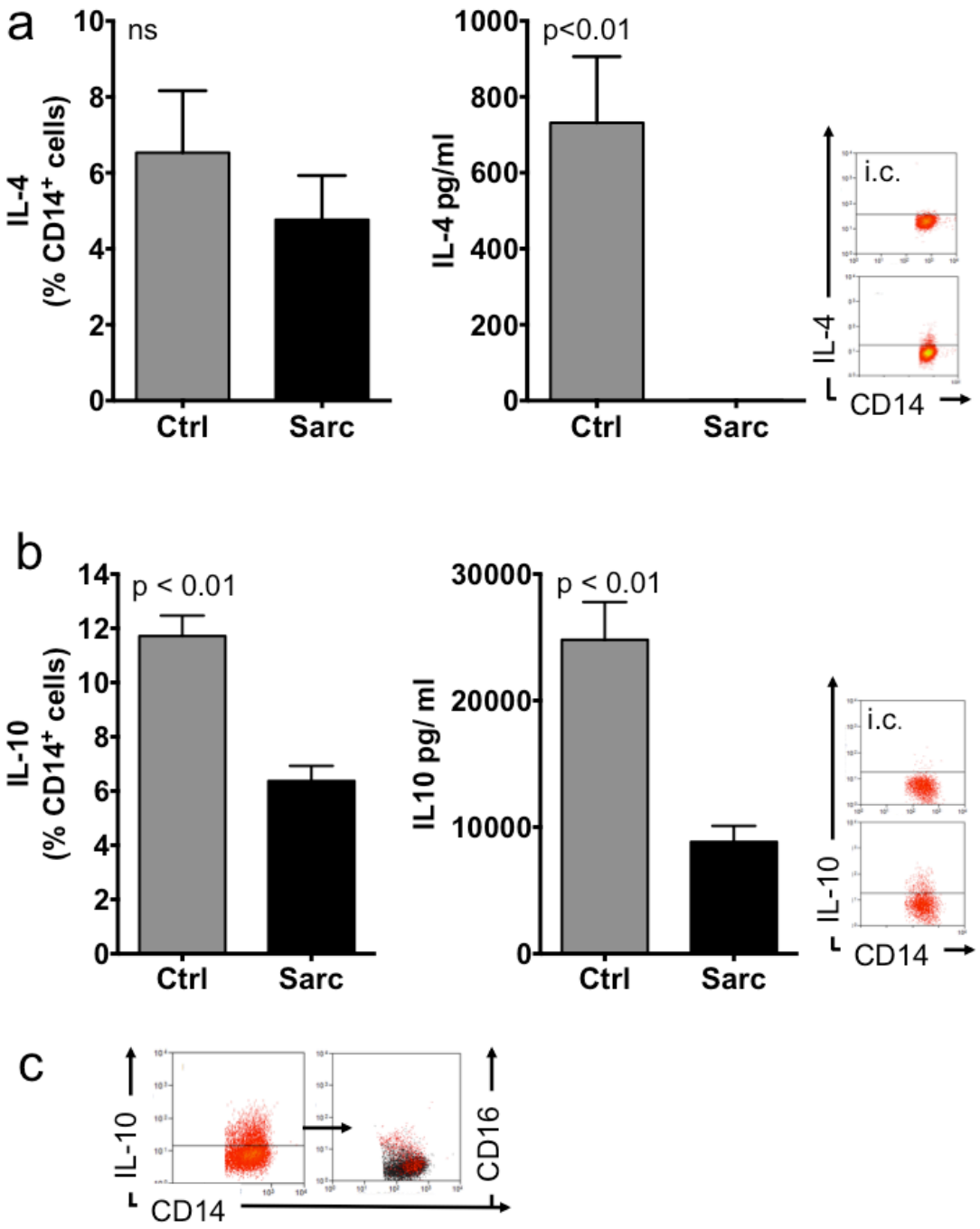


Figure 3.9. Sarcoidosis monocytes produced less IL-4 and IL-10 than control monocytes.

Sarcoidosis monocyte cytokine production by ICS (% total $CD14^+$ cells) and ELISA (pg/ml in culture supernatant (125 000 monocytes) at 18 hours): (a) IL-4 ICS: sarcoidosis 4.77 ± 1.16, control 6.53 ± 1.63, ELISA: sarcoidosis 0.79 ± 0.05, control 731.6 ± 174.4 (n=6 per group), (b) IL-10: sarcoidosis 6.37 ± 0.55, control 11.71 ± 0.75, ELISA: sarcoidosis 8839 ± 1265, control 24808 ± 2973 (n=25 per group). (c) IL-10^+ monocytes gated onto plot demonstrating subsets by CD16 expression. Mean ± SEM. D'Agostino and Pearson omnibus normality test and a Student T test was used for statistical analysis.

Interestingly, in the context of the surface profiling demonstrating increased CCR2 (Figure 3.5b), there was no significant difference in the percentage of LPS-stimulated monocytes expressing the chemokine CCL2 nor the total amount produced between sarcoidosis and control monocytes (Figure 3.10a).

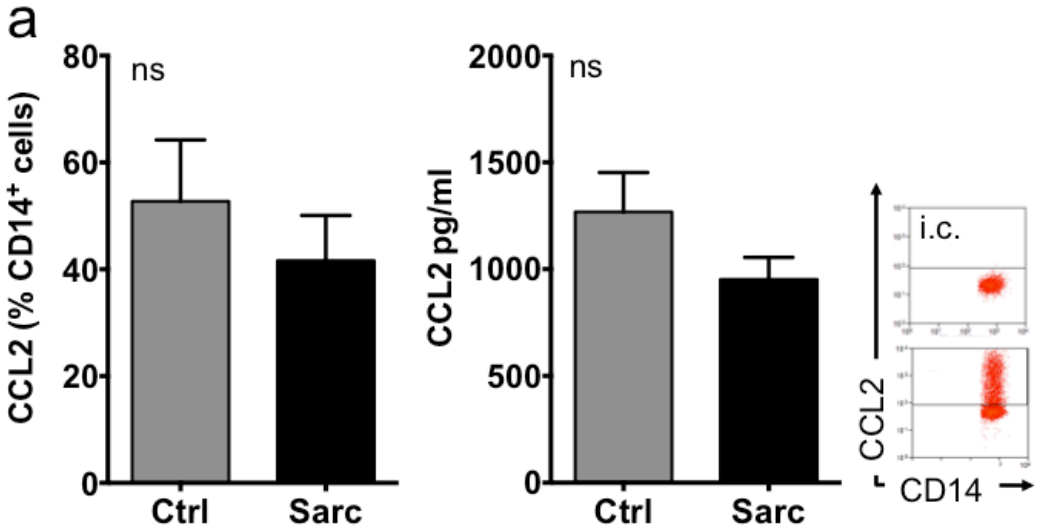


Figure 3.10. Sarcoidosis produced less CCL2 than control monocytes.
(a) Monocyte CCL2 production by ICS (% total $CD14^+$ cells) sarcoidosis 43.45 [13.60-43.45], control 48.95 [25.03-83.40] and ELISA (pg/ml in culture supernatant (125 000 monocytes) at 18 hours) sarcoidosis 914 [760-1129], control 1306 [812-1670] (n=6 per group) (n=6 per group). CCL2 quantity refers to amount found in culture supernatant (125 000 monocytes) at 12 hours as determined by ELISA. D'Agostino and Pearson omnibus normality test and a Mann-Whitney test were used for statistical analysis.

Collectively this suggests that the monocytes are activated in sarcoidosis, with a heightened TNFα and IL-12 response compounded by muted expression of IL-10.

3.5. Discussion

Monocytes are easily activated and previous reports suggest that standard isolation techniques activate monocytes in vitro and altered activation markers

are seen: increased CD16 and CD11b expression (129, 264, 276), decreasing CD62L expression (276). Despite this, density gradient separation remains widely used, even when monocytes are the cells of interest. I confirmed these previous reports and found that there was an increase in the percentage of inflammatory ($CD14^{++}CD16^{+}$) seen when blood is separated using Ficoll as the density separation medium compared with when erythrocytes were lysed directly. There was no difference in the total number of monocytes or the percentage of monocytes that comprise the total PBMC, suggesting that isolation method is crucial when an evaluation of monocyte subset frequency and activity is required. Erythrocyte lysis was therefore used for the surface and cytokine phenotyping studies.

Increased proportions of monocytes in sarcoidosis PBMCs have been previously observed (277, 278) and were confirmed in this study; the percentage of monocytes comprising the PBMC compartment may as much as double in active disease. The relative proportions of the classical and inflammatory monocyte subsets are known to be altered in sarcoidosis as well as in other inflammatory conditions such rheumatoid arthritis, psoriatic arthritis and Crohn's disease where in increase in the proportion of the inflammatory ($CD14^{++}CD16^{+}$) subset is reported (144-146, 188). I confirmed that the inflammatory subset was increased in the study population and that this increase was even greater than that found in the context of severe lung inflammation (H1N1 influenza infection). Interestingly, the proportion of the patrolling ($CD14^{+}CD16^{++}$) subset was increased in sarcoidosis (mean ± SEM control 2.59 ± 0.18 v 5.62 ± 1.0 sarcoidosis; $p < 0.01$), a finding not previously

reported in the literature. Patrolling monocytes are weak phagocytes that crawl along the vascular endothelium in a lymphocyte function-associated antigen-1-dependent manner (LFA-1) (129). They are known to produce TNFα and IL-1β, in response immune complexes containing nucleic acids, via a pro-inflammatory TLR7-TLR 8-MyD88-MEK pathway. They are involved in the innate local surveillance of tissues and the pathogenesis of autoimmune diseases and their increase may relate to antigen sensing in the early pathogenesis of sarcoidosis.

I next explored monocyte surface antigen phenotype. Consistent with previous reports (188, 190, 279), I found monocytes to be more activated in sarcoidosis than in healthy controls. In addition to increased CD16 expression, used to define the inflammatory monocyte subset in humans, I demonstrated that a greater proportion sarcoidosis than control monocytes express the activation markers CD15, CD115, CD163 and possibly HLADR. The carbohydrate adhesion molecule, CD15, most commonly associated with neutrophil phagocytosis and chemotaxis, is also expressed on monocytes contributing to activation and resultant TNFα production (280, 281). CD115 (macrophage colony-stimulating factor receptor, M-CSFR) is used, in conjunction with Ly6C, to identify murine inflammatory monocytes (138, 282) and has been implicated in human monocyte activation, maturation and proliferation (283). The finding that the percentage of monocytes in sarcoidosis expressing CD115 is significantly greater than in healthy controls is consistent with potential application as an inflammatory marker for human monocytes. Increased expression of the myeloid scavenger receptor CD163,

is seen in inflammatory conditions and by IL-6 and IL-10 stimulation (284-287) and is further evidence of the inflammatory monocyte profile in sarcoidois. Cells of the monocyte-macrophage lineage may be classified as M1-like or M2-like. Sarcoidosis monocytes expressed increased surface CD163 but less CD206 compared with controls and so their M1/ M2 potential is not clear. M2 macrophages are important in repair and may have profibrotic features (288). Interestingly, it has been shown that IL-10 overexpression is associated with increased M2 macrophages recruitment in a CCL2 dependent fashion, leading to increased fibrosis in a murine lung model (289). IL-10 production is reduced by the sarcoidosis monocyte, which may initially hinder the reparative response, however, it is likely that IL-4 production by T_H2 cells more latterly results in an M2/ profibrotic environment.

Increased expression of the MHC class II cell surface receptor, HLA-DR might have been expected on sarcoidosis monocytes (290), however this was not observed in this study. Additionally, there was no significant difference in the expression of CD62L or CD43, both of which might have been expected from previous in vitro cell line and murine studies (276, 291, 292). The trend of CD62L expression is however slightly higher in sarcoidosis monocytes and in combination with CD14, CD16 and CD115 may be used to identify inflammatory monocytes and exclude neutrophils ($CD62L^-CD15^+CD14^-$).

There was increased expression of the fractalkine receptor CX_3CR1 and the MCP-1 receptor CCR2 (CD192) consistent with enhanced monocyte migration to the site of inflammation in sarcoidosis (150, 155, 293, 294). The

finding that monocytes are either high or low CCR2 expressing is consistent with previous findings (153, 282). The chemokine receptors CCR1, CCR5, CCR6, CCR7, CCR7 and CXCR2 have been previously implicated in monocyte migration and arrest at the site of pathology (295-299); exploration of the differential expression in sarcoidosis monocytes may provide further information about monocyte recruitment in sarcoidosis. Additionally, the increased expression of CX_3CR1, which provides a cell survival signal to monocytes and CD115, which promotes maturation and proliferation of monocytes is consistent with the finding that monocytes are increased in number in sarcoidosis (283, 294).

Once at the site of pathology, monocytes may be retained by the increased levels of the integrin CD11b, although interestingly, there was no difference in the expression of either CD43 or CD62L, both of which serve as monocytic adhesion molecules. It would be interesting to examine the differential expression levels in sarcoidosis monocytes of other known monocyte adhesion molecules such as VLA-4 (involved in monocyte adhesion to inflamed endothelium, LFA1 (required for steady-state patrolling), MAC1 (adhesion during acute inflammation), PECAM1 and PSGL1 (transendothelial migration of monocytes) and M-DC8 (Human 6-sulfo LacNAc, SLAN) (129, 151, 300, 301). M-DC8 is of particular interest in the context of sarcoidosis as it is preferentially expressed by the patrolling subset, which I have shown to be increased in the condition.

The phagocytosis, degradation, and clearance of antigens depends on the function of receptors for Fc fragment of immunoglobulin G: FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16) and/or complement receptors: CR1 (CD35), CR3 (CD11b), CR4 (CD11c). Although the level of CD16, implicated in phagocytic ability (302), is increased in sarcoidosis, due to an increase in both $CD14^{++}CD16^{+}$ and $CD14^{+}CD16^{++}$ subsets, it is interesting to note that the monocytes with highest CD16 expression, the patrolling monocytes are poor phagocytes. The scavenger receptor CD206 (mannose receptor) is important for effective phagocytosis and is significantly reduced in sarcoidosis, potentially having a negative impact on phagocytic ability (303). The expression of two other phagocytic markers were measured, CD163 and the low affinity IgG Fc receptor (FcγRII) CD32 (303, 304). Whilst CD163 expression was increased, CD32, previously reported to be down-regulated on monocytes in sarcoidosis was not significantly different (302). Overall this suggests that monocyte phagocytic capacity may be altered in sarcoidosis. Other monocyte receptors potentially involved in phagocytosis, including CD35, CD64 and the macrophage antigen CD169, require further delineation in sarcoidosis. CD169 and its interaction with iNKT cells is of particular interest in the context of the iNKT deficiency in sarcoidosis, discussed in the next chapter (305-307).

It has been reported that TLR2 and TLR4 expression on monocytes is higher in sarcoidosis, both ex vivo and after stimulation and that increased TLR2 expression in particular, in combination with NOD2 signaling results in an increase in the pro-inflammatory cytokines TNFα and IL-1β (101). I was not

able to replicate this finding either ex vivo or following stimulation for a similar duration with the same concentration LPS. There was a small, non-significant increase in the level of TLR2 expressed (mean ± SEM control 4.23 ± 1.49, sarcoidosis 7.4 ± 0.5), but no difference in TLR4. This may be due to differences in cell separation, Ficoll was used in the study by Wiken and colleagues (101), with possible activation and alteration of surface phenotype, as discussed previously. Alternatively differing patient characteristics in the study compared with those recruited for this thesis, for example, the authors included patients with Löfgren's, an immunologically and phenotypically distinct form of sarcoidosis, and patients on steroids, which may affect the immune profile. TLR2/ NOD2 synergism in the induction of IL-10 has been described in the inflammatory condition Crohn's disease (308), and in the context of aberrant IL-10 production in monocytes, discussed later in this chapter.

PD-1 (programmed cell death protein 1) is a cell surface protein expressed on monocytes and is part of the extended CD28/CTLA-4 family of T cell regulators. Ligation of PD-1 on monocytes has been shown to inhibit T cell expansion in HIV expansion by inducing IL-10 production by monocytes (309). The finding that PD-1 expression on monocytes is significantly reduced in sarcoidosis monocytes compared with healthy control monocytes provides a potential mechanism for reduction in IL-10 production and signaling.

To summarise these findings, monocytes in sarcoidosis expressed markers suggesting an increased activation state with increased migratory,

proliferative, survival and adhesive properties, all of which would be consistent with a role in the early antigen sensing at the site of pathology in sarcoidosis and would help to explain their increased frequency. The lack of a suitable mouse model in sarcoidosis hampers the ability to tease out the functionality of these altered antigen profiles. Further studies, could however, be performed in vitro using blocking and migratory assays to explore how these profiles later monocyte kinetics.

Monocyte cytokine function was examined both ex-vivo and after overnight stimulation (18 hours) with LPS. The ex-vivo studies revealed very little in terms of intracellular cytokine staining. Of the cytokines tested, only TNFα was detectable by ICS, but there was no statistical difference between sarcoidosis and controls. Perhaps a more suitable way to determine the resting state activity of monocytes in sarcoidosis is through gene expression analysis.

I found that the monocyte production of TNFα and IL-12, both pro-inflammatory cytokines, are increased in sarcoidosis compared with healthy controls. Increased TNFα, originating from T cells and AM, has previously reported in sarcoidosis where it contributes to the maintenance of granuloma and correlates with disease progression (3, 18, 98, 273, 275, 310, 311). This has lead to the use of monoclonal antibodies against TNFα such as Infliximab, in the treatment steroid refractory disease. This approach is supported by small studies, case reports and a randomized double-blind placebo-controlled trial showing infliximab to be superior to placebo in treating

pulmonary sarcoidosis (98, 126, 311). Surprisingly, despite the body of evidence supporting the role of TNFα in the pathogenesis of sarcoidosis, its production by peripheral monocytes in sarcoidosis is less well documented (312).

TNFα exerts its function by binding to and signaling via two different receptors: TNFR1 (CD120a), constitutively expressed at low levels on nearly all nucleated cell types, and TNFR2 (CD120b) inducible and expressed by cells of myeloid lineage, peripheral T cells and alveolar lymphocytes and macrophages. Recent reports suggest that TNFR1 and TNFR2 are differentially expressed on the different monocyte subsets in sarcoidosis (279) with TNFR1 being primarily expressed on inflammatory monocytes and TNFR2 on the patrolling monocytes. Given the importance of TNFα in the immune pathology of sarcoidosis, it would be interesting to further study of these receptors in sarcoidosis.

IL-12 is produced by monocytes after IFNγ priming or phagocytosis (313, 314) and is involved in the induction of T_H1 responses. It activates T cells to produce IFNγ and TNFα, further enhancing IFNγ activity by reducing IL-4 mediated suppression (315). IL-12 expression has been widely reported to be increased in sarcoidosis AM and BALF (46, 47, 316) although monocyte IL-12 activity is less well understood. Interestingly, it is suggested to have a role in granuloma formation in the lung condition hypersensitivity pneumonitis (317). The finding that monocyte production of this cytokine increased in addition to TNFα, with which is acts synergistically, adds weight to the hypothesis that

monocytes can contribute to early pathogenesis, including granuloma formation.

IL-10 is an anti-inflammatory cytokine understood to have a non-redundant role in controlling inflammatory responses in autoimmune conditions, infection and tumours in vivo (318). Numerous cells of both the innate and adaptive immune system are capable of producing IL-10 (319-321). The suggestion that it can reduce inflammatory response in a rat model of experimental granuloma sparked interest in the potential role of IL-10 in disease pathogenesis in sarcoidosis (322). All subsets of human monocytes are known to produce IL-10 upon LPS stimulation in a dose-dependent manner (323-325). Evidence for levels of this cytokine in sarcoidosis have been conflicting with different studies suggesting that AM and BALF IL-10 is reduced in sarcoidosis (47), and others suggesting that AM produce more IL-10 in sarcoidosis (326). This may be in part due to the study of undifferentiated cell populations in the BALF, which can include T cells and B cells capable of producing IL-10 (327, 328). Monocyte IL-10 production has previously been unreported in sarcoidosis and the finding that they produce less IL-10 and the implications of this for the pathogenesis of sarcoidosis is the focus of the next chapter in this thesis.

The fact that the scavenger receptor CD163 was found to be significantly increased in sarcoidosis is interesting because, whilst this is consistent with observations in other inflammatory conditions (285), this does not fit with the fact that expression IL-10 is low and TNFα is high from sarcoidosis

monocytes. Previous reports suggest that CD163 is up-regulated by IL-10 (286, 287) and down-regulated by LPS stimulation and TNFα (285, 329). These conclusions were not drawn however from work on primary human monocytes, suggesting that either the regulation of CD163 is altered either in primary human monocytes, in sarcoidosis or in both. The regulation of this receptor and its implications for sarcoidosis immune pathology may warrant further study.

IL-4 can regulate the production of both IL-10 and IL-12 and promote the alternative activation and fusion of macrophages (72, 330-333). Whilst sarcoidosis monocytes seem capable, from ICS studies, of producing IL-4, there is a complete absence of IL-4 in the culture supernatant, a phenomenon previously noted in sarcoidosis T cells (334). This suggests that monocytes in sarcoidosis may have a decreased M2 'protective' phenotype and may contribute to the low monocyte IL-10 levels observed.

IL-13 is a T_H2 cytokine which, in conjunction with IL-4, promotes an M2 macrophage phenotype. It has previously been suggested on the basis of BAL cell and PBMC mRNA that IL-13 is elevated in sarcoidosis (335). I found that there was a small but non-significant rise in the amount of IL-13 produced by monocytes in sarcoidosis (ICS) (mean ± SEM control 4.40 ±1.02, sarcoidosis, 6.18 ± 1.88). It would be interesting to investigate IL-13 levels in the subset of sarcoidosis patients with bronchial hyper-reactivity because IL-13 is associated with bronchial hyper-reactivity in asthma and a monoclonal antibody to IL-13 (Lebrikizumab) is now available, albeit with debatable

efficacy (336).

IL-17A has been implicated in granuloma formation in sarcoidosis (52, 337). Previous studies have focused on T cells, AM, BAL and granuloma tissue, but not monocytes. I examined monocyte IL-17A and also its isoform IL17F in sarcoidosis and found that there was a small, non-significant increase in the amount of IL-17A detected by ICS (mean ± SEM control 3.5 ±0.49, sarcoidosis, 4.5 ± 1.46). Interestingly there was a significant increase in the proportion of monocytes producing IL-17F detected by ICS ($p < 0.005$, (mean ± SEM control 0.24 ±0.05, sarcoidosis, 0.62 ± 0.12). However, this represents a tiny proportion of circulating monocytes and requires confirmation by either gene expression analysis or supernatant ELISA. It would also be interesting to look for IL-17F in the monocytes associated with the granulomata.

CCL2 (MCP-1) is a chemokine is involved in the release of inflammatory monocytes from the bone marrow and their recruitment to sites of inflammation (132, 133); it has been reported to be increased in the BALF of sarcoidosis patients (338, 339). I found no difference in the amount of CCL2 produced by monocytes in sarcoidosis.

The pro-inflammatory cytokines TNFα (percentage cells and quantity) and IL-12 (quantity) were increased in sarcoidosis, whilst the regulatory cytokines IL-10 (percentage cells and quantity) and IL-4 (quantity) were decreased. It is important to note that these differences were observed in LPS stimulated

monocytes after 18 hours of culture. It may be that the differences are more or less significant at alternative time-points, and that due to varying cytokine stability that differential expression may have been missed. Of note, apart from TNFα, there was no differential expression in sarcoidosis monocytes ex vivo. Further analysis of differential expression at alternative time-points, concentrations of LPS (both optimised for TNFα production in view of its known importance in sarcoidosis), as well as response to alternative TLR agonists such as Pam3CSK4 or Zymosan are also required to increase our understanding of sarcoidosis monocyte activity. The relative timing of peak cytokine production may influence how these monocytes interact and differentiate. This study suggested that monocytes in sarcoidosis are pro-inflammatory producing increased TNFα and IL-12 and less IL-4 and IL-10. It may be, however at different time-points, the balance of cytokine production switches to a more regulatory phenotype. This is known to be the case in established fibrotic sarcoidosis. Establishing the differential kinetics of monocyte cytokine production would be crucial in further studies looking to delineate mechanistic pathways.

It would be interesting to examine sarcoidosis monocyte IL-6 expression, particularly in the context of the microarray study in chapter 7, which found it to be one of the most significantly up regulated genes in sarcoidosis monocytes. It has previously been found to be increased in the gene transcripts of AM in sarcoidosis and is known to switch monocyte differentiation towards macrophages rather than DCs (340, 341). Additional cytokines that it would be interesting to profile in sarcoidosis include: the

monocyte chemotactic cytokine CXCL10 (IP-10); reduced production by monocyte derived macrophages in sarcoidosis after treatment with Infliximab has been reported (342); CCL18, increased levels of which have been found in the serum, BAL fluid and AM culture supernatants of inflammatory lung diseases, including sarcoidosis, and have been implicated in transition towards granulomatous macrophages; CCL20, increased levels of which have been reported in sarcoidosis (92); and IL-18 a pro-inflammatory cytokine increased in sarcoidosis with unclear implications (343).

3.6. Summary

In this chapter I demonstrate that there is an increased number of both inflammatory ($CD14^{++}CD16^{+}$) and patrolling ($CD14^{+}CD16+^{+}$) monocytes in sarcoidosis in comparison with healthy controls. Functionally, they produce increased amounts of TNFα and IL-12 and reduced levels of IL-10. These findings show that monocytes from sarcoidosis patients are distinctly different from those in healthy controls.

Chapter 4: Regulation of IL-10 producing monocytes by iNKT cells

4.1. Introduction

In the last chapter, I showed that monocytes are both increased in number and phenotypically abnormal in sarcoidosis. Since I was interested in the role of monocytes in the pathogenesis of sarcoidosis, and sarcoidosis is characterised by T cell over-activity in the early phase of disease, I was interested in whether defective IL-10 production by sarcoidosis monocytes is related to this aberrant T cell response. Genome-wide association studies have previously identified BTNL-2, a butyrophilin/B7-like molecule and a purported negative co-stimulatory molecule for T cell proliferation, as a potential susceptibility factor (38). However, the function of BTNL-2 in sarcoidosis is poorly understood and little functional work has been done to support this hypothesis. Another possible cause for the large CD4 T cell expansion is a defect in IL-10 producing cells, since IL-10 has distinct T cell suppressive effect (344, 345). These could be IL-10 producing Fox-P3 regulatory T cells (T_{regs}), regulatory B cells (B_{regs}) or the less studied, IL-10 producing monocytes (63, 65, 323). Both T_{regs} and B_{regs} have been investigated in sarcoidosis but paradoxically shown to be elevated in numbers (58, 68). Very little is known of 'regulatory' monocyte activity. IL-10-deficient macrophages have been reported to fail to reproduce the characteristic repression on inflammatory mediators or T cell proliferation (309, 346).

There is growing evidence in murine and in vitro models to support iNKT-monocyte cross talk; the group has previously shown that (i) in an iNKT-deficient ($J\alpha18^{-/-}$) murine model of severe inflammatory lung injury (Influenza A infection), monocytes accumulate at the site of pathology in association with increased CCL2 (347); (ii) in a model of multiple sclerosis, activation of iNKT cells promotes the differentiation of monocytes to non-inflammatory, M2 macrophages with an improvement in outcome (140, 347). Additionally, iNKT cells have been shown to demonstrate CD1d-dependent killing of $CD68^{+}$ tumor-associated monocytes/macrophages (TAMs) and to instruct human peripheral blood monocytes to differentiate into myeloid APCs (immature DCs), via GM-CSF and IL-13 (255, 256).

I hypothesised that, in the context of observed cross talk between iNKT cells and monocytes, that in sarcoidosis, iNKT cell deficiency represents the loss of an upstream monocyte regulatory factor. I hypothesised that reduction in IL-10 production by monocytes in sarcoidosis results in increased T cell proliferation and activity in this condition, and that the defect in IL-10 production is related to impaired iNKT-monocyte interaction. I therefore wanted to explore the effect of iNKT cells on monocyte function, including cytokine expression and suppression of T cell proliferation in the context of sarcoidosis.

4.2. Aim

The aim of this chapter was to investigate the phenotypically altered sarcoidosis monocytes capacity to suppress T cell expansion and to determine the impact of defective monocyte IL-10 production on this activity

4.3. Methods

Donors were recruited as previously described; demographics are listed in Table 4.1.

	Sarcoidosis				Control		
	# (#Ex Pulm/SA)	Age (SD)	Male	CAS (0/1/2/3/4)	# (SA)	Age (SD)	Male
Fig 4.1e	7 (3/0)	45 (13.0)	3	(0/1/3/2/1)	7 (1)	32 (4.0)	4
Fig 4.1f	6 (2/0)	43 (13.0)	3	(0/1/3/1/1)			
Fig 4.2d&e	40 (4/0)	48 (10.6)	17	(0/12/18/7/2)	12 (2)	41 (13.0)	23
Fig 4.3a-f, 4.4a-f & 4.5a-e	6 (0/0)	43 (6.2)	3	(0/3/3/0/0)	6 (2)	33 (5.7)	4
Fig 4.6a	50 (2/0)	44 (12.7)	23	(3/17/15/12/4)	25 (2)	41 (11.0)	14
Fig 4.6b	18 (1/0)	48(8.0)	7	(1/5/6/6/0)	12 (1)	35 (7.0)	6
Fig 4.7c-d	36(3/1)	46 (7.7)	15	(0/18/10/6/2)			
Fig 4.16a-b & Fig 4.17a-f	6 (0/0)	44 (10.7)	2	(0/3/2/1/0)	6 (1)	31 (6.7)	3
Fig 4.18b, d	18 (4/0)	45 (10.5)	8	(0/4/9/4/1)	18 (2)	32 (5.0)	10
Fig 4.19b, d, f	10 (3/0)	47 (12.1)	3	(0/4/5/1/0)	10 (1)	33 (4.3)	6
Fig 4.20b, d	6 (0/0)	46 (10.4)	2	(0/2/4/0/0)	6 (1)	30 (5.8)	3

Table 4.1. Donor demographics
Number of participants per figure indicated in table, number with extra-pulmonary involvement (Ex Pulm) or South Asian (SA) origin indicated in parenthesis. CAS indicates serum biomarker clinical activity score: Lymphocyte count $<1.0x10^9$/l = 1; $>1.0x10^9$/l = 0. Serum ACE: >100 U/l = 2; 55-100U/l = 1; <55 U/l = 0. IgG >13g/l = 1; ≤ 13g/l = 0. Maximum score = 4.

T cell proliferation was assessed by a reduction in CFSE fluorescence and tritiated thymidine [3H-Tdr] uptake using a standard 5-day proliferation assay where T cells (peripheral blood leucocytes), were stimulated with allogenic monocyte-derived DCs (described in sections 2.2.12-13). Cell division was indicated by a reduction in CFSE staining intensity, as determined by flow cytometry or by thymidine incorporation. Monocyte suppression of proliferation was determined by the addition of monocytes to the assay. B

cells, isolated using CD20 positive magnetic selection, were used to confirm that any suppressive effect seen was due to monocyte activity and not due to restricted compartment size.

Surface phenotype and cytokine profiles were examined as previously described.

iNKT-monocyte interaction was examined using an in vitro co-culture assay where iNKT cells were cultured with negatively selected monocytes for 18 hours in H10 at a final ratio of 1:1 (250 000 cells total) (section 2.2.11). The super antigen αGalCer was not used to stimulate the iNKT cells in this system, because it is not found in humans and therefore does not accurately represent the situation in vivo where iNKT cells are thought to respond to bacterial and endogenous glycolipid as well as cytokines such as IL-12 and IL-18.

'iNKT cells' in this study refers to an established and previously phenotyped $CD4^+$ clone (LH22) (265) unless otherwise specified, in which case an iNKT line (iNKTLi) generated by FACS sorting $CD3^+6B11^+$ (described in section 2.2.4) was used. The iNKTLi was phenotyped to confirm surface and cytokine profile before use in the study.

iNKT-monocyte interaction studies were conducted using pre-treatment of monocytes with blocking antibodies, the doses of which were optimised for maximal functional blocking. The effect of direct contact was assessed by

separating monocytes in a Transwell™ insert from iNKT cells in the plate base (12 well plate). Imagestream™ flow cytometry was used to further visualise the interaction (section 2.2.8.13).

4.4. Results

4.4.1. Sarcoidosis monocytes were unable to suppress T cell proliferation

I commenced my investigation into the functional consequences of abnormal monocyte phenotype in sarcoidosis by examining the effect of sarcoidosis monocytes on T cell proliferation. A standard CFSE proliferation assay was used to determine proliferation as indicated by a reduction in fluorescence intensity (Figure 4.1a). The addition of monocytes from healthy controls suppressed T cell proliferation. This was concurrently verified by a tritiated thymidine [3H-Tdr] uptake assay (Figure 4.1b and c).

To ensure that the observed suppression was not due to an increased number of cells resulting in a lack of 'immune space', monocytes were substituted by the same number of irradiated $CD20^+$ B cells; this confirmed that, unlike monocytes, the same numbers of B cells do not suppress T cell proliferation (Figure 4.1d).

Sarcoidosis monocytes were then added to the assay (sarcoidosis monocytes, healthy control T cells and DCs). This demonstrated that sarcoidosis monocytes are not capable of suppressing DC-induced T cell

proliferation (Figure 4.1e). As expected, allogeneic T cell proliferation in the periphery is significantly lower in sarcoidosis than healthy controls, the addition of control monocytes does not further suppress proliferation (Figure 4.1f).

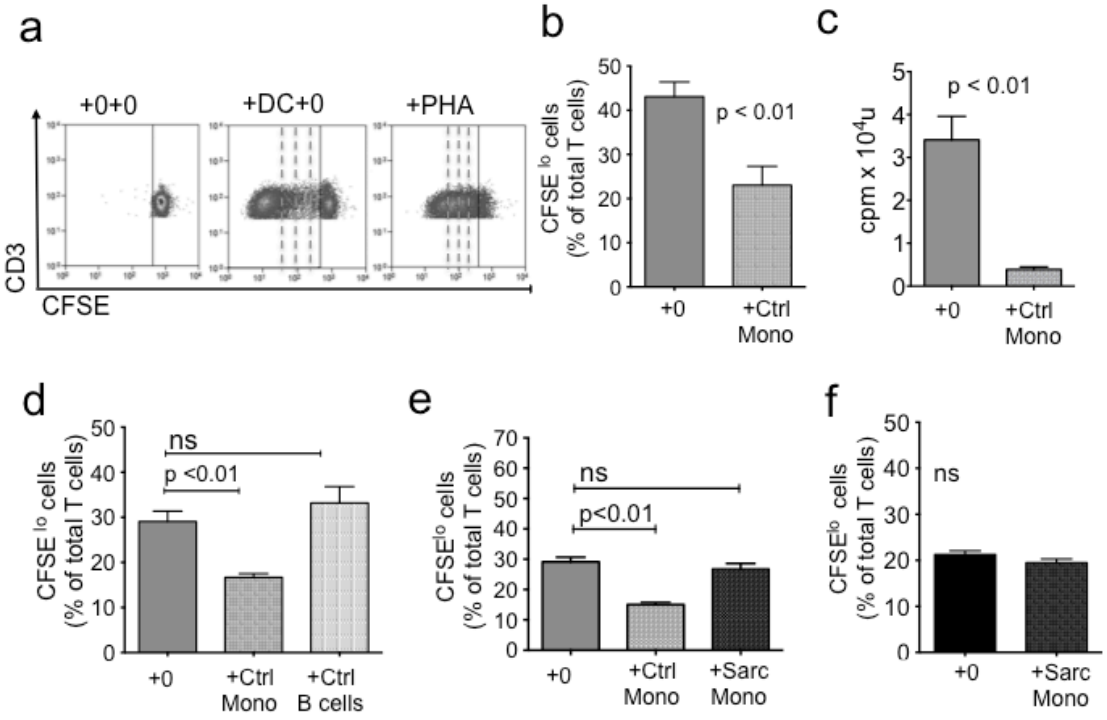


Figure 4.1. Sarcoidosis monocytes were unable to suppress T cell proliferation.

CFSE labeled T were stimulated with allogenic DCs; proliferation was measured at 5 days by reduction in fluorescence intensity. $CFSE^{lo}$ represents CFSE staining lower than non-proliferating baseline (staining to the left of solid vertical line), broken vertical lines divide daughter cells. (a) Representative plots illustrating CFSE staining (PHA stimulated cells as a control). (b) Monocytes suppress T cell proliferation: +0: % $CFSE^{+}$ cells 43.07 ±3.32 $CFSE^{lo}$ cells; + monocytes: 23.03 ± 4.27 (n = 15 per group, 5 independent experiments). (c) Proliferation additionally assessed by thymidine uptake by dividing cells: +0: 3.41 ± 0.55 counts per minute (cpm); + monocytes: 0.39 ± 0.06 (n = 15 per group, 5 independent experiments). (d) Substitution of monocytes by B cells did not result in suppression of proliferation: +0: 29.08 ± 2.3 $CFSE^{lo}$ cells; + B cells: 33.20 ± 3.613 (ns) (n = 7 per group, 2 independent experiments). (e) Sarcoidosis monocytes do not suppress T cell proliferation: +0: 29.13 ± 1.49 $CFSE^{lo}$ cells; + control monocytes: 15.03 ± 0.66; + sarcoidosis monocytes: 26.83 ± 1.74 (n = 7 per group, 2 independent experiments). (f) Sarcoidosis T cell proliferation is reduced and is not further suppressed by the addition of control monocytes. The baseline level of T cell proliferation varies between experiments therefore suppression is assessed against baseline for each experiment. Mean ± SEM illustrated. D'Agostino and Pearson omnibus normality test and Student t test or one-way ANOVA with Tukey post-test comparison were used for statistical analysis.

4.4.2. Monocyte suppression capacity was IL-10 dependent

Exogenous IL-10 is known to suppress T cell proliferation and, having established that sarcoidosis monocytes are deficient in IL-10 production, the relationship between monocyte IL-10 production and suppressive capacity was examined. In order to determine the contribution of monocyte IL-10 to suppression, the presence and quantity of IL-10 found in the proliferation assay supernatant were first determined on day 1, 3 and 5 (Figure 4.2a). This was maximal on day 1 (18 hours after commencing the assay), the quantity steadily decreased over the time course and a mean of 3.3ng/ml of IL-10 was still present in the co-culture supernatant at day 5; IL-10 was not detected in the absence of monocytes.

IL-10 receptor blockade (CD210) on days 1, 2 and 4 after initiation of culture restored the T cell proliferative capacity to levels observed without addition of monocytes (Figure 4.2b), implying that IL-10 was responsible for the T cell suppression. Although, in this assay, it was most likely that IL-10 originated from monocytes, there was a theoretical possibility that leukocyte-monocyte interaction could generate IL-10 producing lymphocytes. Since it was not possible to selectively block IL-10 originating from monocytes in this system, the IL-10 producing fraction of monocytes was isolated by using a IL-10 capture magnetic bead assay, which enriches the IL-10 producing subset from 15-20% of monocytes to 50-60%. Addition of IL-10-enriched monocytes reduced T cell proliferation by nearly the same percentage (mean 38% reduction) as that when non-enriched monocytes were added (mean 47% reduction) implying that nearly all the suppressive effect was accounted for by

IL-10 producing monocytes (Figure 4.2c). Suppression was not observed with addition of IL10 negative monocytes. Together, these in vitro findings support the notion that monocytes in the vicinity of activated T cells could control T cell proliferation via the production of IL-10.

To assess whether monocyte IL-10 production impacts on the level of disease activity as assessed clinically. Using a predefined, composite measure of clinical activity designed and validated by other members of the group (Appendix 2 and Table 4.2), patients were recruited spanning the entire predefined clinical activity score (0 to 12). Monocytes from patients exhibiting the lowest clinical disease activity score had the highest number of IL-10 producing monocytes and the greatest capacity for IL-10 production after LPS stimulation (Figure 4.2d and e). This suggests a potential link in vivo between IL-10 production by monocytes and clinical manifestation of disease at presentation, and indirectly with T cell activity and granuloma burden.

CXR Score
i. Normal = 0 ii. (ii) Bilateral hilar lymphadenopathy or reticulation only (suggesting established fibrosis) = 1 iii. Nodules or consolidation ± (ii) = 2 Disease extent Score 1 for each CXR zone affected (upper, mid and lower zones; right and left)
Cellular Score
i. Lymphocyte count <1.0x10^9/L = 1; >1.0x10^9/L (normal) = 0 ii. Serum ACE >100U/L =2; 55-100U/L = 1; <55 U/L (normal) = 0 iii. IgG >13g/L = 1; ≤ 13g/L = 0
Total maximum score 12

Table 4.2. Clinical Activity Score used to provide a measure of disease activity.
CXR 'zones' refer to conventional radiographic definition: above anterior aspect of 2nd rib is upper zone; between anterior aspect of 2nd and 4th rib is mid zone; below 4th rib is lower zone.

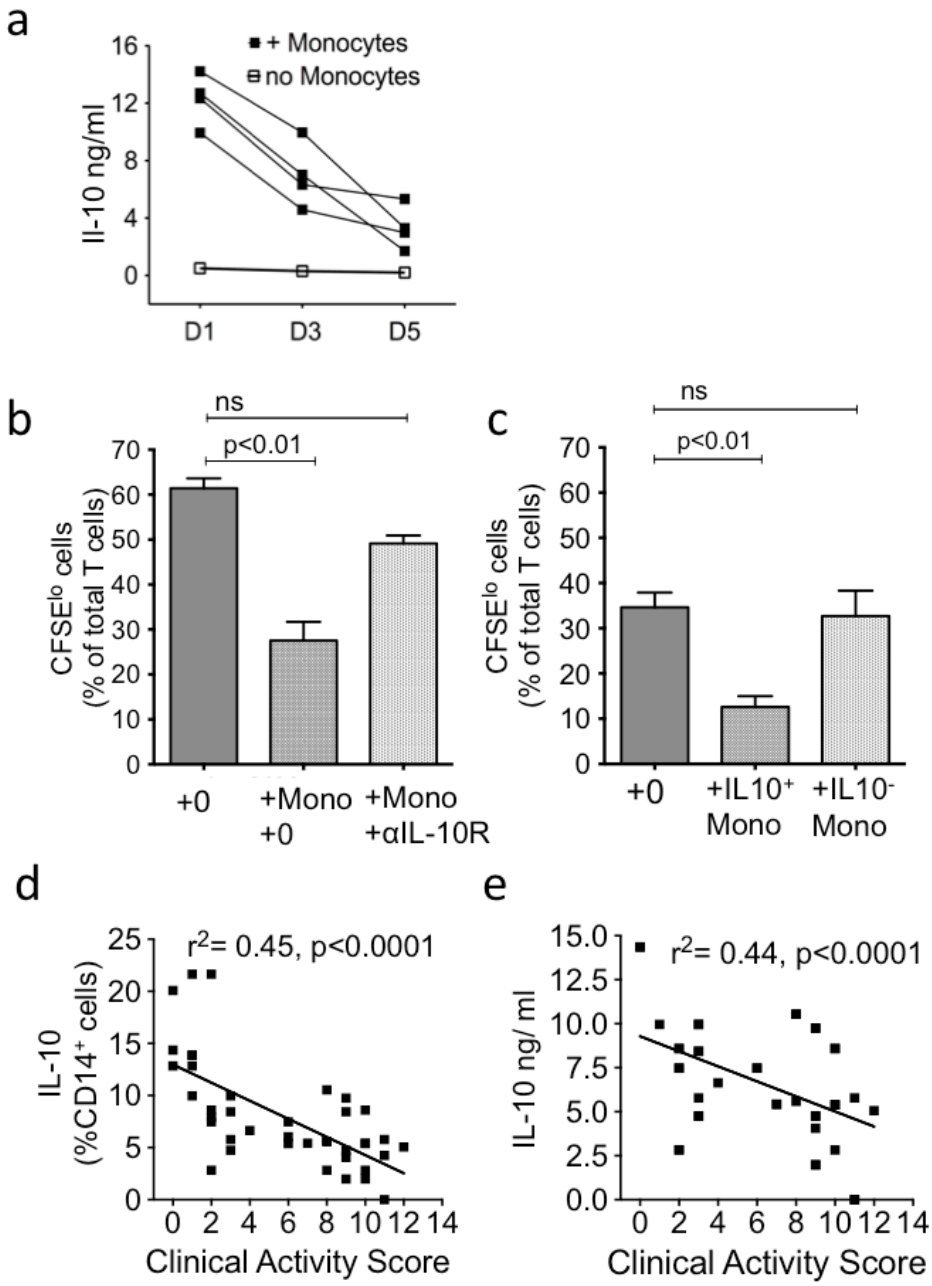


Figure 4.2. Monocyte suppression of T cell proliferation was IL-10 dependent.

(a) IL-10 production by monocytes in the proliferation assay supernatant is maximal at 18 hours. No IL-10 was detectable in the supernatant in the absence of monocytes. (b) Suppression of T cell proliferation was abrogated by the addition of IL-10R blocking antibody (αIL-10R): +0: 61.44 ± 2.18 $CFSE^{lo}$ cells; + monocytes alone: 27.54 ± 4.16; + monocytes + αIL-10R: 49.15 ± 1.76. (c) Similarly, $IL\text{-}10^{-}$ monocytes lacked the capacity to suppress T cell proliferation: +0: 34.63± 3.26 $CFSE^{lo}$ cells; $+IL\text{-}10^{+}$ monocytes 12.62 ± 2.37; + $IL\text{-}10^{-}$ monocytes: 32.68 ± 5.63 (n=9 per group, 3 independent experiments). Correlation between disease activity as measured using a pre-defined composite score (Table 4.2) and (d) proportion of IL-10 producing sarcoidosis monocytes and (e) IL-10 levels in supernatant of 125 000 LPS-stimulated monocytes (n=40). Mean ± SEM illustrated. Kruskal-Wallis with Dunn's multiple comparisons post-test analysis was performed. Correlation was assessed using Spearman's Rank test.

4.4.3. IL-10 producing monocyte phenotype

In order to try and identify the IL-10 producing monocyte subset without requiring fixing and permeablisation, which prevents them for being selectively used in assays, and to further understand the altered activity of this subset in sarcoidosis, I examined their surface phenotype. Very few (1%) of the IL-10 producing monocytes express CD16 and there was no difference in CD16 expression between the two groups (Figure 4.3a). The activation markers CD15, CD11b and CD115, were significantly down regulated in the sarcoidosis IL-10 producing subset (Figure 4.3b, c and d). The surface molecules PD1, which negatively regulates T cell activation by APCs, and CD1d, were both significantly down regulated in the sarcoidosis IL-10 producing monocyte (90% expression to 15% and 96% to 62% respectively) (Figure 4.3e and f). The mannose receptor (CD206) was significantly down regulated and negligible in the sarcoidosis IL-10 monocyte (Figure 4.4a). There was no difference in the expression of the scavenger receptor and activation marker CD163 a scavenger receptor (Figure 4.4b). There was a down regulation in the expression of the other phagocytic marker examined, CD32 (Figure 4.4c). The expression of the adhesion molecules CD62L and CX3CR1 were both down regulated in sarcoidosis IL-10 producing monocytes (Figure 4.4d and e). There was no significant difference in the monocyte chemoattractant receptor CCR2 (Figure 4.4f). Therefore, the IL-10 expressing monocytes were $CD14^{+}CD16^{-}CD206^{-}CD115^{+}CD15^{-}$. They expressed CD1d, were MCP-1^{lo} and TNFα^{lo}. No single or combination of markers tested could reliably identify the IL-10 producing subset.

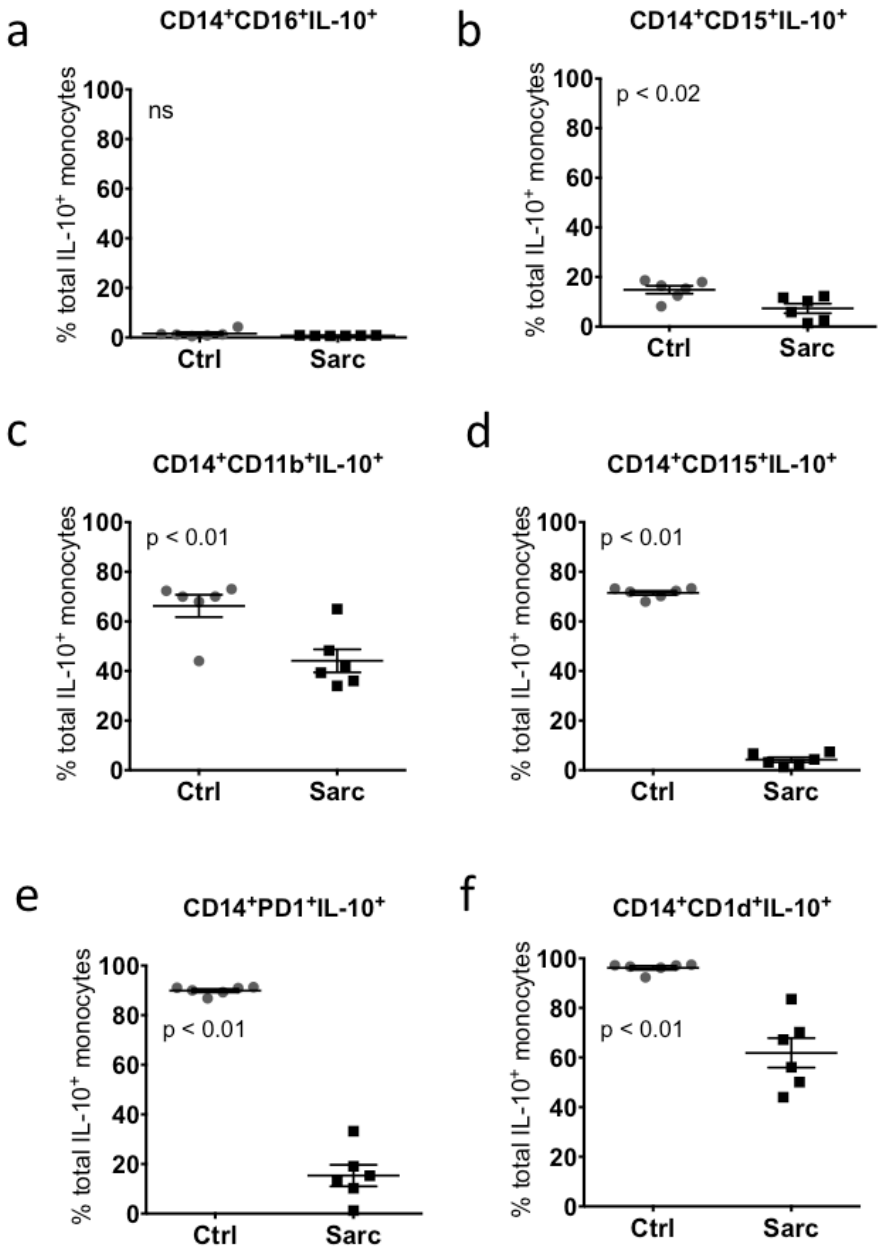


Figure 4.3. IL-10+ monocyte surface antigen phenotype.
IL-10 monocyte surface antigen phenotype was assessed by flow cytometry after 18 hours stimulation with LPS in order to identify the IL-10$^+$ cells. Antigen expression is given as % of total CD14$^+$ cells. (a) There was no significant difference in CD16 expression between sarcoidosis 1.19 [0.77-2.07] and controls 0.74 [0.68-0.86]. (b) CD15 expression was lower in sarcoidosis 8.01 [2.27-11.80] than controls 15.90 [11.39-18.12] as was expression of (c) CD11b sarcoidosis 40.66 [35.50-52.42] controls 70.00 [62.00-72.50], (d) CD115 sarcoidosis 3.80 [2.16-6.86], controls 72.11 [69.66-73.23], (e) PD1 sarcoidosis 14.14 [7.98-22.63], controls 90.48 [88.62-91.11] and (f) CD1d sarcoidosis 61.66 [48.59-73.56], controls 96.87 [95.24-97.26]. (n=6 all groups). Mean ± SEM illustrated on graphs. Median [IQR] supplied text. D'Agostino and Pearson omnibus normality test a Mann-Whitney test was used for statistical analysis.

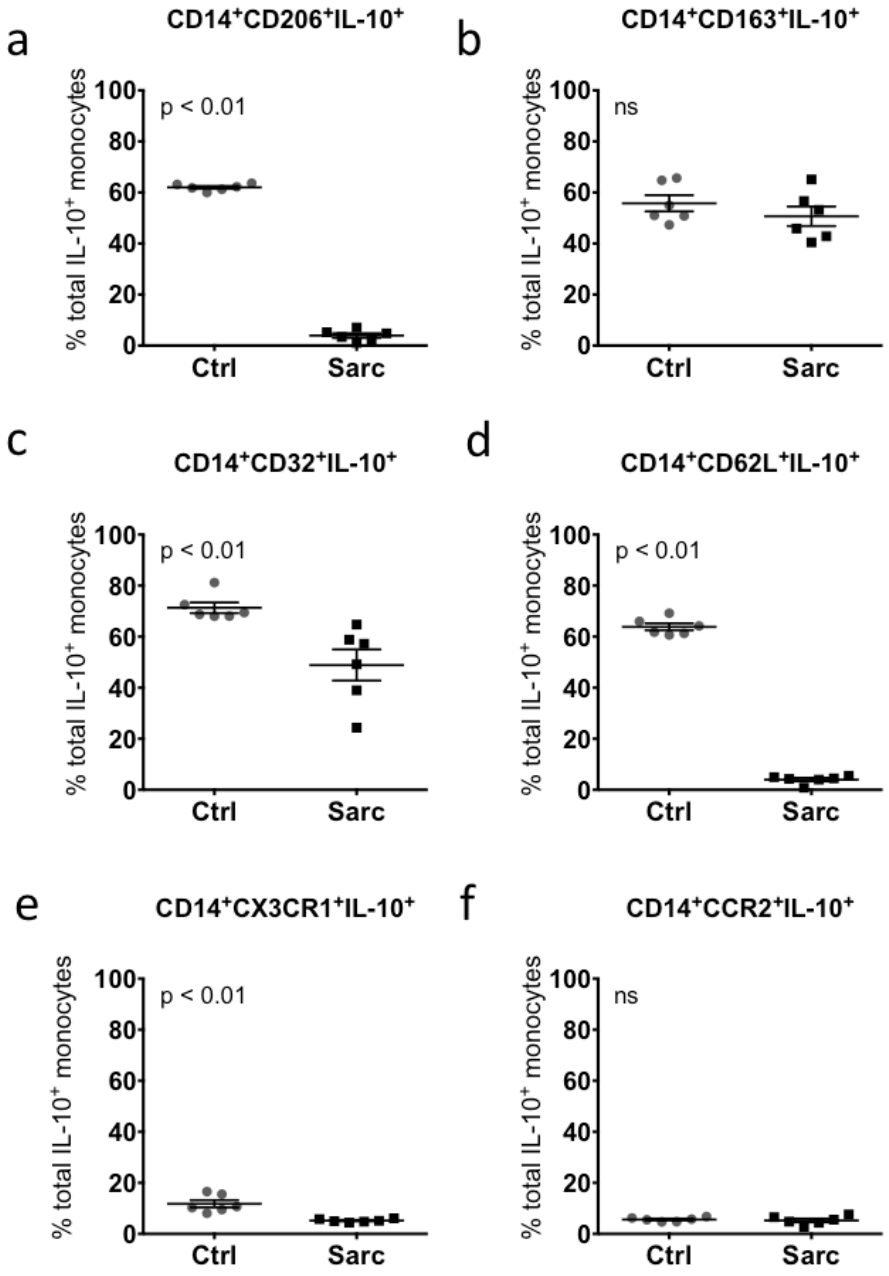


Figure 4.4. IL-10+ monocyte surface antigen phenotype.
IL-10 monocyte surface antigen phenotype was assessed by flow cytometry after 18 hours stimulation with LPS in order to identify the IL-10$^+$ cells. Antigen expression given as % of total CD14$^+$ cells. (a) CD206 expression was much lower in sarcoidosis 4.05 [1.71-5.68] than controls 62.01 [60.89-63.23] as was (c) CD32 sarcoidosis 53.22 [35.34-60.33], controls 68.09 [68.06-74.73], (d) CD62L sarcoidosis 4.39 [3.21-5.11], controls 63.00 [61.14-66.81] and (e) CX3CR1 sarcoidosis 5.06 [4.69-5.86] controls 10.49 [9.14-15.81]. There was no significant difference in the expression of (b) CD163 sarcoidosis 49.50 [42.19-58.76], controls 52.97 [49.91-64.98] or (f) CCR2 sarcoidosis 5.19 [3.92-6.83], controls 5.66 [4.74-6.37] (n=6 all groups) Mean ± SEM illustrated on graphs. Median [IQR] supplied text. D'Agostino and Pearson omnibus normality test and a Mann-Whitney test were used for statistical analysis.

The additional cytokine activity of the IL-10 producing subset was then characterised. There were fewer IL10-TNFα double producing monocytes in sarcoidosis than in controls (Figure 4.5a), no difference in the proportion of monocytes expressing the pro-inflammatory cytokines IL-12 and IL-17 (Figure 4.5b and c) nor the regulatory IL-4 (Figure 4.5d). Despite there being minimal difference in the overall proportion of monocytes expressing CCR2, the relative proportion of its ligand CCL2 was significantly decreased in the sarcoidosis monocyte subset (Figure 4.5e).

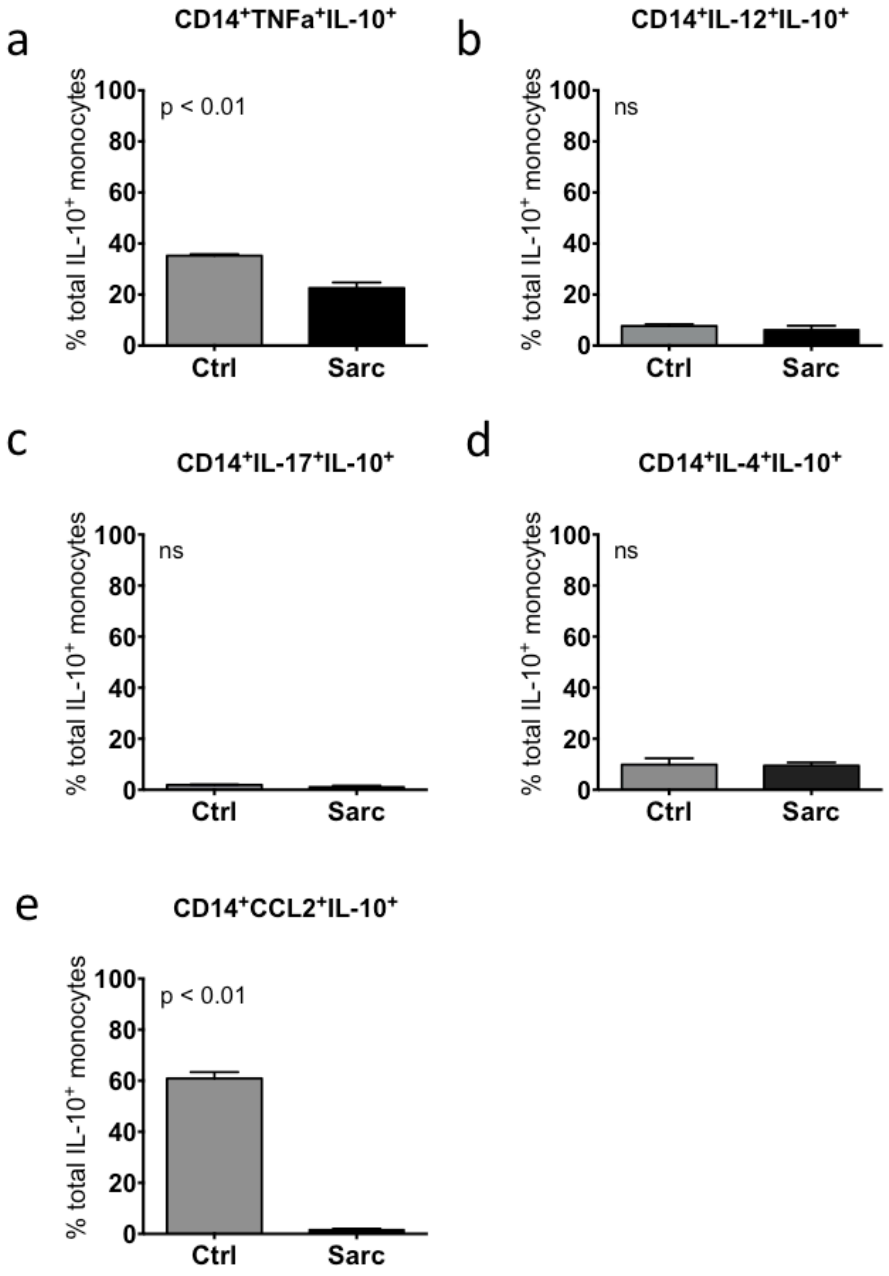


Figure 4.5. IL-10+ monocyte cytokine phenotype.
Co-expression of additional cytokines by IL-10 monocyte was assessed by ICS after 18 hours stimulation with LPS. Cytokine expression given as % of total $CD14^+$ cells. (a) TNFα expression was lower in sarcoidosis 21.83 [18.77-26.91] than controls 35.08 [33.86-36.18] as was (e) CCL2 sarcoidosis 1.73 [0.89-2.33], controls 62.65 [57.40-65.63]. There was no difference in the co-expression of (b) IL-12 sarcoidosis 6.26 [4.64-7.34], controls 7.91 [6.99-8.12], (c) IL-17 sarcoidosis 0.73 [0.59-2.46], controls 1.75 [1.68-2.77] or (d) IL-4 sarcoidosis 9.33 [8.53-10.25], controls 10.12 [7.87-12.03]. Mean ± SEM illustrated on graphs. Median [IQR] supplied text. D'Agostino and Pearson omnibus normality test and a Mann-Whitney test were used for statistical analysis.

4.4.4. iNKT cells augmented monocyte suppression T cell proliferation by inducing monocyte IL-10

In order to assess the impact of reduced iNKT number in sarcoidosis, I first confirmed that the number of iNKT cells in the study population was significantly reduced (Figure 4.6a and b). I then examined the relationship between iNKT cell number and monocyte IL-10 expression and found that the number of circulating iNKT cells correlated with IL-10 secretion by monocytes (Figure 4.6c).

I then investigated whether human iNKT cells can influence monocyte IL-10 production. LPS- stimulated monocytes were co-cultured with iNKT cells at a ratio of 1:1. iNKT cells were not additionally stimulated using agents such as α-GalCer or β-glucocerebroside, as it was found that the use of the former led to decreased monocyte survival in the culture system. IL-10 expression was determined by both ICS and ELISA for secreted protein in supernatant of fixed numbers of monocytes. For the first six samples, it was found that addition of iNKT cells increased IL-10 expression on monocytes by ICS with no significant difference between the ratios used and so a ratio of 1:1 was used in the remaining experiments (Figure 4.6d). iNKT cells have previously been reported to produce IL-10 under certain conditions, I therefore I confirmed by flow cytometry that, in this co-culture system that monocytes ($CD14^+$) were the sole source of IL-10 in the supernatant (Figure 4.6e).

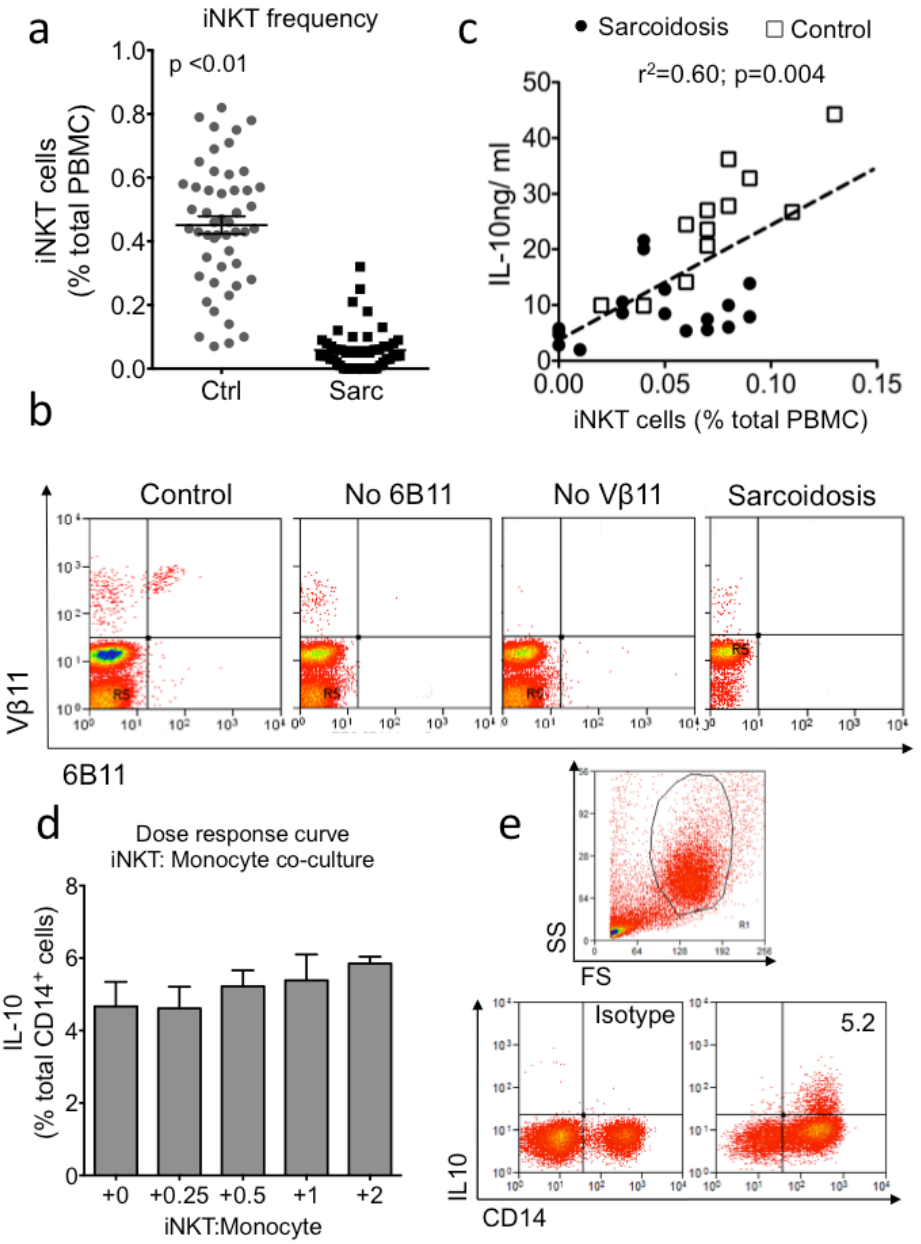


Figure 4.6. iNKT cell number was reduced in sarcoidosis, corresponding to a reduced monocyte IL-10 capacity.
iNKT cells assessed using flow cytometry & identified as $CD3^{+}6B11^{+}V\beta11^{+}$ cells. 6B11 identifies the invariant CDR3 region of the Vα24-Jα18 human iNKT TCR, Vβ11 identifies TCR-β chain. (a) iNKT cells are reduced in sarcoidosis (% total PBMC): [sarcoidosis 0.06 ± 0.01; controls 0.45 ± 0.03] (n = 50 per group, mean ±SEM, Student T test). (b) Representative staining with FMO controls. (c) Correlation of circulating iNKT cell number with IL-10 level in supernatant of LPS-stimulated monocytes (18 hours) from sarcoidosis (n=18) and healthy controls (n=12). Values shown are correlation using all data (i.e. sarcoidosis and controls). If these were analysed separately, correlation between iNKT levels and IL-10 from monocytes was stronger (r2=0.86; $p < 0.0001$); there was no significant correlation between the two parameters for sarcoidosis patients (Spearman's Rank correlation test). (d) No significant difference was seen in monocyte IL-10 expression when co-cultured with monocytes at different ratios (Friedman test). (e) $CD14^{+}$ monocytes were the sole producers of IL-10 in the co-culture system.

There was a significant increase in both the proportion of IL-10-expressing monocytes and the amount of IL-10 produced after co-culture with iNKT cells (Figure 4.7a and b). Indeed, the addition of iNKT cells to sarcoidosis monocytes restored the proportion of IL-10 producing cells to that observed in control monocytes (Figure 4.7c), and increased the levels of IL-10 in the supernatant of these co-cultures (Figure 4.7d).

iNKT cells are known to produce a wide range of cytokines and chemokines under different circumstances, it is likely that not every iNKT cell can achieve everything reported in the iNKT cell repertoire. The iNKT clone used (LH22) has previously been extensively phenotyped using CD1d tetramers. This has been described in detail previously (265). Confirmation of maintenance of the clone phenotype was regularly performed using αGalCer presented by DCs. To ensure that the iNKT mediated increase in monocyte IL-10 was not clone-specific, I generated an iNKT line (iNKTLi) (section 2.2.4). Preliminary iNKT line phenotyping was conducted in parallel with the established clone at 4 and 18 hours ± using αGalCer presented by DCs. The line comprised $CD4^+CD8^+$, $CD4^+CD8^-$ and $CD4^-CD8^+$ subsets (Figure 4.8a) and produced TNFα, IFNγ and IL-4 with differing kinetics to the iNKT22 clone. There was very little IL-2, IL-10, GM-CSF, IL-17A and IL-12 production from either (Figure 4.8a-b and 4.9a). More comprehensive phenotyping including cytokine profiling at different time-points and response to different agonists presented by CD1d tetramer, such as βGluCer are planned. The ability of the iNKT line to induce monocyte IL-10 was assessed against the clone (iNKT22). Co-culture with

the line increased the proportion of monocytes expressing IL-10 both compared with control monocytes and those co-cultured with iNKT22 (4.7e).

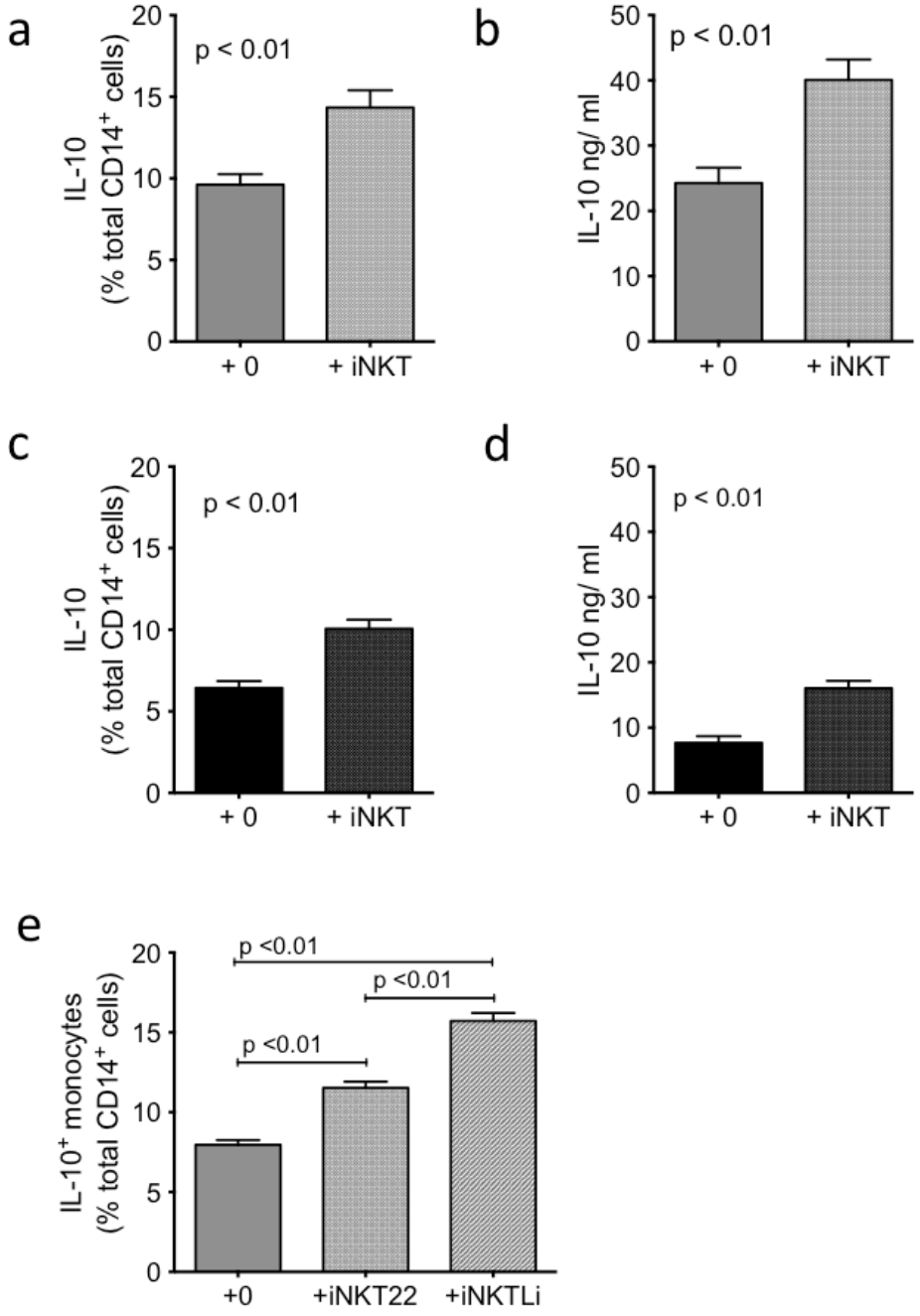


Figure 4.7. Monocyte IL-10 production was augmented by co-culture with iNKT cells.

In both control and sarcoidosis monocytes, there was a significant increase in both (a) the proportion of IL-10-expressing monocytes [control: +0: 9.61 ± 0.64 (% $CD14^+$ cells), +iNKT: 14.34 ± 1.01] (c) [sarcoidosis: +0: 6.43 ± 0.41, +iNKT: 10.07 ± 0.55] and (b) the amount of IL-10 produced [controls: +0: 24.28 ± 2.33 (ng/ml), +iNKT: 40.07 ± 3.13] (d) [Sarcoidosis +0: 7.68 ± 1.01, +iNKT: 16.03 ± 1.15] after co-culture with iNKT cells (n = 36 sarcoidosis, 27 controls). (e) iNKT induced increase in monocyte IL-10 production was verified by co-culture with iNKTLi: +0: 7.96 ± 0.29 (% $CD14^+$ cells); +iNKT22: 11.53 ± 0.38; +iNKTLi: 15.72 ± 0.51. (n=12). Mean ± SEM. D'Agostino and Pearson omnibus normality test and Student paired T test or Anova with Tukey post-test comparisons were performed.

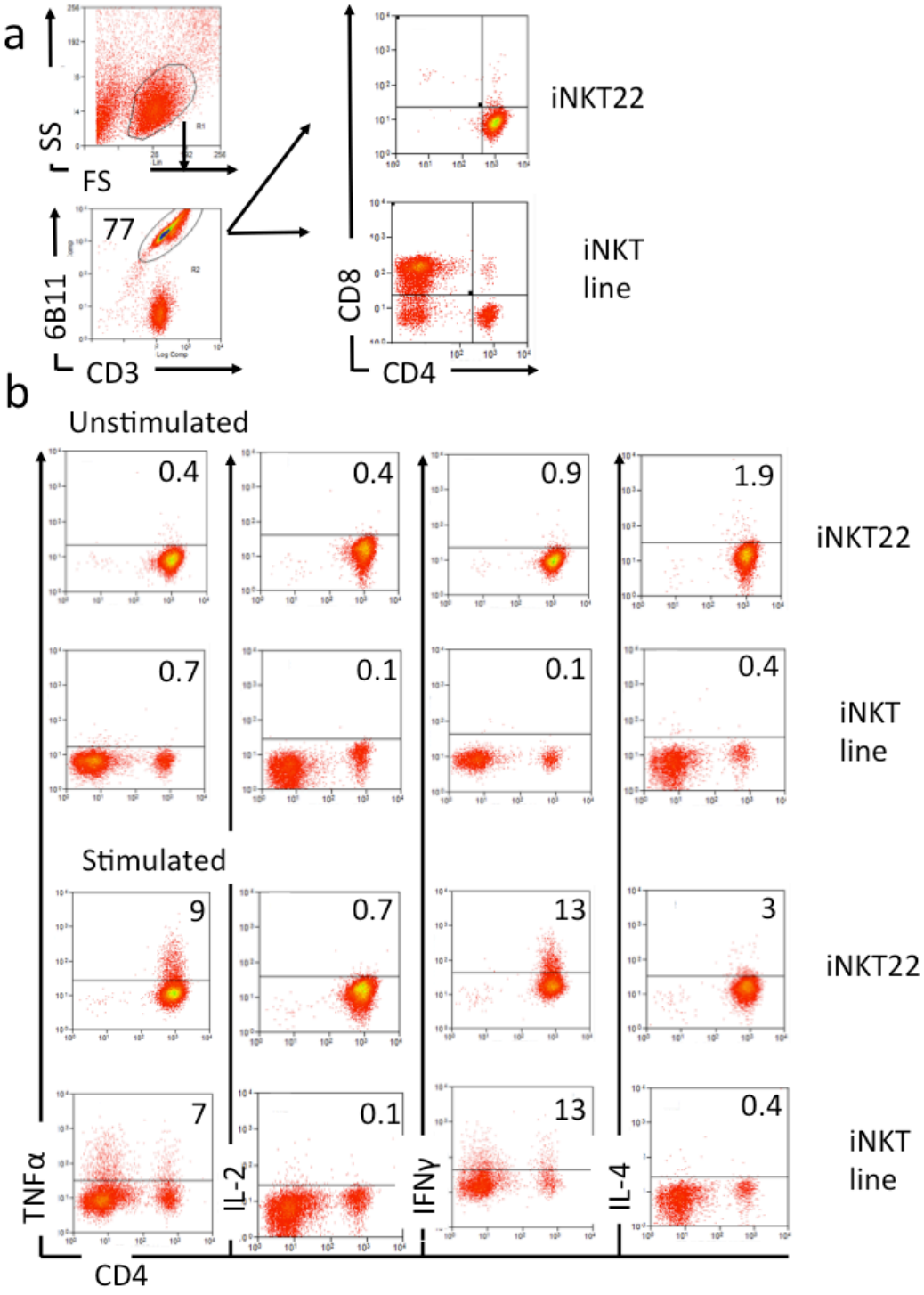


Figure 4.8. iNKT line phenotyping
iNKTLi phenotyping by flow cytometery ± 6 hour αGalCer stimulation [presented by DCs], iNKT22 staining illustrated for comparison. Figures indicate percentage of total cells represented in the gate. (a) iNKT line comprises $CD4^+$, $CD8^+$ and $CD4^+CD8^+$ subsets. (b) iNKTLi produces TNFα and IFNγ with similar kinetics to iNKT22. No IL-2 or IL-4 staining is seen.

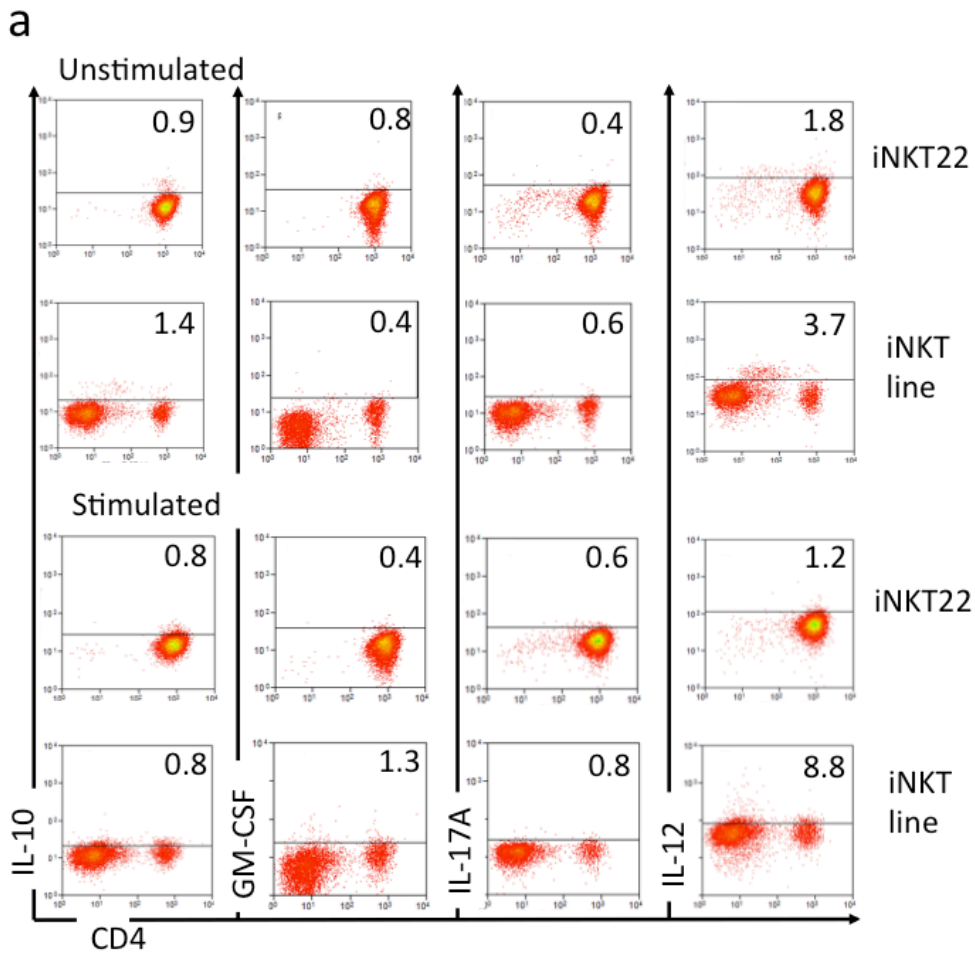


Figure 4.9. iNKT line phenotyping
iNKTLi phenotyping by flow cytometery ± 6 hour αGalCer stimulation. iNKT22 staining illustrated for comparison. Figures indicate percentage of total cells represented in the gate. (a) The $CD4^-$ iNKTLi produces IL-12, in contrast to the $CD4^+$ iNKTLi or iNKT22 (exclusively $CD4^+$). No IL-10, GM-CSF or IL-17A is seen from either iNKTLi or iNKT22.

When iNKT cells (but not B cells) were added to both the CFSE assay (as described in Figure 4.1d) and the thymidine uptake assay, the suppressive capacity of the control monocytes was augmented (Figure 4.10a and b) and that of sarcoidosis monocytes was restored to levels observed for control monocytes (Figure 4.10c).

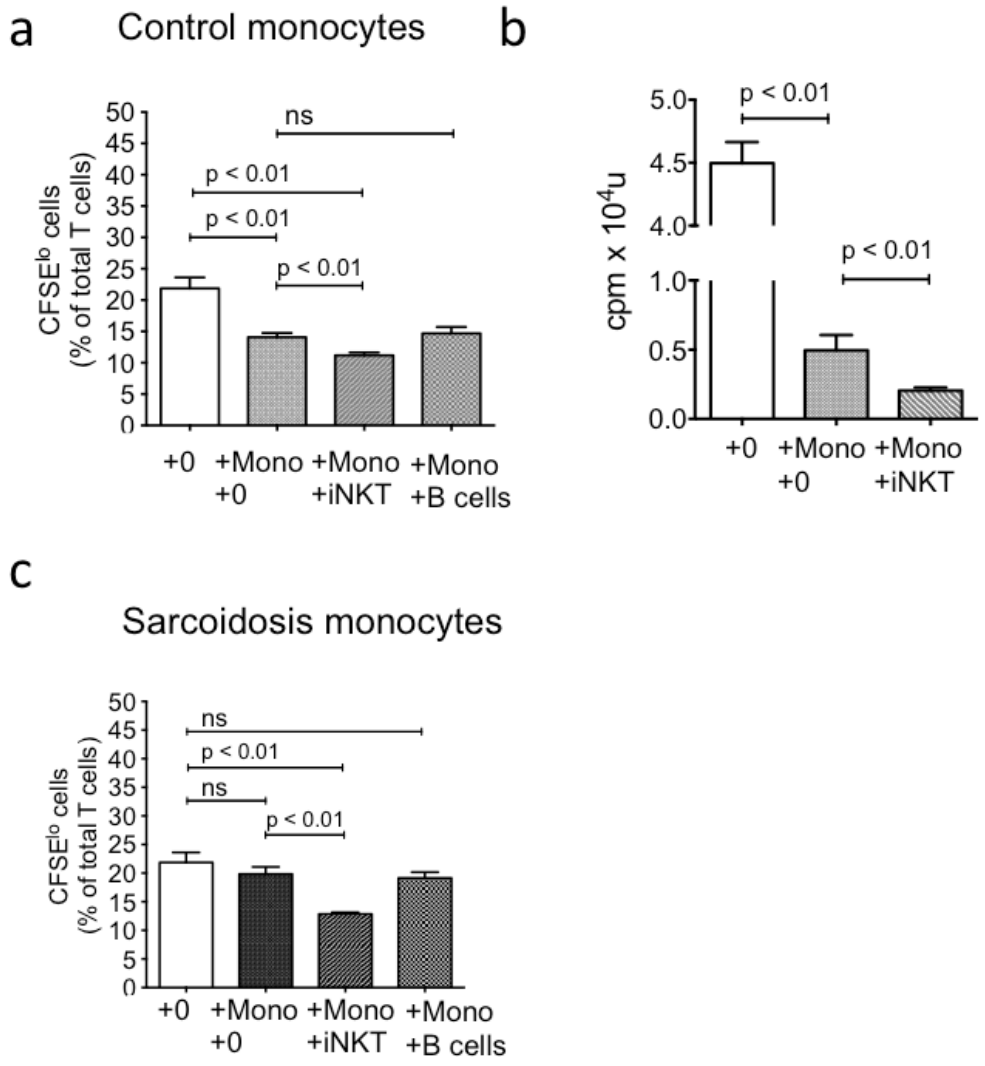


Figure 4.10. iNKT cells augmented control monocyte suppression of T cell proliferation.
(a) Addition of iNKT cells to the CFSE proliferation assay further increases monocyte suppression of T cell proliferation. $CD20^+$ B cells were added as a control to ensure proliferation was not being curtailed by lack of space: +0: 21.87 ± 1.74 $CFSE^{lo}$ cells (% total T cells); +Monocytes alone: 14.07± 0.69; +Monocytes +iNKT: 11.17 ±0.46; +Monocytes +B cells: 14.69 ±1.01. (b) Augmentation of monocyte suppression of T cell proliferation confirmed by thymidine uptake: +0: 4.50 ± 0.17 cpm; +Monocytes alone: 0.50 ± 0.11; +Monocytes +iNKT: 0.20 ± 0.02. (c) Sarcoidosis monocyte suppression of T cell proliferation is rescued by the addition of iNKT cells but not B cells: +0: 21.87 ± 1.74 $CFSE^{lo}$ cells (% total T cells); +Monocytes alone: 19.84 ± 1.25; +Monocytes +iNKT: 12.86 ± 0.30; +Monocytes +B cells: 19.16 ±1.03. (n = 7 all groups, 2 independent experiments per condition). Mean ± SEM illustrated. D'Agostino and Pearson omnibus normality test and one-way ANOVA with Tukey post test comparisons were used for statistical analysis.

4.4.5. iNKT cell induction of monocyte IL-10 was partially contact dependent

To determine if CD1d engagement was required for production of IL-10 from monocytes, monocytes were pulsed with 25μg/ml CD1d blocking antibody (CD1d41.2) before co-culture. This significantly reduced monocyte IL-10 expression, although did not return expression to baseline (Figure 4.11a). To determine physical contact was required for monocyte IL-10 production, monocytes were cultured with complete physical separation by a Transwell™ insert. This abolished the increase in monocyte IL-10 production induced by iNKT cells (Figure 4.11b and c). This suggests that direct physical signals, in addition to CD1d, such from adhesion and co-stimulatory molecules, may play a role in 'licensing' monocytes to produce IL-10 when interacting with iNKT cells.

To visualise this conclusion, I used Imagestream™ flow cytometry single-cell visualisation of single cell interaction. I observed that at any one point, 2% of cells are in contact with each other as doublets, regardless of the ratio of iNKT to monocytes. By adding fluorescent markers for monocytes and iNKT cells, it was observed that the doublets were single iNKT cells in direct contact with another monocyte (rather than non-specific aggregations of cells) (Figure 4.11d - f).

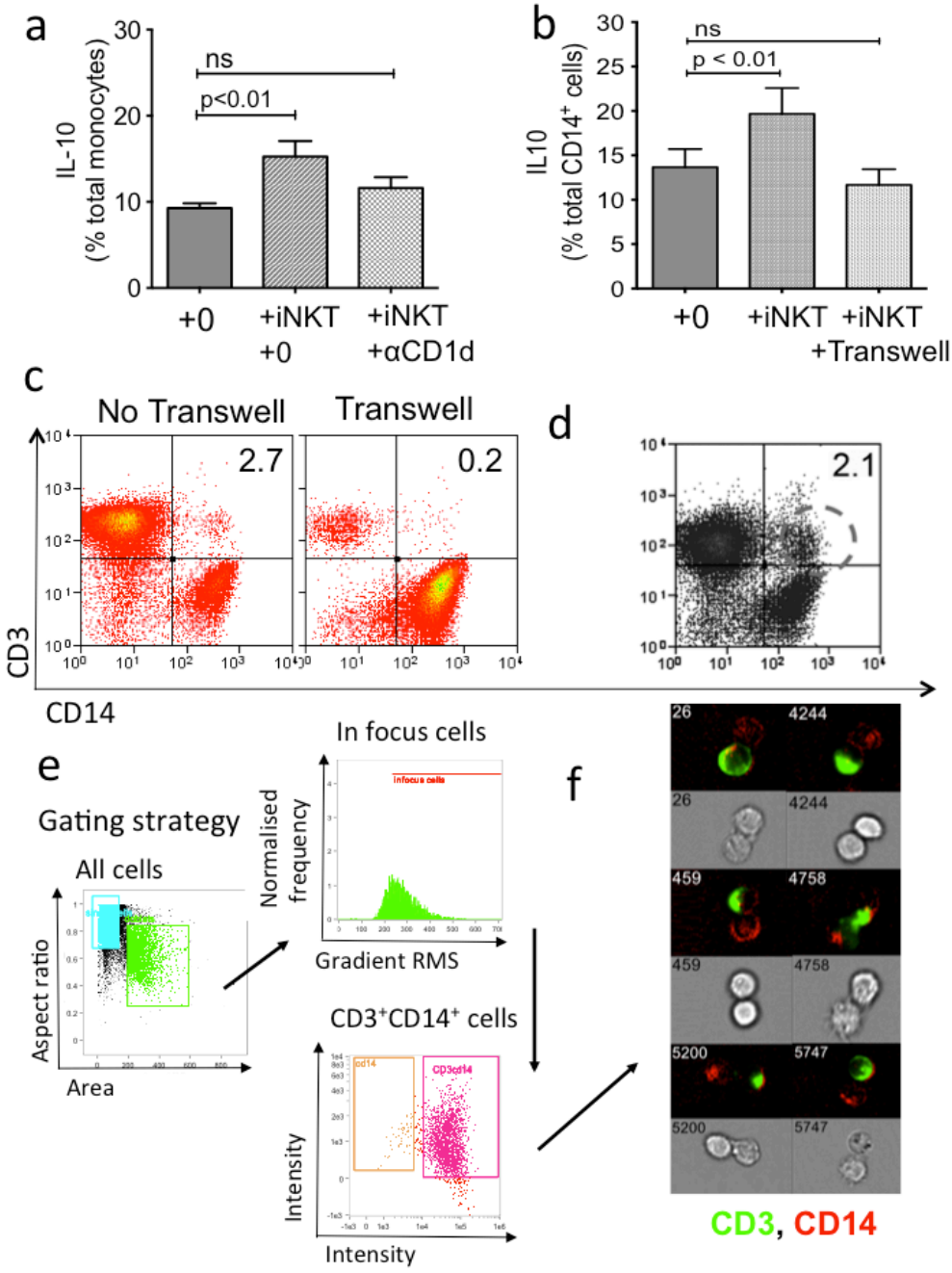


Figure 4.11. iNKT augmentation of IL-10 production was impaired by blocking CD1d.

The proportion of monocytes expressing IL-10 is increased by contact with IL-10 and reduced by (a) Cd1d blockade with 25μg/ml CD1d blocking antibody (CD1d41.2, αCD1d): +0: 9.27 ± 0.56 (IL-10$^+$CD14$^+$ cells [%total CD14$^+$ cells]; +iNKT: 15.26 ±1.81; +iNKT+ αCD1d: 11.61±1.26 and (b) physical separation of iNKT and monocytes with a Transwell insert (monocytes in upper chamber): +0: 13.67 ±2.03; +iNKT: 19.67 ±2.91; +iNKT +Transwell: 11.67 ±1.76. (n=7 per group, 2 independent experiments). D'Agostino and Pearson omnibus normality test and one-way ANOVA with Tukey post-test comparisons were used for statistical analysis. (c) Plots illustrating CD3$^+$CD14$^+$ (interacting) cells in iNKT-monocyte co-culture ± Transwell insert, detected by flow cytometry. (d-e) Plots indicate cells gated for Imagestream analysis: CD3$^+$CD14$^+$ in focus cells. (f) Illustrates monocyte-iNKT interaction detected by imagestream.

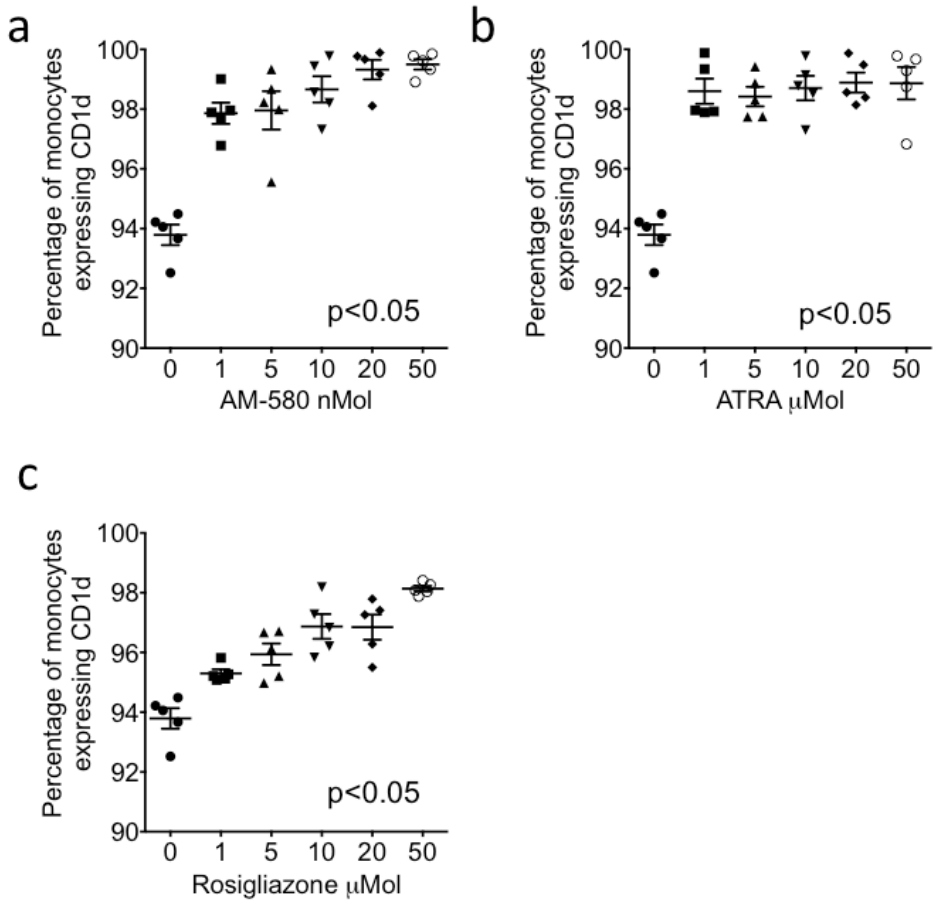


Figure 4.12. Monocyte CD1d expression was increased in a dose-dependent manner RAR signaling pathway agonists AM-580, ATRA and Rosiglitazone.
The percentage of monocytes expressing CD1d was increased after treatment with (a) AM-580 (b) ATRA and (c) Rosiglitazone in a dose dependent manner. D'Agostino and Pearson omnibus normality test and one-way ANOVA with Tukey post-test comparisons were used for statistical analysis.

I have shown that there was no difference in the surface expression of CD1d on control and sarcoidosis monocytes (Figure 3.6d), however, since the number of iNKT cells is reduced in sarcoidosis, I reasoned that an increase in the amount of CD1d expressed on monocytes could enhance the likelihood of interaction. CD1d expression on monocyte-derived DCs can be modulated by PPARγ (Rosiglitazone) and RARα ligands (AM-580 and ATRA) via the vitamin A pathway (348). After dose optimisation, AM-580, ATRA and Rosiglitazone were added to LPS stimulated monocytes for 18 hours. Whilst all these

agents increased CD1d expression on monocytes (4.12a-c) and independently suppress monocyte IL-10, they did not increase the amount of IL-10 expression after contact with iNKT cells (Figure 4.13a-f).

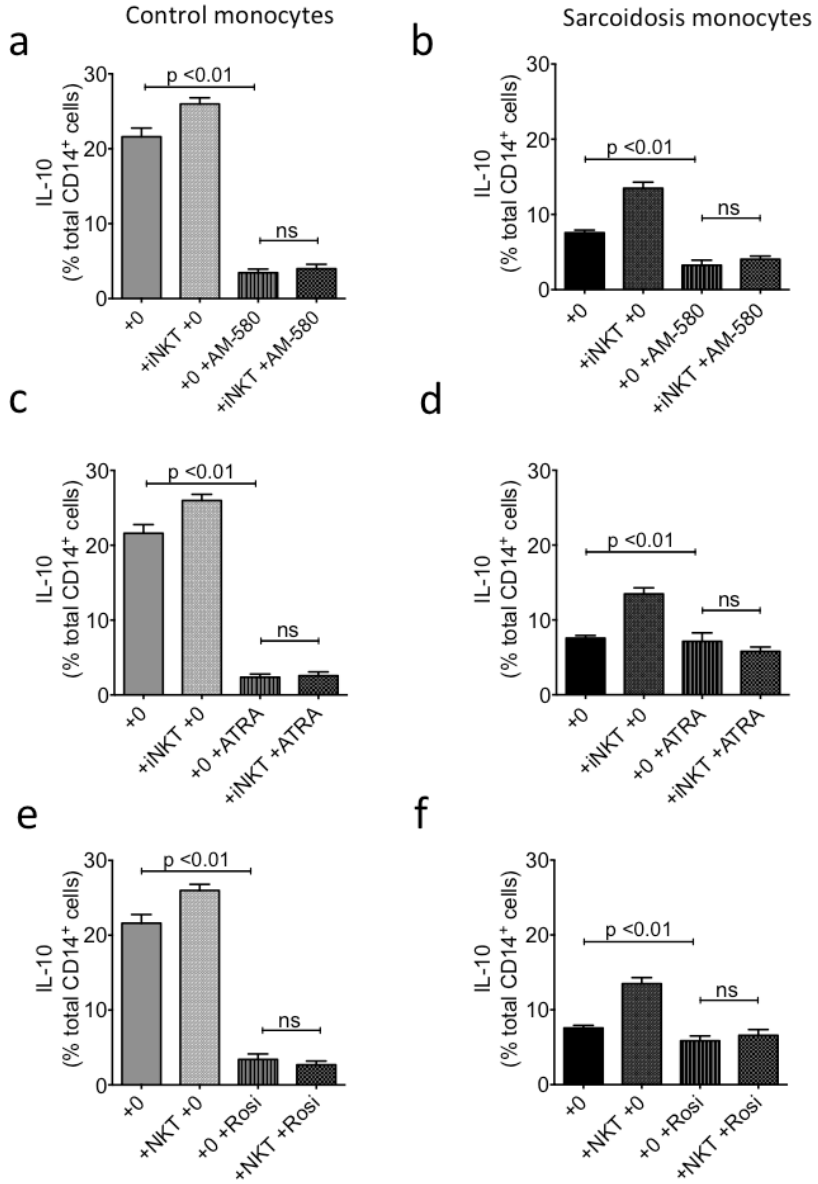


Figure 4.13. RAR pathway agonists independently suppressed monocyte IL-10 but did not increase expression after contact with iNKT cells.

The effect of RAR pathway agonists on monocyte IL-10 expression was assessed by flow cytometry. AM-50 (a-b), ATRA (c-d) and Rosiglitazone (e-f) all independently suppress IL-10 expression by both sarcoidosis and control monocytes. Despite increasing CD1d expression on monocytes, no further increase was seen after the RAR agonist treated monocytes were co-cultured with iNKT cells. Mean ± SEM illustrated. D'Agostino and Pearson omnibus normality test and one-way ANOVA with Tukey posttest comparisons were used for statistical analysis.

4.4.6. How do iNKT cells signal to monocytes?

Blocking CD1d only partially reverses the iNKT cell mediated induction of IL-10 whilst separating the two cell types by a Transwell™ insert results in a more complete reversal. It is therefore clear that CD1d mediated iNKT-monocyte interaction only contributes partly to the production of IL-10 from monocytes. I was interested in other potential pathways as additional signaling pathways for iNKT-monocytes cross talk: ICAM-1 and/or Dectin-1-mediated mechanisms.

ICAM-1 (Intercellular Adhesion Molecule-1) is an immunoglobulin supergene family and functions as a ligand for LFA-1 (Lymphocyte Function-Associated Antigen-1), a cell-cell and cell-matrix receptor and are both expressed on iNKT cells and monocytes. In a recent propionobacter murine model, purported by the authors to represent the situation in sarcoidosis (though this is contentious), a deficiency in ICAM-1 was found to result in decreased IL-10 production by T-regs (349). I therefore wanted to investigate whether ICAM-1 might be involved in iNKT-mediated induction of IL-10.

Monocytes were pre-pulsed with an ICAM-1 blocking antibody for 1 hour prior to the addition of iNKT cells (αI-CAM was left in for the duration of the 18 hour co-culture and was therefore available to block iNKT ICAM-1). There was no difference in IL-10 expression with the addition of αICAM-1, which remained at the increased levels observed with the addition of iNKT cells alone (4.14a). To ensure that there was adequate blocking of any potentially two-way interaction, the TNFα and CD107a response to iNKT cells were assessed.

The monocyte TNFα response to iNKT cell was abrogated by iCAM-1 blocking, as was the CD107a response (4.14b-c).

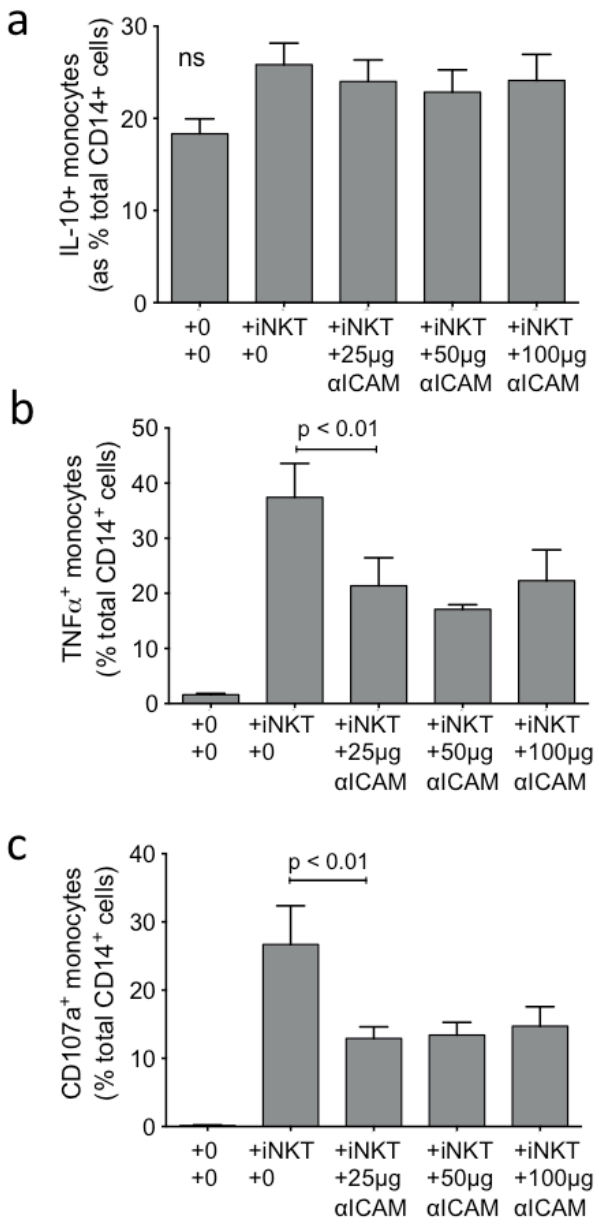


Figure 4.14. ICAM blockade did not affect monocyte iNKT induced IL-10 production.
Blocking ICAM on monocytes with increasing doses of ICAM blocking antibody (αICAM) did not alter iNKT cell induced monocyte IL-10 production (b) but did reduce monocyte expression of (b) TNFα [+iNKT: 30.76 ± 2.80 IL-10$^+$ monocytes (% total CD14$^+$ cells); +iNKT+ αICAM: 21.37 ±5.09] and (c) CD107a TNFα [+iNKT: 26.70 ± 5.66 CD107a monocytes (% total CD14$^+$ cells); +iNKT+ αICAM: 12.93 ±1.69], (controls to ensure ICAM blocking was occurring). (n=9). Mean ± SEM. D'Agostino and Pearson omnibus normality test and one-way ANOVA with Tukey post-test comparisons were used for statistical analysis.

The beta-glucan receptor Dectin-1 (CLEC7A, C-type lectin domain family 7 member A) is a non Toll-like pattern recognition receptor (PRR), expressed predominantly on monocytes, macrophages, and DCs. Signaling through Dectin-1 has been shown to up regulate the synthesis of IL-10 by human monocytes and murine DCs (350, 351). I thus wanted to ascertain whether Dectin-1 may be involved in the iNKT mediated induction of monocyte IL-10. Dectin-1 expression was confirmed on negatively selected monocytes (Figure 4.15a) and the expression was found to be up regulated after monocyte culture for 18 hours with iNKT cells in both controls and sarcoidosis (Figure 14.15b and c).

The relationship between iNKT cells, monocyte IL-10 and Dectin-1 was then explored by the addition of a Dectin blocking antibody. This suggested that the blocking Dectin-1 on monocytes reduces the ability of iNKT cells to induce IL-10 production in a dose dependent manner (Figure 14.15d) and the observation confirmed by use of the iNKT line (iNKTLi) (Figure 14.15e).

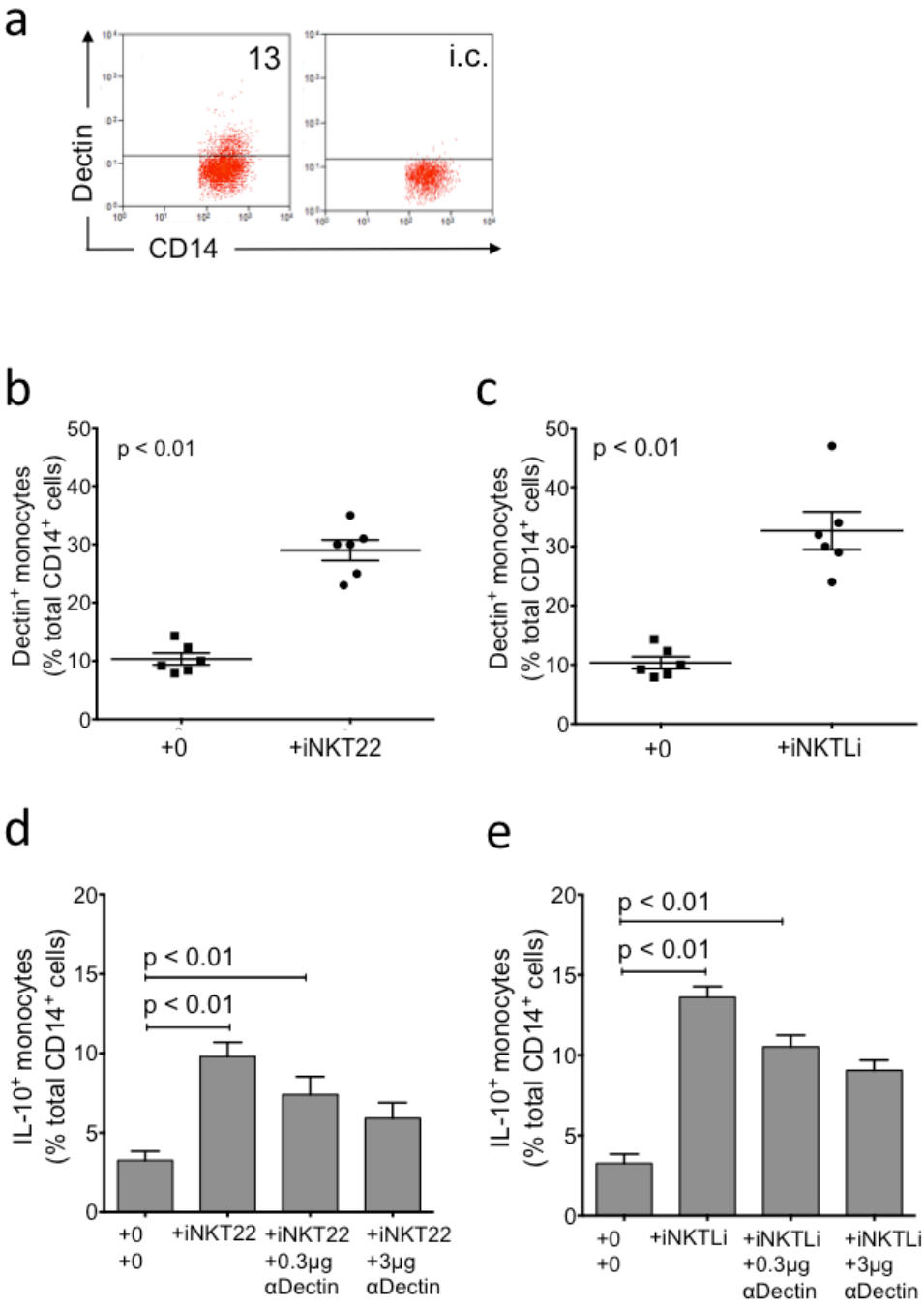


Figure 4.15. Dectin-1 blockade inhibited monocyte iNKT induced IL-10 production.

(a) Monocyte expression of Dectin-1 was confirmed by flow cytometry. Monocyte dectin-1 expression was increased by co-culture with either (b) iNKT22 (+0: 10.35 ±1.01 (dectin-1$^+$cells as % total CD14$^+$ cells); +iNKT22: 29.0 ± 1.77) or (c) iNKTLi (+iNKTLi: 32.67 ± 3.18). Dectin-1 blockade inhibits both (d) iNKT22 (+0:3.27 ± 0.57 IL-10$^+$cells as % total CD14$^+$ cells); +iNKT22: 9.8 ±0.88; +iNKT22 +0.3μg αDectin-1: 7.40 ± 1.13; +iNKT22 + 3μg αDectin-1: 5.92 ±0.98; and (e)iNKTLi: +0: 3.27 ± 0.57; +iNKTLi: 13.62 ±0.67; +iNKT22 +0.3μg αDectin-1: 10.52 ± 0.72; +iNKT22 + 3μg αDectin-1: 9.06 ± 0.62; induced IL-10 production. (n=9). Mean ± SEM illustrated. D'Agostino and Pearson omnibus normality test and Student's T test or one-way ANOVA were used for statistical analysis.

In summary, iNKT-monocyte cross talk requires physical interaction between the two cells, but is not only mediated via engagement of CD1d. One possible additional interaction pathway is Dectin-1.

4.4.7. Influence of iNKT cells on monocyte secretion of other cytokines

I next examined whether iNKT cells also affected monocyte production of cytokines other than IL-10. Co-culture with iNKT cells for 18 hours did not affect either control or sarcoidosis monocyte IL-4 expression as measured by ICS (Figure 4.16a-b). There was a modest, but significant increase in the amount of IL-4 detected in the culture supernatant of control monocytes (Figure 4.16c). Sarcoidosis monocytes produce very little IL-4 and there was no alteration in this after contact with iNKT cells (Figure 4.16d). Additionally, in controls, but not sarcoidosis, there was an increase in the proportion of monocytes expressing IL-13 after co-culture with iNKT.

iNKT cell contact did not influence monocyte TNFα production in either controls or sarcoidosis either in terms of the proportion of monocytes expressing surface TNFα (Figure 4.17 and b) or the amount of protein in the supernatant (Figure 4.17c and d). AM are well known to produce TNFαat increased levels in sarcoidosis, and I have shown in the previous chapter that this observation can be extended to peripheral circulating monocytes. Importantly, the TNFα producing cells are not the same monocytes as the IL-10 producing subset (Figure 4.18e).

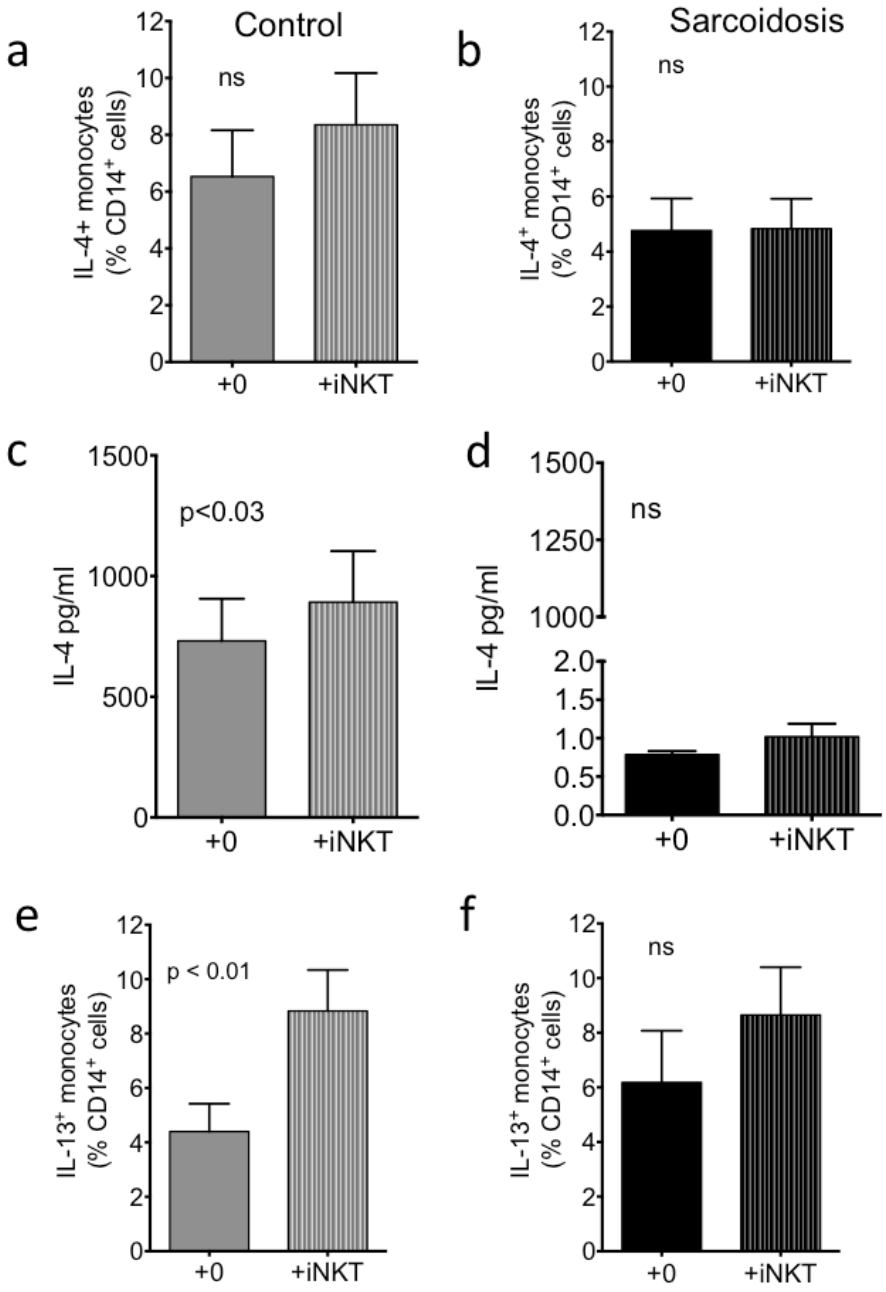


Figure 4.16. iNKT cell interaction increased control monocyte IL-13 expression.

Co-culture with iNKT cells did not affect the percentage of monocytes expressing IL-4 in (a) controls (+0: 6.53 ±1.63 [IL-4$^+$ cells as % total CD14$^+$ cells], +iNKT: 8.35 ±1.82) or (b) sarcoidosis (+0: 4.77 ±1.16, +iNKT: 4.83 ±1.08), nor the quantity of IL4 produced (c,d) Control: +0: 731.6 ±174.4 pg/ml, +iNKT: 892.0 ±211.5; sarcoidosis: +0: 0.79 ± 0.05, +iNKT: 1.02 ±0.17. IL-13 expression was increased on (e) control (+0: 4.40 ±1.02 [IL-13$^+$ cells as % total CD14$^+$ cells], +iNKT: 8.83 ±1.51) but not (f) sarcoidosis monocytes (+0: 6.18 ±1.88, +iNKT: 8.65 ±1.75). (n=9 per group). D'Agostino and Pearson omnibus normality test and paired Student's T test were used for statistical analysis.

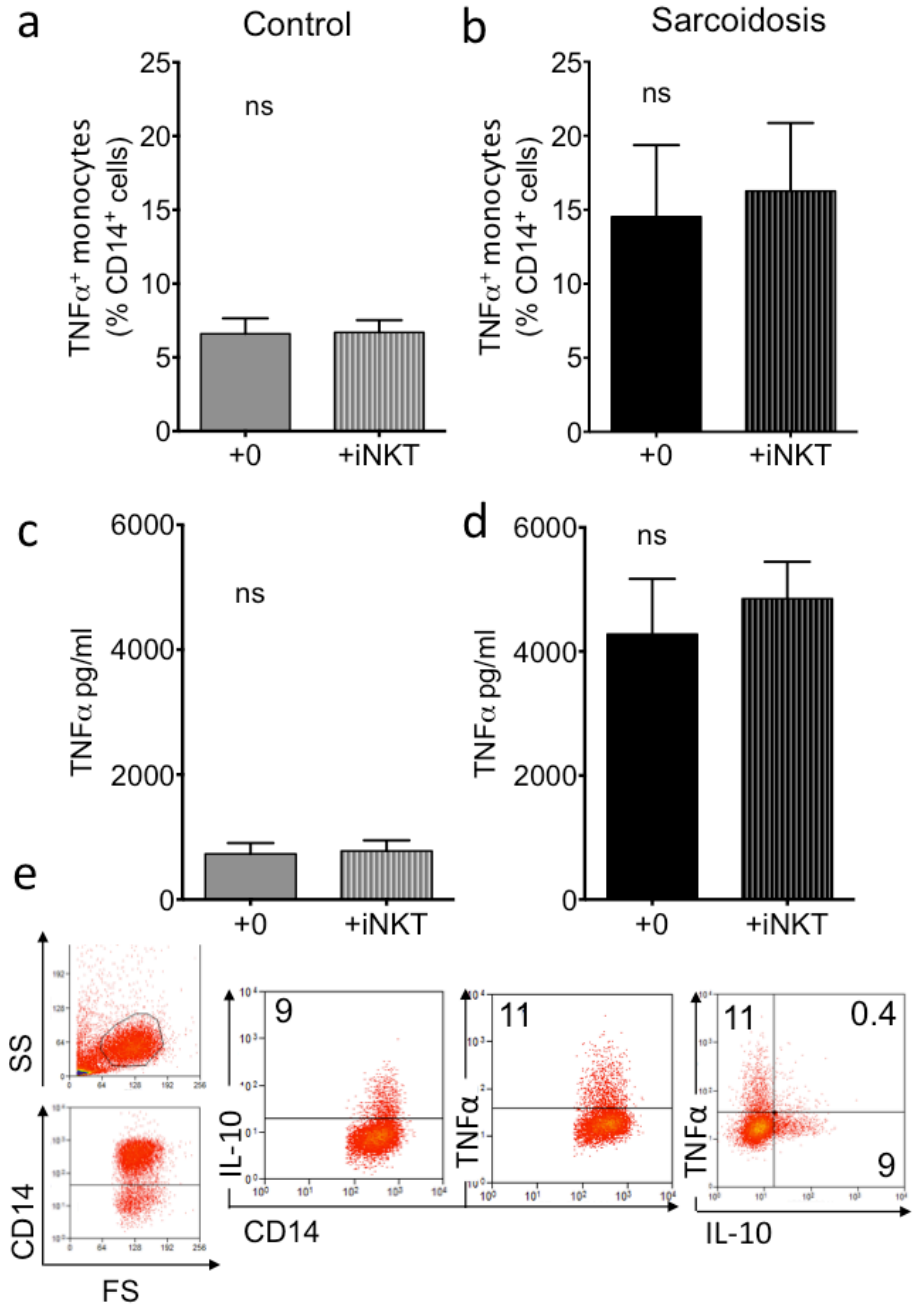


Figure 4.17. iNKT cell interaction did not alter monocyte TNFα expression.
Co-culture with iNKT cells did not affect the percentage of monocytes expressing TNFα in (a) controls (+0: 6.59 ±1.05 [$TNFα^+$ cells as % total $CD14^+$ cells], +iNKT: 6.69 ±4.24) or (b) sarcoidosis (+0: 14.77 ±4.84, +iNKT: 16.27 ±4.60), nor the quantity of TNFα produced (c,d) control: +0: 731.6 ±174.4 pg/ml, +iNKT: 778.1 ±169.4; Sarcoidosis: +0: 4280 ±890.8, +iNKT: 4852 ±594.4. (n=18 per group). (e) Plots illustrating that IL-10 and TNFα originate from different monocytes. D'Agostino and Pearson omnibus normality test and paired Student's T test were used for statistical analysis.

iNKT contact increased both the proportion of IL-12 producing monocytes and the amount of IL-12 produced in both controls and sarcoidosis (Figure 4.18a-d). The quantity of IL-18 in the culture supernatant was increased when control monocytes were cultured with iNKT cells, however, there was a decrease in the amount of IL-18 in the sarcoidosis monocyte supernatant (ICS was not performed for IL-18) (Figure 4.18e-f). IL-12 and IL-18 are both implicated in the activation of iNKT cells (232) and these data suggest that the monocytes, upon contact with iNKT cells can further activate the iNKT cells (or others in the vicinity) by producing IL-12 and IL-18 through positive feedback.

iNKT cells did not have any influence on the proportion of monocytes producing the monocyte chemotactic cytokine CCL2 or the quantity produced by either sarcoidosis or control monocytes (Figure 4.19a-d).

In summary contact with iNKT cells did not alter monocyte TNFα or CCL2 production. The proportions of control monocytes expressing IL-4, IL-12, IL-13 and IL-18 were all increased. iNKT contact increased monocyte IL-12 and decreased monocyte IL-18.

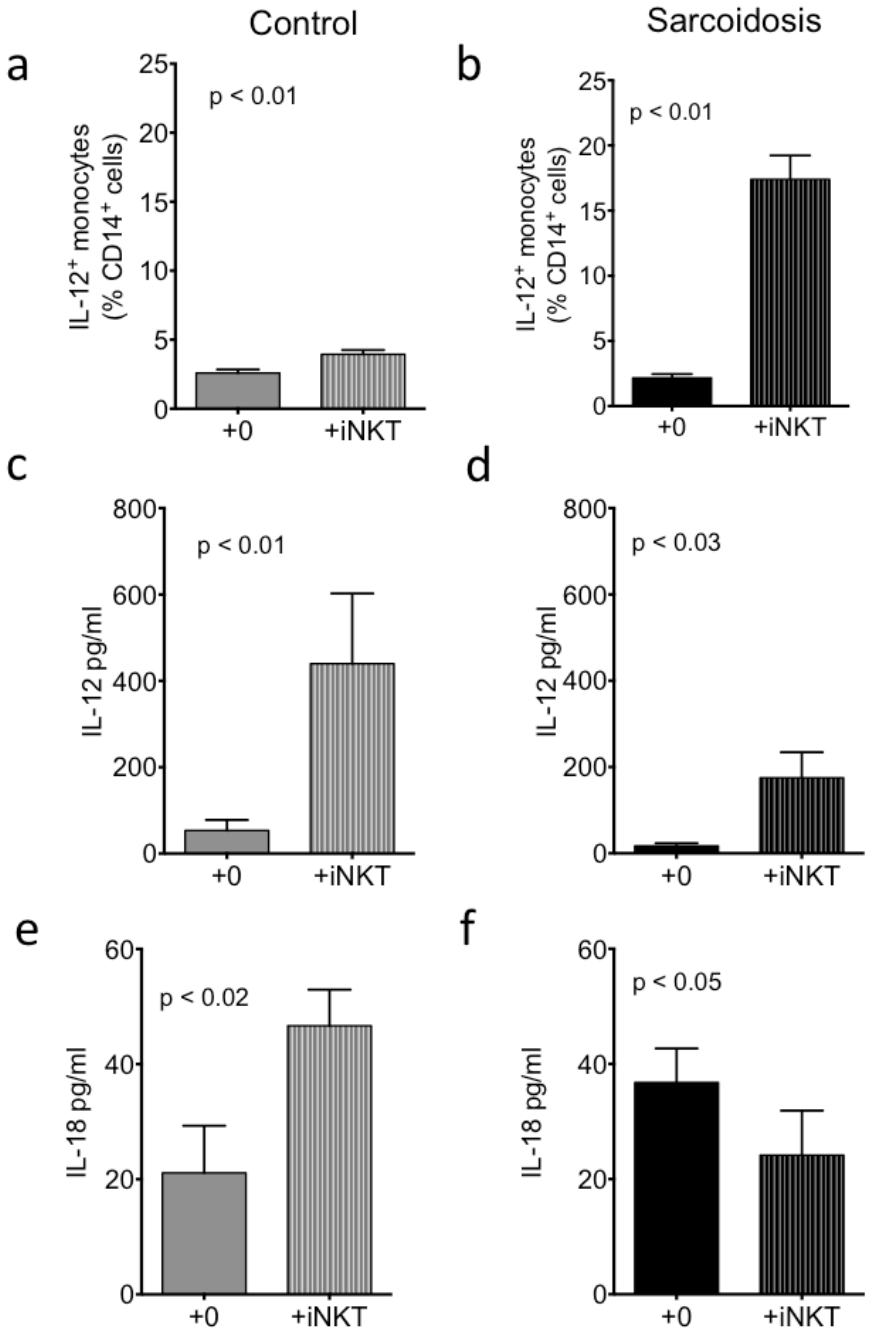


Figure 4.18. iNKT cell interaction increased monocyte IL-12 expression.

Co-culture with iNKT cells increases the percentage of monocytes expressing IL-12 in (a) controls (+0: 2.59 ±0.25 [IL-12$^+$ cells as % total CD14$^+$ cells], +iNKT: 3.94 ±0.31) and (b) sarcoidosis (+0: 2.168 ±0.29, +iNKT: 17.41 ±1.83), and the quantity of IL12 produced (c,d) control: +0: 53.49 ±24.29 pg/ml, +iNKT: 439.6 ±162.9; sarcoidosis: +0: 17.29 ± 6.17, +iNKT: 174.9 ±59.33. IL-18 expression was increased by (e) control (+0: 21.10 ±8.19pg/ml, +iNKT: 46.67 ±6.27) but the reverse was seen in sarcoidosis monocytes (f) +0: 36.80 ±5.89, +iNKT: 25.17 ±7.73). (n=10 per group). D'Agostino and Pearson omnibus normality test and paired Student's T test were used for statistical analysis.

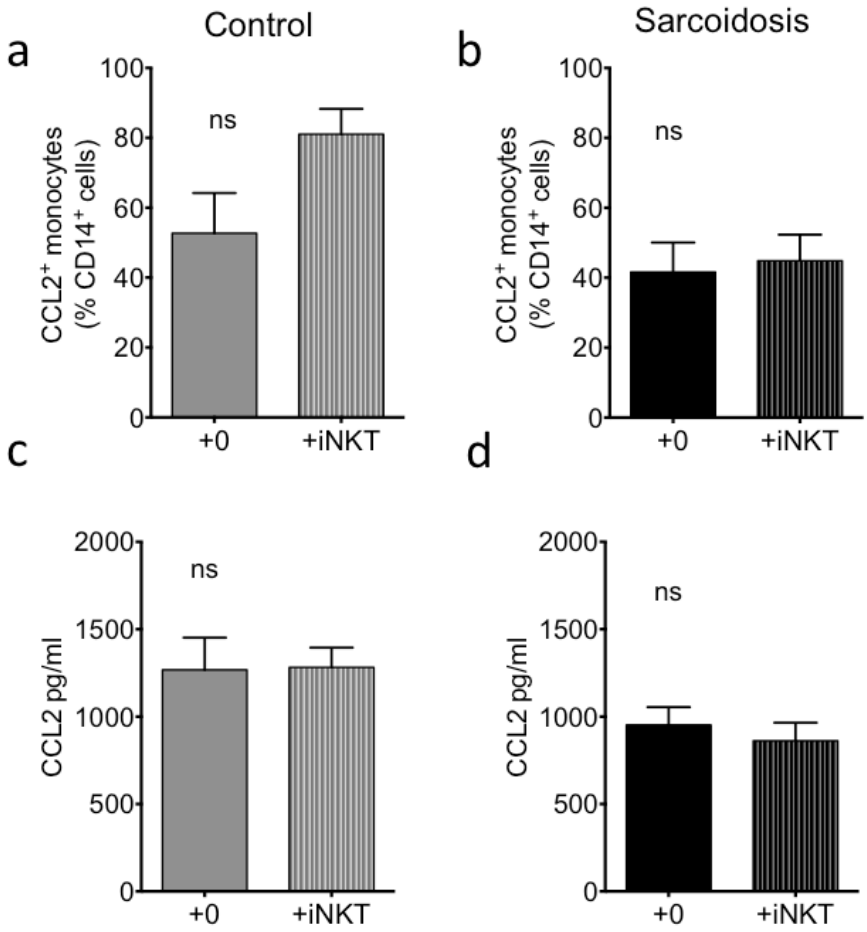


Figure 4.19. iNKT cell interaction did not alter monocyte CCL2 expression.
Co-culture with iNKT cells did not affect the percentage of monocytes expressing CCL2 in (a) controls (+0: 52.70 ±11.50 [$CCL2^+$ cells as % total $CD14^+$ cells], +iNKT: 81.03 ±7.26) or (b) sarcoidosis (+0: 41.62 ±8.46, +iNKT: 44.87 ±7.46), nor the quantity of CCL2 produced (c,d) control: +0: 1268 ±184.2 pg/ml, +iNKT: 1282 ±112.8; sarcoidosis: +0: 952.1 ± 102.5, +iNKT: 861.7 ±103.8. D'Agostino and Pearson omnibus normality test and paired Student T test were used for statistical analysis.

4.5. Discussion

Having established that monocytes are increased in number and phenotypically abnormal in sarcoidosis, I was motivated to investigate the implication of these abnormalities. I was prompted to commence with a study of monocyte IL-10 production in sarcoidosis because, although previous evidence for serum IL-10 in sarcoidosis is conflicting, it is reportedly low in

sarcoidosis BALF and isolated AMs (47). Little is known of the IL-10 activity of peripheral monocytes in sarcoidosis. In view of an overriding T_H1 response and a number of previous reports supporting the roles of both MDSC and IL-10 in the suppression of T-cell proliferation (309, 345, 346, 352-354), the suppressive activity of monocytes and the relationship to IL-10 was assessed.

I confirmed the suppressive role of control monocytes and the defective suppressive capacity of sarcoidosis monocytes. This suppressive capacity was shown to be IL-10 dependent, supported by very recent work reporting that IL-10-deficient macrophages are less able to suppress T cell proliferation (309, 346). That monocytes might be regulatory is not without precedent, cells of the myeloid lineage are known to suppress T cell proliferation both under resting and disease conditions, where they become sub-specialised and are referred to as myeloid-derived suppressor cells (352-354). In sarcoidosis, monocyte IL-10 production is deficient and I propose that the loss of this regulatory pathway, resulting in less IL-10 at the site of pathology, contributes to the hyperactive T cell activity that is a key feature of sarcoidosis immunology. Importantly, there was a negative correlation between monocyte IL-10 levels and patient clinical disease activity, indirectly associating low IL-10 with high T cell activity was demonstrated.

I confirmed the previous finding that number of iNKT cells was reduced and that iNKT cells were in fact were entirely undetectable in nearly a third of individuals with sarcoidosis (61, 62). Studies in mouse models of the autoimmune condition systemic lupus erythematosus, where autoantibodies

are triggered by an increased load of apoptotic cells (including self antigen, perhaps suggesting a parallel to sarcoidosis), suggest that the observed iNKT deficiency results in increased autoreactive B cell activation contributing to the inflammatory response (355, 356). I examined the potential of iNKT cells to regulate (augment) monocyte suppression of T cell proliferation since this is a key feature of the immune pathology in sarcoidosis. I hypothesised that this suppression may be mediated through induction of IL-10. I found that iNKT cells, derived both from an established clone and a recently generated line, increase monocyte IL-10 production in both control and sarcoidosis. Interestingly, culture with the iNKT line increased monocyte IL-10 production to a greater extent than that seen with the iNKT clone. This difference is not explained by the iNKT phenotyping, which demonstrated that, in terms of cytokine profiling, that the line and the clone were broadly similar. The difference may be explained by the additional activity of $CD4^{-}CD8^{+}$ and $CD4^{+}CD8^{+}$ subsets in the line. Alternatively, this difference may be attributable to differences in iNKT TCR affinity, mediated by variations in the CDR3α loop of the TCR, which results in differential cell affinity for CD1d (357). Differences in iNKT affinity have been observed in early rheumatoid arthritis (358) and study of TCR affinity in both the line and clone used in this study, as well as the remaining iNKT cell compartment in sarcoidosis, may help our understanding of how iNKT cells influence monocyte IL-10 production and contribute to disease pathogenesis.

The increase in IL-10 production subsequent to iNKT contact suggests that iNKT cells may be involved in attenuating excess T cell proliferation by

inducing monocyte IL-10 production. The link between iNKT cells and excessive T cell proliferation is supported by reports that iNKT cell deficiency correlates with BALF lymphocytosis and exaggerated $CD4^+$ T cell responses in sarcoidosis (62). Residual iNKT cell activity in sarcoidosis was not explored in this study, but previous reports suggest that they display markers of functional exhaustion and loss of IFN-γ production indicating that even with the small numbers remaining, the cells are deficient functionally (359). Together, these findings support the hypothesis that loss of T cell regulation during the priming phase is a critical factor in disease initiation and additionally may provide an explanation for the 'immunologically exhausted' nature of Fox-P3 TREGs observed in sarcoidosis since these cells are functioning without the contribution from IL-10 producing monocytes (58, 85).

The ability of iNKT cells to influence monocyte IL-10 production has not been previously demonstrated, although macrophage and monocyte-derived DCs interaction with iNKT cells has been reported. Monocyte-derived DCs co-cultured with iNKT cells produce IL-10, supporting my findings in non-conditioned monocytes (256). This suggests that iNKT cells might 'licence' monocytes to specifically produce IL-10.

Modification of the RARα pathway with the PPAR-γ agonist Rosiglitazone or the synthetic RARα ligand AM-580 can increase CD1d expression on human monocyte-derived DCs and cells respectively (348, 360). Since iNKT cells are absent or low in sarcoidosis, the therapeutic option of activating or increasing the iNKT number may not be viable. An alternative is to target the

sensitivity of monocytes to iNKT cell interaction. Whilst I was able to demonstrate that Rosiglitazone, AM-580 and ATRA all increased CD1d expression on monocytes and independently suppress monocyte IL-10, they did not increase the amount of IL-10 expression after contact with iNKT cells.

One interesting aspect of this finding is that iNKT cells were able to increase monocyte IL-10 production without an exogenous glycolipid such as αGalCer, widely used to specifically activate iNKT cells. In addition, CD1d blockade caused reduction but not complete abrogation of IL-10 production, suggesting that the iNKT-monocyte interaction is not solely CD1d mediated. Therefore, the ability to increase IL-10 production depends on signals generated by presentation of a self/endogenous-antigen on CD1d. However is likely that other paths of cross-talk occur to enhance this endogenous stimulation since optimal CD1d blockade caused reduction but not abrogation of IL-10 production.

To address the question of alternative cross-talk regulation, the effect of ICAM-1 and Dectin-1 were both investigated as potential co-regulatory factors in monocyte-iNKT interaction; the choice of these factors being based on recent reports that they are involved in APC-T cell interaction mediated IL-10 production (349-351). Whilst no link between ICAM-1 and iNKT-directed monocyte IL-10 production was demonstrated, the results suggesting a potential role for Dectin-1 are more interesting. I confirmed that Dectin-1 expression on both control and sarcoidosis monocytes is up regulated after iNKT cell contact. This is supported by previous work from the group

demonstrating that iNKT cells can increase Dectin-1 expression on murine monocytes (140).

In addition to activation by αGalCer, it has been shown that three distinct pathways may activate iNKT cells, which is discussed in more detail in chapter 1 (section 1.4.2). Two of these pathways involve IL-12 and IL-18 with or without an additional contribution from CD1d presented glycolipid antigen (230, 232-235). It is therefore interesting that iNKT cell contact increased both the proportion of IL-12 producing monocytes and the amount of IL-12 produced in both controls and sarcoidosis. Additionally, whilst there was an increase in the amount of IL-18 in control monocyte supernatant, there was a decrease in the amount in the sarcoidosis monocyte supernatant. These data suggest that the monocytes, upon contact with iNKT cells have the potential to activate the iNKT cells (or others in the vicinity) by producing IL-12 and IL-18 through positive feedback. This finding is pertinent in view of the fact that, in this study, iNKT cells were able to increase monocyte IL-10 production without exogenous glycolipid.

It is not clear where the interaction between iNKT cells and monocytes might occur, although it is possible that this takes place in the peripheral blood, the sheer force of movement of circulating cells and the infrequency of iNKT cells makes this unlikely. Having demonstrated that physical contact between iNKT cells and monocytes is required for the iNKT directed monocyte IL-10 production, it seems more likely that this occurs in spleen, lymph nodes or bone marrow, where monocytes and iNKT cells maybe in contact for longer

and ideally locates them to impact on T cell proliferation (361). In this study, imagestream analysis demonstrated that whilst contact is required, only a small proportion (2%) need to be in contact at any point to increase in IL-10 production.

Previous work from my supervisor's group has demonstrated that in a murine inflammatory model, iNKT cell deficiency is associated with an increase in monocyte number at the site of pathology driven by CCL2 (347) such that it might be expected that iNKT cells would reduce monocyte CCL2, particularly in sarcoidosis where the monocytes are preconditioned by low iNKT cell numbers in a potentially similar way to the iNKT deficient mouse. I did not, however, see an alteration in monocyte CCL2 expression after iNKT cell contact; it could be that the culture conditions were not optimal for study of this cytokine, that the finding in murine monocytes is not translatable to human monocytes or most likely, that some other factor is mediating CCL2 expression.

Contact with iNKT cells produced a modest increase in the proportion of control monocytes that express IL-4, whilst this increase was not statistically significant, it was supported by a significant increase in the quantity of IL-4 detected in the supernatant of control monocytes. There was no detectable change in monocyte IL-4 after contact with iNKT cells in sarcoidosis monocytes. Additionally, contact with iNKT cells increased the proportion of monocytes expressing IL-13 in controls but not sarcoidosis. That iNKT cells may direct healthy control monocytes to produce IL-4 and IL-13, suggests that

iNKT cells may have a role in biasing control monocytes towards M2 differentiation. This is supported by previous work from the group where it was shown that in a murine inflammatory model, iNKT cells promote differentiation of monocytes to M2 macrophages in an IL-4 and CD1d-dependent process (140).

4.6. Summary

In summary, this chapter shows that IL-10 (but not other cytokine) production and the T cell regulation capability of monocytes are defective in patients with sarcoidosis. The *in vitro* data showed that iNKT cells enhanced monocytic IL-10 production, and as a minimum this required CD1d and Dectin-1 expression on iNKT and monocytes respectively.

Chapter 5: Monocyte phagocytosis and fusion as indicators of early granulomagenesis in sarcoidosis

5.1. Introduction

The granuloma in sarcoidosis is a highly active immune structure that causes pathology both through physical distortion of the tissues and through the production of inflammatory mediators thereby perpetuating disease activity. The granuloma in sarcoidosis is immunologically similar to that seen in TB, however, despite extensive study, as discussed in section 1.1.4.4, the identity of the antigenic stimulus remains elusive. It is possible likely that a non-specific nidus of poorly soluble material provides a persistent inflammatory stimulus.

The events leading to granulomagenesis can be approximated by study of the schistosomiasis egg model (362), dextran (363, 364), cytokine-coated beads (365), antigen-coated beads (366), or with the use of antigens such as mKatG or SAA. These models contribute to the understanding of the downstream effects of abnormal immune activation but have not explained the 'upstream' factors and the aetiology of granulomagenesis.

In earlier chapters of this thesis, I have shown that monocyte surface antigen and cytokine profiles are altered in sarcoidosis monocytes. I have demonstrated that this has implications for the ability of monocytes to suppress T cell proliferation, and discussed how this may contribute to

pathogenesis in sarcoidosis. In this chapter I examine how altered monocyte phenotype and function might contribute to granulomagenesis, a key pathological abnormality in sarcoidosis. Since persistence of an unknown antigen is a putative early step in granulomagenesis, I hypothesised that abnormal monocyte activity in sarcoidosis may impact on early antigen clearance by phagocytosis, thereby leading to granuloma initiation.

Granuloma formation requires fusion of monocytes and macrophages into multinucleate giant cells (MGC). The presence of MGC in the tuberculous granuloma was first described by Langhans in 1868 and has since been described in a variety of granulomatous disorders including sarcoidosis. There are several ideas for the functional role of MGC in granulomas, including a disposal function for exhausted macrophages and specific functions in the phagocytosis of pathogens involved in granuloma formation (367). I therefore examined monocyte phagocytosis and fusion capacity in sarcoidosis monocytes.

5.2. Aim

The aim of this chapter was to investigate monocyte phagocytosis of latex beads and fusion capacity as surrogate makers of granuloma formation in sarcoidosis.

5.3. Methods

Donors were recruited as previously described, demographics are listed in Table 5.1.

	Sarcoidosis				Control		
	# (#Ex Pulm/SA)	Age (SD)	Male	CAS (0/1/2/3/4)	# (SA)	Age (SD)	Male
Fig 5.1e	6 (0/0)	42 (8.9)	2	(0/1/4/1/0)	6 (0)	33 (4.4)	3
Fig 5.3c & 5.4a	3 (0/0)	36 96.7)	1	(0/1/2/0/0)	3 (0)	33 (6.7)	2
Fig 5.6a-f	4 (0/0)	46 (11.3)	2	(0/3/1/0/0)	4 (0)	34 (5.1)	2
Fig 5.7a-b & Fig 5.8a-c	6 (1/0)	45 (12.3)	2	(0/3/2/1/0)	6 (1)	32 (4.3)	4

Table 5.1. Donor demographics
Number of participants per figure indicated in table, number with extra-pulmonary involvement (Ex Pulm) or South Asian (SA) origin indicated in parenthesis. CAS indicates serum biomarker clinical activity score: Lymphocyte count $<1.0x10^9$/l = 1; $>1.0x10^9$/l = 0. Serum ACE: >100 U/l = 2; 55-100U/l = 1; <55 U/l = 0. IgG >13g/l = 1; ≤ 13g/l = 0. Maximum score = 4.

In order to study phagocytic capacity, negatively selected monocytes were incubated with latex beads for 2 hours at a monocyte: bead ratio of 1:20. Latex beads were chosen for this study rather than, for example fluorescently labeled opsonised bacteria, because it probably represents the antigen in sarcoidosis more closely since the antigen is likely to be a non-specific inert particle. Uptake of fluorescent beads was initially assessed by conventional flow cytometry and then further delineated using the Imagestream flow cytometer, which allowed individual cells to be visualised.

Monocyte fusion capacity was assessed using an established fusion assay (described in section 2.2.16 (368)). Monocytes were cultured at varying ratios and time points in X-Vivo medium containing pooled decomplemented human serum on a permissive culture surface (Permanox). Fusion occurs without additional stimulus, but can be enhanced by the addition of IL-4 on day 4 of a 5-7 day assay. The fusion ratio was determined by dividing the number of nuclei in fused cells by the total number of nuclei. The greater the ratio, the

more fusion has occurred. ELISA was used to determine the quantity of IL-4 and TNFα in the fusion assay supernatant at the termination of the assay (day 5 or 7).

5.4. Results

5.4.1. Sarcoidosis monocytes are less efficient phagocytes than control monocytes.

Monocytes, isolated by CD14 negative magnetic selection, were stimulated with 1μg/ ml LPS and incubated with fluorescent latex bead ratios of 1:10, 1:20, 1:100, 1:500 and 1:1000 to establish a suitable bead concentration (Figure 5.1a, c). Data were acquired by standard flow cytometry, bead positive events were taken to represent monocytes that has phagocytosed at least one bead. From this titration, I selected a monocyte: bead ratio of 1:20 for the remaining experiments. The time course titration is illustrated in Figure 5.1b; 2 hours was chosen for further study as it allowed suboptimal phagocytosis, which could be further manipulated if required. Representative staining illustrating $CD14^+$ cells ± beads is shown in Figure 5.1d. The percentage of bead positive cells as determined by flow cytometry is less in sarcoidois monocytes than control monocytes (Figure 5.1e).

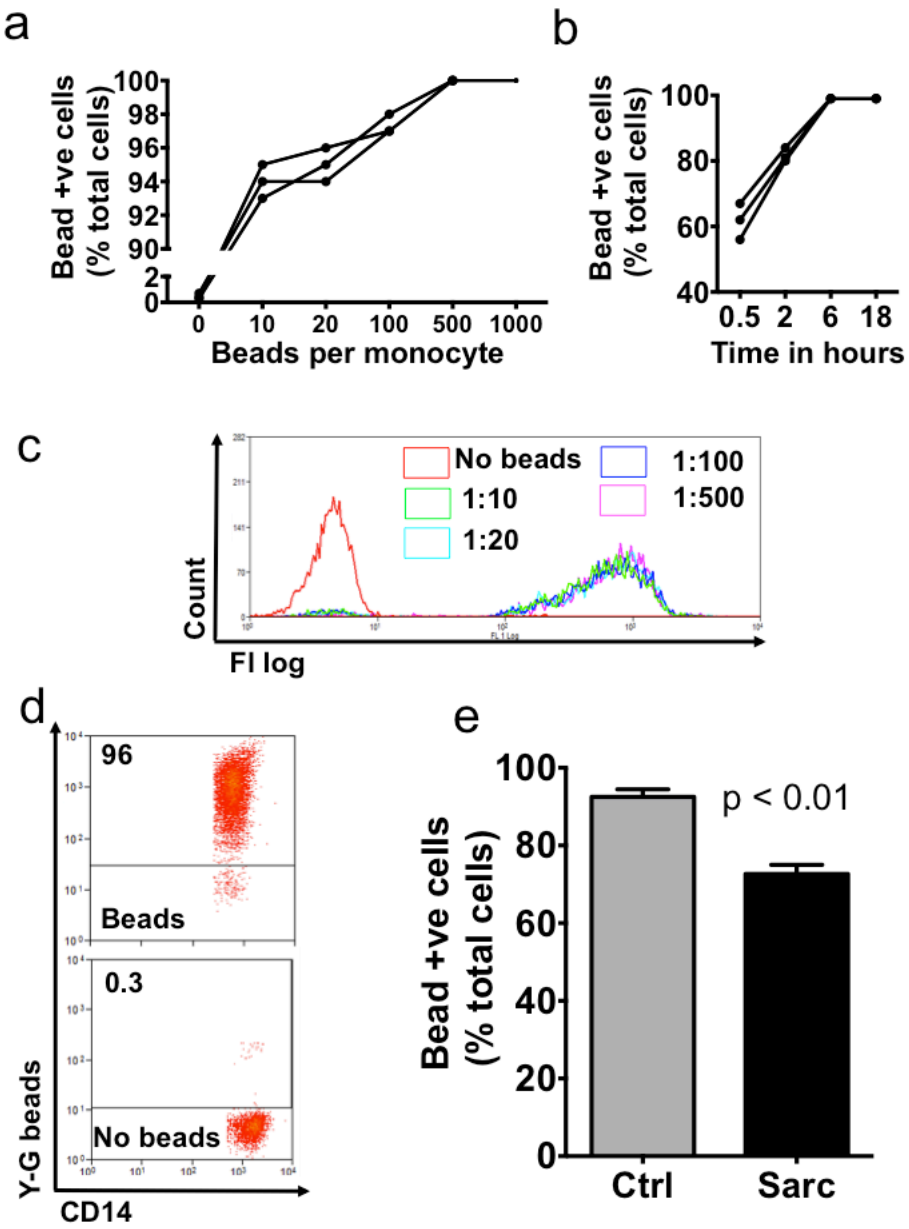


Figure 5.1. Fluorescent bead uptake is reduced by sarcoidosis monocytes compared with healthy control monocytes.
Monocyte phagocytic uptake of 1µm yellow–green fluorescent beads was assessed by standard flow cytometry. (a) Bead concentration was determined by examining phagocytosis after 2 hours; 250 000 monocytes per well, total volume 200µl. Monocyte: bead ratio 1:20 was used for the remaining experiments. (b) Percentage of bead positive cells at varying time points. 2 hours was used for remaining experiments. (c) Representative histogram (d) FACS staining ± beads, numbers indicate percentage of total cells represented. (e) Comparison of bead uptake between sarcoidosis (72.67% ± 0.95) and control monocytes (92.50 ± 0.81) (n = 6 per group). Mean ±SEM. D'Agostino and Pearson omnibus normality test and Student t test were used for statistical analysis.

5.4.2. ImageStream™ gating

Single cells that were in focus were used for imagestream analysis and account for a mean of 25% of the cells acquired per sample (Figure 5.2a-b). A tight gating strategy was used, as per standard protocol, because out of focus cells are uninterpretable (Figure 5.2c). This does result in exclusion of many cells, which may have implications for the interpretation of the data.

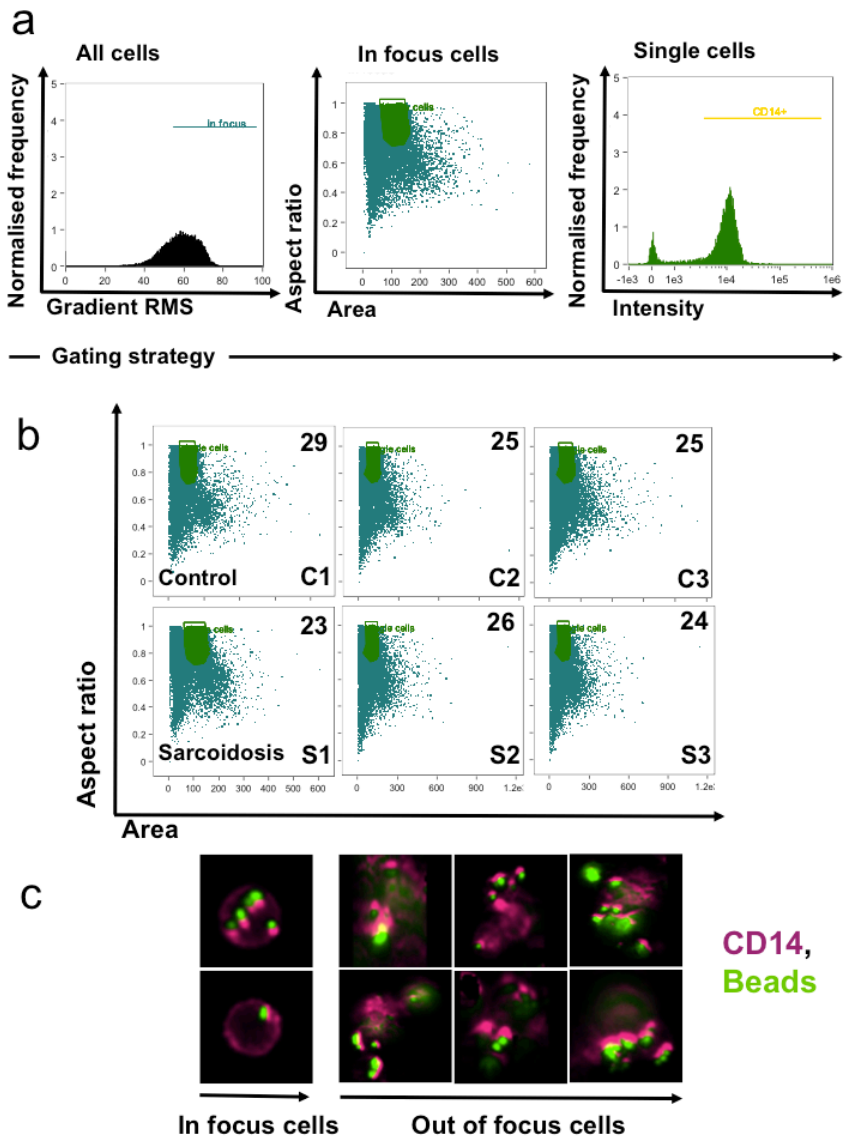


Figure 5.2. Gating strategy for imagestream phagocytosis assay
(a) In focus single cells were used in imagestream analysis. (b) Aspect ratio: area plots for each sample; highlighted area used in analysis. Number refers to percentage of total cells included in gated area. (c) Representative cells from both inside (in focus) and outside the gated area. Surface staining for CD14 represented by magenta, fluorescent beads green.

5.4.3. Sarcoidosis monocytes are less efficient at phagocytosing latex beads than control monocytes.

Representative images indicating cells with varying bead counts confirm that the CD14+ cells have phagocytosed the beads rather than them remaining externally adherent (Figure 5.3a). Spot count was determined using the Amnis analysis software (Figure 5.3b) and was verified manually. Spot count can be represented as binary outputs (number of cells having undertaken phagocytosis versus the number that have not), the mean spot count per cell or the number of cells with 0, 1, 2, 3, 4, 5+ spots. The mean spot count per cell was used as this is the standard method of reporting such data and allows clear comparisons to be made. The mean spot count in sarcoidosis monocytes was lower than in control monocytes, indicating reduced phagocytosis by sarcoidosis monocytes (Figure 5.3c), which reflects the observations from flow cytometry (5.1b). A greater number of monocytes in sarcoidosis do not participate in phagocytosis and fewer are seen to contain 5 or more cells after the incubation period, suggesting decreased efficiency (Figure 5.3d).

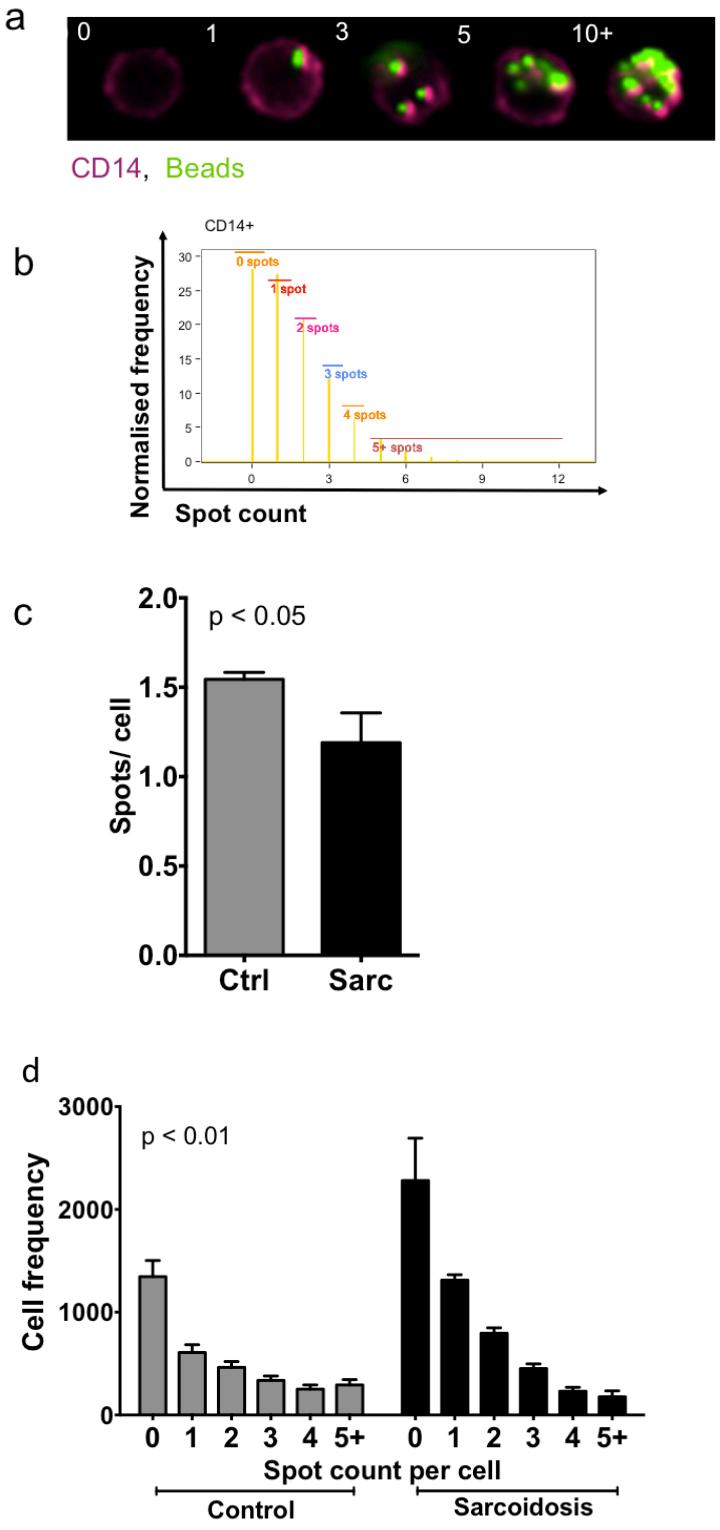


Figure 5.3. Sarcoidosis monocytes phagocytose less efficiently than those from healthy controls.
(a) Representative surface staining for CD14 (magenta), beads (green). (b) Representative spot count per cell (Amnis IDEAS automated spot count). (c) Mean number of spots per cell: sarcoidois 1.19 ±0.11, control 1.54 ±0.02. Mean ± SEM. (d) Spot count per cell; total cell count 20000 per sample, n=3 per group. D'Agostino and Pearson omnibus normality test & Student t test (c) or one-way ANOVA (d) were used.

5.4.4. Sarcoidosis monocytes fuse to form MGCs in vitro more readily than control monocytes.

I found that monocytes fuse with both Langhans-type (circumferential arrangement of nuclei) and foreign-body type (random arrangement of nuclei) morphology (Figure 5.4a). After 5 days culture, low-level fusion by sarcoidosis monocytes and minimal fusion by control monocytes was seen (Figure 5.6a).

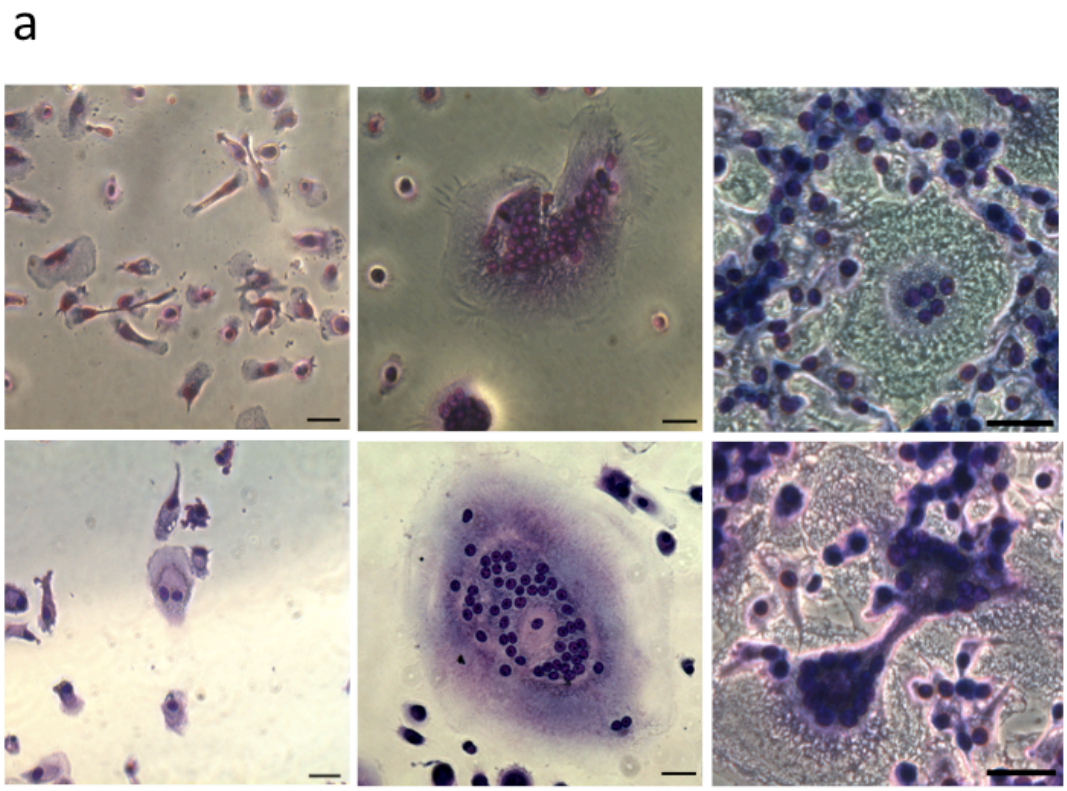


Figure 5.4. Monocytes fuse to form multinucleate giant cells in vitro.
(a) Monocytes fuse in vitro to form multinucleate giant cells. Membrane contact between two joining MGCs is clearly seen in the bottom right image. Scale represents 50μm. Monocytes plated on Permanox chamber slides; 100 000 cells per chamber. Cells were assessed at D7 (Haematoxylin & Methylene blue staining).

When IL-4 was added on day 4 (24 hours before completion of the assay) fusion was increased in both sarcoidosis and controls, with fusion of sarcoidois monocytes being near maximal (fusion ratio 0.96) (5.5a-b). Fusion of control monocytes was unchanged at 7 days in the absence of IL-4, but at 7 days (72 hours after adding IL-4), control monocytes had also reached near maximal fusion (Figure 5.5a). Interestingly, sarcoidosis monocytes had undergone further fusion at day 7 even in the absence of IL-4. Because IL-10 is known to be anti-inflammatory and its secretion is reduced in sarcoidosis monocytes, the effect of IL-10 on fusion was assessed. IL-4 mediated fusion of both control and sarcoidosis monocytes was reduced by the addition of IL-10 and reversed with IL-10 blocking (5.5c-d).

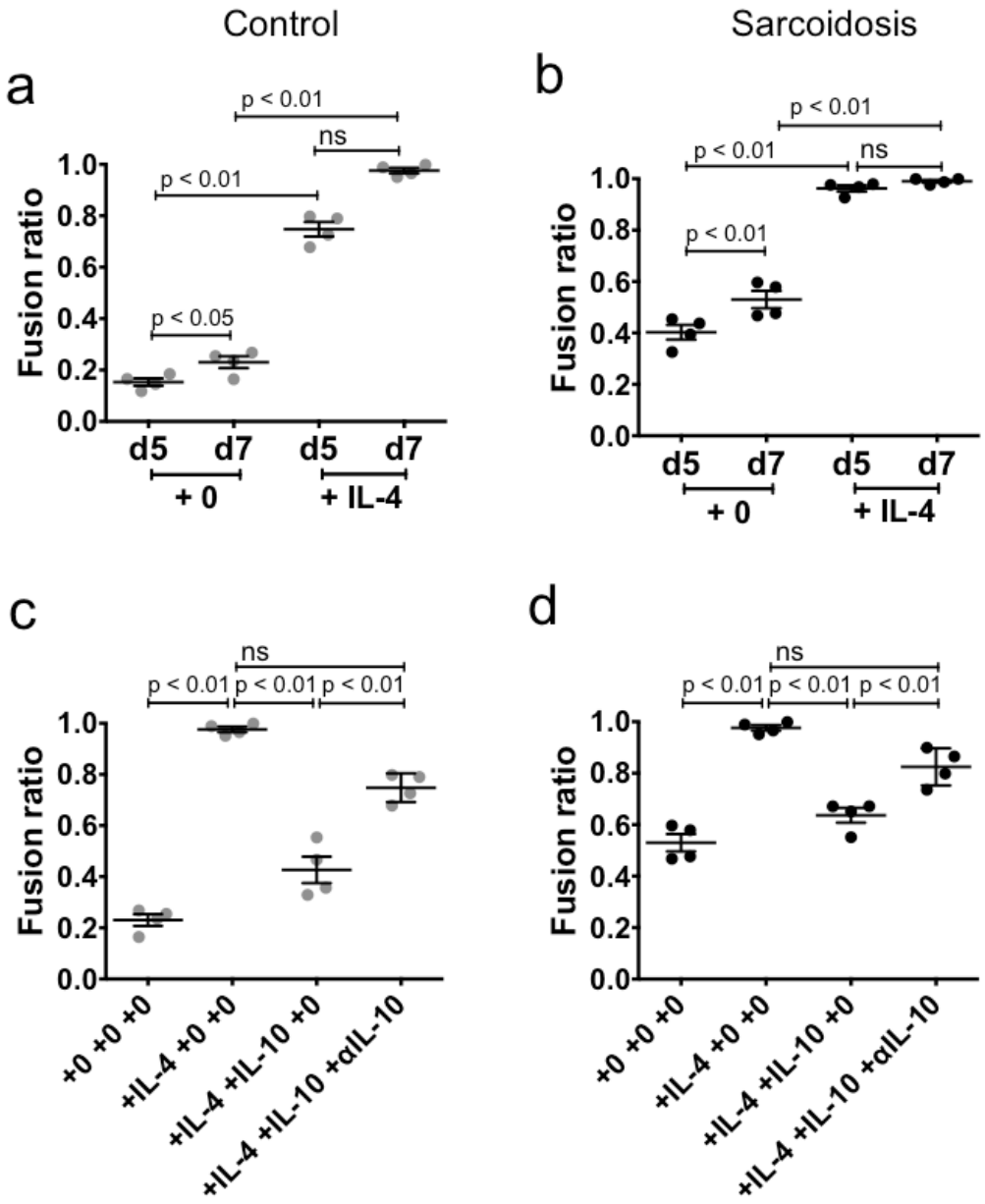


Figure 5.5. Sarcoidosis monocytes fuse more readily than controls.

Monocyte fusion ± IL-4 (20ng/ml). Fusion calculated as the number of fused cells (cells with more than one nucleus) divided by the total number of cells. Sarcoidosis monocytes fused more readily than controls. Fusion ratios for (a) control and (b) sarcoidosis monocytes. Fusion is reduced by IL-10. Reduction of fusion by IL-10 is reversed by IL-10 blocking antibody (αIL-10). Fusion ratios after ± IL-4 (20ng/ml) ± IL-10 (40ng/ml) ± αIL-10 (4ng/ml) in (c) control (ratio +0: 0.23 ±0.02, +IL-4: 0.97 ±0.01, +IL-4+IL-10: 0.43 ±0.05, +IL-4+IL-10+αIL-10: 0.75 ±0.03) and (d) sarcoidosis (ratio +0: 0.53 ±0.03, +IL-4: 0.98 ±0.01, +IL-4+IL-10: 0.64 ±0.02, +IL-4+IL-10+αIL-10: 0.82 ±0.07) monocytes. Mean ± SEM. One-way ANOVA with Tukey post-test comparisons were used for statistical analysis.

5.4.5. Both intrinsic cellular properties and serum factors contribute to increased fusion in sarcoidosis monocytes.

Sarcoidosis monocytes fused more readily than controls both with and without the addition of IL-4 as a fusogenic stimulus (Figure 5.6a). When control monocytes were cultured, in pooled sarcoidosis serum as opposed to control serum, increased fusion was seen without additional IL-4 (Figure 5.6b).

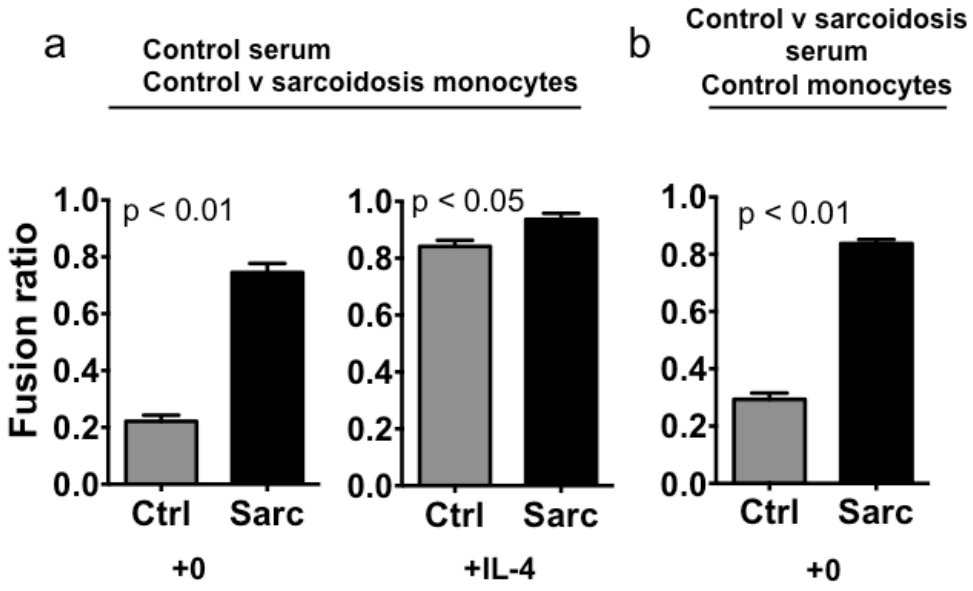


Figure 5.6. Serum and cell intrinsic factors drive increased monocytes fusion in sarcoidosis.
(a) Fusion ratios at day 7 for control (ratio -IL-4 0.24 ± 0.02, +IL-4 0.97 ±0.01) and sarcoidosis (ratio -IL-4 0.53 ± 0.03, +IL-4 0.99 ±0.01) monocytes in pooled healthy control donor serum ± IL-4 (20ng/ml) and (b) for control monocytes in pooled healthy control (mean ratio 0.29 ± 0.02) or pooled sarcoidosis serum (0.83 ±0.01) without additional IL-4. (n = 6 per group). Mean ± SEM. D'Agostino and Pearson omnibus normality test and Student T test was used for statistical analysis.

5.4.6. IL-4 levels are lower in the fusion culture supernatant of sarcoidosis monocytes than controls at 7 days.

IL-4 was detectable in the supernatant of control monocytes cultured in control serum, even when none had been added to the assay, suggesting that the monocytes were producing IL-4 (Figure 5.7a), an observation supported by the earlier profiling studies (Figure 3.9a). Interestingly, when control monocytes were cultured in sarcoidosis serum rather than control serum, the level of IL-4 seen was lower, suggesting that the IL-4 was being neutralised by some other serum factor (Figure 5.7b). When IL-4 was added to the culture supernatant at day 4, by day 7, the amount in the supernatant of control monocytes cultured in sarcoidosis serum is much less than those cultured in control serum (Figure 5.7c).

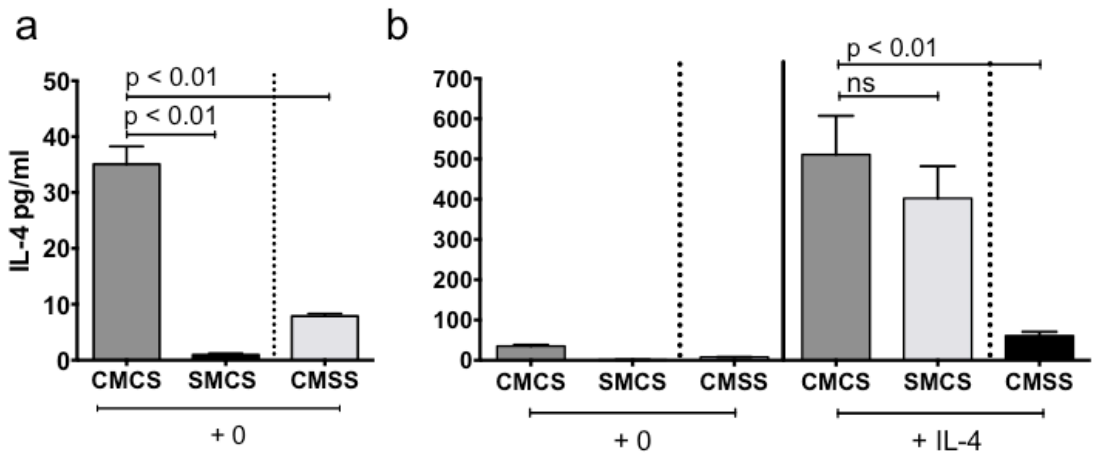


Figure 5.7. IL-4 was reduced in fusion supernatant at 7 days.
CMCS = control monocytes cultured in control serum, SMCS = sarcoidosis monocytes in control serum and CMSS = control monocytes in sarcoidosis serum. IL-4 quantity was determined by ELISA and was reduced in the fusion supernatant at 7 days: (a) IL-4 (CMCS: 35.06pg/ml ± 3.19, CMSS: 7.89 ± 0.40, SMCS: 0.97 ± 0.26); no additional IL-4 added during culture period. IL-4 is reduced in the fusion assay supernatant when monocytes are cultured in sarcoidosis serum: (b) IL-4 concentrations in the supernatant at 7 days ± additional IL-4 added on day 4. Note differing ordinate scale. Mean ± SEM. D'Agostino and Pearson omnibus normality test and a one-way ANOVA were used for statistical analysis.

5.5.5. Discussion

This study demonstrated that sarcoidosis monocytes phagocytosed less efficiently than control monocytes, reflected by both a decrease in the total percentage of cells undergoing phagocytosis and by the number of beads phagocytosed per cell. Studies have shown that in the human airway, phagocytosis is a rapid process with 50–75% of the particles being phagocytosed within 2–3 h, 90% or more by 10 h, and nearly 100% by 24 h after particle deposition (369). Macrophages with high particle load are cleared more rapidly compared with those with fewer particles (370, 371). It may thus be hypothesised that sarcoidosis monocytes having decreased capacity to ingest multiple poorly degradable particles remain in the interstitium for longer where a permissive cytokine milieu promotes early granulomagenesis. Abnormal phagocytosis has been reported in other inflammatory lung conditions such as COPD, asthma and cystic fibrosis, where both phagocytosis of bacterial pathogens and efferocytosis of apoptotic cells by AM are impaired (372). Indeed, particle size, geometry and degradability have all been shown to influence efficiency of phagocytosis with poorly biodegradable material being most inefficiently cleared (373-375). It would be interesting to continue this line of inquiry by examining the ability of sarcoidosis monocytes to phagocytose other particles such as opsonized bacteria, senescent neutrophils, zymosan, SAA or mKatG to confirm the observation of altered phagocytosis.

This study does not address the mechanism by which altered phagocytosis by sarcoidosis monocytes occurred. Numerous receptors are known to be

involved in mediating phagocytosis, including complement receptors; TLR2, TLR4; Fc receptors such as CD16, CD32, CD64, CD23, CD89; scavenger receptors such as CD204, the mannose receptor (CD206), CD163 and MARCO (macrophage receptor with collagen structure). Fc receptors mediate phagocytosis through recognition of immunoglobulin-coated particles while scavenger receptors are important in uptake and clearance of modified host molecules and apoptotic cells. The latter bind and internalise micro-organisms and their products; and are involved in lipid metabolism and bind modified LDL. The class A scavenger receptor MARCO, present on BAL and other tissue macrophages in humans, has been shown to be responsible for the uptake of un-opsonized particles such as titanium dioxide, iron oxide and polystyrene latex (PSL) (376). The next stage in this work would be to characterise differential receptor expression and use this to identify any signaling pathway anomaly. I have already established that CD206 expression is less on sarcoidosis monocytes than controls (Figure 3.6c), whilst this may simply reflect differing stages of maturation or differentiation; it further supports the finding that sarcoidosis monocytes have a decreased capacity to phagocytose. Additionally, I would be interested to tease out the functional implications of the altered expression of CD163 observed (Figure 3.6a) and to identify any altered expression of MARCO in characterising scavenger receptor anomalies in sarcoidosis (377) as the immediate next step in analysing the mechanism behind the abnormal phagocytosis capacity.

In contrast to this finding, a recent study of AM in sarcoidosis using quantitative proteomics found phospoliphase D (PLD), important in the

regulation of phagocytosis by human macrophages, and the adaptor protein Ras-related protein Rab-11A (RAB11A) which regulates membrane traffic and fusion of intracellular transport vesicles during phagocytosis in human macrophages to be up-regulated (259, 378-381). The authors concluded that this suggests that phagocytic pathways are up regulated in sarcoidosis, however, the patient sample was small and heterogeneous, making conclusions difficult. In addition, they only looked at AM in the alveolar space, which is in direct contact with the outside world. As discussed in chapter 1, the extent to which the existing AM are involved in early interstitial granulomagenesis is unclear and debatable. In sarcoidosis, it is more likely that monocytes and the interstitial macrophages are the initial phagocytes central to the formation of the granuloma, upstream of the AM. It may be that by the time the AM come into play that altered or even heightened phagocytosis is no longer immunologically pertinent.

Further studies including examination of alternative antigen ingestion, phagocytic receptor kinetics and signaling pathways are planned. A potentially exciting method of examining altered phagocytic activity in sarcoidosis would be to employ humanised mice in adoptive transfer studies of sarcoidosis and control monocytes (382). Such work may help delineate any contribution that altered phagocytosis may have to the immune pathogenesis of sarcoidosis.

The fusion of monocytes to form MGC has been widely reported in response to a variety of stimuli including IL-4, IL-13, IL-17A, IFNγ, Concavalin A (ConA), Muramyl dipeptide (MDP), PHA, PMA, GM-CSF or M-CSF in addition to IL-4

and MGC culture supernatant (368, 383-393). Here, use of a permissive culture surface (Permanox plastic) which allows fusion to occur in the presence of decomplemented pooled human serum without additional agents (332), I have shown that in contrast to decreased phagocytosis, sarcoidosis monocytes fuse more readily than control monocytes.

It is known that MGC in tissue biopsy samples from sarcoidosis demonstrate expression of monocyte-macrophage markers such as CD14, CD11a and 3A5, but not macrophage markers such as MAC387 and macrophage galactose-type C-type lectin [MGL] (386). This suggests that the MGC in sarcoidosis granulomata are derived from monocytes rather than from tissue macrophages. Additionally, it has been shown that, in tissue culture, older monocyte-derived macrophages (day 8) fuse less well than newly differentiated macrophages; fusion capacity can be restored by contact with the addition of freshly isolated monocytes (367). Taken together, these findings support the hypothesis that it is the monocyte and the monocyte-like interstitial macrophage, rather than the AM that are important in early granulomagenesis.

The dynamics of monocyte fusion is relatively under-studied in comparison with its initiation. Whilst it is likely that the monocytes in the in vitro model used are transitioning through a macrophage-like state in acquiring fusion competence (368), the lack of macrophage markers in sarcoidosis MGC suggests that direct monocyte fusion may be possible and relevant. Macrophage fusion requires first, the induction of a fusion-competent status

(STAT6 signaling); then CCL2 mediated macrophage chemotaxis; cell–cell adhesion mediated by E-cadherin, substrate adhesion by adhesion factors including β1 and β2 integrins; RAC1 mediated cytoskeletal rearrangements; actin polymerization; CD36 mediated lipid recognition; and finally membrane fusion.

In this study, I confirmed the previous finding that monocytes can fuse in the Permanox system in the absence of IL-4 (368) and that sarcoidosis monocytes fuse more quickly and completely. By day 7, even in the absence of IL-4 almost complete fusion occurs such that all monocytes in the well are fused into one giant cell. It is therefore unclear what is mediating the fusion as I have already demonstrated that sarcoidosis monocytes produce very little IL-4 and IL-13 in culture. Interestingly, when looking at the quantity of IL-4 in the fusion culture supernatant, although control monocytes, cultured in control serum without exogenous IL-4, produce detectable levels of IL-4, the same monocytes, when cultured in sarcoidosis serum have far less (3-fold reduction). Furthermore, it was seen that when IL-4 is added to the system, far less is detected in the supernatant of control monocytes cultured in sarcoidosis serum. This suggests the presence of an IL-4 neutralising factor in the sarcoidosis serum confirming that IL-4 is not required for the fusion of sarcoidosis monocytes in this system.

IL-13 is known to induce multinucleate giant cell formation both *in vitro* and *in vivo*, independently of IL-4, via STAT6 signaling (332, 394). It has previously been suggested on the basis of BAL cell and PBMC mRNA that IL-13 is

elevated in sarcoidosis (335). I found that there was a small but non-significant increase in the amount of IL-13 produced by monocytes in sarcoidosis (ICS) (mean ± SEM control 4.40 ±1.02, sarcoidosis, 6.18 ± 1.88). Although IL-13 was not directly measured in this system, I have shown that the amount produced by sarcoidosis monocytes in culture is minimal in LPS stimulated monocytes after culture for 18 hours. It may be that these were not the optimum conditions for monocyte IL-13 production. The role of IL-13 in sarcoidosis monocyte fusion, which occurs over a longer period, would benefit from further examination. Examination of the fusion assay supernatant at varying time-points or blocking with an IL-13 blocking antibody may help define the relative contribution of IL-13 in sarcoidosis monocyte fusion. Whilst it is possible that levels increase after this period, it is more likely that some other factor such as IL-17A or IFNγ is driving fusion in this system and examination of the fusion assay supernatant would be informative. *In vivo* in sarcoidosis, bacterial agents such as MDP remain a possibility.

Having established that IL-10 production in sarcoidois monocytes is reduced; I wanted to ascertain whether IL-10 affected monocyte fusion capacity. IL-10 was added to the IL-4 mediated fusion system and the amount of fusion measured at 7 days. I found that when IL-10 was added to the fusion assay, at the same time as the IL-4 on day 4, this reduced the amount of fusion seen in both the control and sarcoidosis groups. This effect was partially restored by adding IL-10 blocking antibody. This suggests that IL-10 may be important in destablising early granulomagenesis and is supported by previous observations that IL-4 and IL-10 have opposing effects on monocytes (333).

Interestingly, these findings are echoed by a similar in vitro study on MGC formation in TB monocytes (395). The authors found IL-10 in their culture supernatant and concluded, as they had used monocyte adherence to isolate them from PBMCs, that the source of the IL-10 must be T cells. It would be interesting to explore this further, including how this may be manipulated, in the context of our understanding of monocyte IL-10 activity.

In the last part of the study, I found that control monocytes, cultured in pooled serum from individuals with active sarcoidosis (not on treatment), fused more readily than when the same monocytes were cultured in pooled control serum. This suggests that factors both intrinsic to the monocyte and soluble factors in the serum contribute to the increased fusion seen in sarcoidosis. One other study has also found that monocytes from individuals with sarcoidosis fused more readily than control monocytes, albeit using fusogenic mediators such as ConA and IL-13 with no difference between control and sarcoidosis serum (386). The disease activity level at the time of sampling was not clear however and exogenous fusion mediators may have confounded the observations. A more complete study of cytokine dynamics (both culture supernatant and serum) and fusion marker expression is required in order to draw conclusions on how this increased fusion is mediated.

Careful exploration of the mediators known to be involved in macrophage fusion would be interesting in order to determine their role in sarcoidosis monocyte fusion. These include the monocyte recruitment factors such as CCL2, discussed earlier and LFA-1 (Lymphocyte function-associated antigen

1) (389); adhesion molecules such as E-cadherin (antibodies against which result in impaired MGC formation(396)), DC-STAMP (dendritic cell-specific trans-membrane protein); regulatory molecules such as (STAT 6 also required (396)), TREM2 (triggering receptor expressed on myeloid cells 2) and its partner DAP12 (DNAX activating protein 12 signaling adaptor), all of which are required for E-cadherin and DC-STAMP expression (397). Additionally, it would be interesting to study the role of signaling molecules, the exact role of which remain unclear even in macrophage fusion, such as RAC1 (Ras-related C3 botulinum toxin substrate 1 signaling molecule), DOCK180 (dedicator of cytokinesis), PtdSer, CD36, SIRPα (signal regulatory protein α) /CD47, P2X7 receptor, CD9/CD81 (332, 385) and MMP9 (matrix metalloproteinase 9).

5.6. Summary

In summary I have found that in sarcoidosis, monocyte phagocytosis is impaired and fusion is increased. These anomalies appear to be secondary to both intrinsic cellular factors and the serum cytokine profile. I hypothesise that in sarcoidosis, a poorly degradable antigen, is encountered by abnormally functioning monocytes in the interstitium. These monocytes are less able to phagocytose and clear the antigen and are slow to clear from the interstitial space. They are increased in number and therefore fuse to form MGC, which go on to participate in granulomagenesis.

Chapter 6: Vitamin D and the monocyte: modulating function

6.1. Introduction

Vitamin D, 1alpha, 25-dihydroxy,1,25$(OH)_2D_3$ [1,25$(OH)_2D_3$, Calcitriol], is a key regulator of immune responses, including monocyte differentiation and maturation, as well as being important in the homeostasis of calcium and phosphate metabolism.

A potential link between sarcoidosis and vitamin D was first noted when it was established that AM from the BAL of individuals with sarcoidosis were able to convert the inactive precursor, 25-hydroxyvitamin D3 ($25OHD_3$, calcidiol) to calcitriol (398-401). The vitamin D receptor (VDR) is constitutively expressed on monocytes and activated macrophages and increased production of calcitriol by macrophages has previously been widely reported in granulomatous disease (398-400, 402). That vitamin D plays an important role in protection against the granulomatous condition tuberculosis has been evident since as far back as the early 20th century, with the use of cod liver oil and sunlight being therapeutically employed. Multiple studies demonstrate that hypovitaminosis D is a risk factor for the development and reactivation of tuberculosis (403, 404).

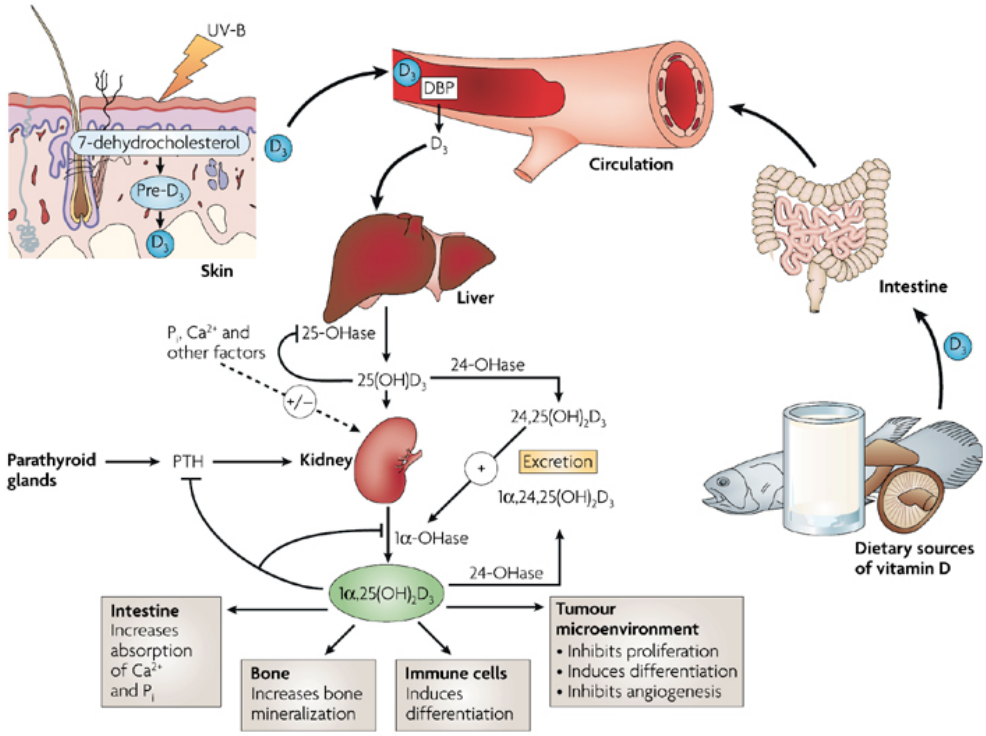


Figure 6.1. Vitamin D metabolism.
From *Deeb et al. Nat Reviews Cancer, 2007* (405). Photochemical synthesis of vitamin D_3 (cholecalciferol, D_3) occurs in the epidermal basal layers where pro-vitamin D_3 (7-dehydrocholesterol) is converted to pre-vitamin D_3 (pre-D_3) in response to ultraviolet B (sunlight) exposure. Vitamin D_3, obtained in this way or via intestinal absorption, binds to vitamin D-binding protein (DBP) in the blood, and is transported to the liver where it is hydroxylated by 25-hydroxylases (25-OHase). The resultant 25-hydroxycholecalciferol ($25(OH)D_3$) is then 1α-hydroxylated in the kidney by 25-hydroxyvitamin D_3-1α-hydroxylase (1α-OHase) to form the active $1\alpha,25(OH)_2D_3$ (calcitriol). The synthesis of $1\alpha,25(OH)_2D_3$ from $25(OH)D_3$ is stimulated by parathyroid hormone (PTH) and suppressed by Ca^{2+}, P_i and $1\alpha,25(OH)_2D_3$ itself.

Vitamin D is important in the monocyte response to bacterial insult. CYP27B1 (25-hydroxyvitamin D-1α-hydroxylase), which converts calcidiol to calcitriol, is induced in human monocytes by TLR stimulation. This action is negatively regulated; treatment of monocytes with calcitriol suppresses TLR2 and TLR4 expression (406). Additionally, monocytes are able to produce calcitriol in response to IFNγ and LPS stimulation. Vitamin D deficiency impairs antimicrobial function by down-regulating macrophage HLADR, lysosomal

enzyme acid phosphatase and the antimicrobial peptide hCAP-18 (Human cathelicidin antimicrobial peptide-18) (406-410).

Much of the work on human monocyte responsiveness to calcitriol has been conducted using cell lines (U937 or THP) rather than primary monocytes. Treatment of monocytes with calcitriol leads to down-regulation of TNFα (407, 409, 411), IL-12 (346) and IL-4 (409, 412). The reported IL-10 response to calcitriol is variable, seemingly highly dependent on cell type and culture duration (346). IL-10 production by the human monocytic cell line THP1 after calcitriol stimulation is initially decreased (at 8 hours) before increasing by 24-48h (413). In chapter 4 I showed that IL-10 deficient primary monocytes failed to suppress T cell proliferation. The proposition of using calcitriol to boost monocyte IL-10 production is attractive in the clinical setting and early studies have shown that vitamin D supplementation can increase $CD4^+$ T cell IL-10 mRNA in asthma (414). It has been anecdotally observed in the Oxford Sarcoidosis Clinic that serum vitamin D levels are low in sarcoidosis.

6.2. Aim

To confirm the anecdotal observation that vitamin D levels are low in individuals with sarcoidosis, explore the primary human monocyte response to calcitriol and determine if this differed between sarcoidosis and controls.

6.3. Methods

Individuals with sarcoidosis were recruited at first presentation to the Oxford Sarcoidosis-ILD service as previously described, and additionally, provided (i) that they had not received treatment prior to attending their first appointment and (ii) that their symptom duration was short and presentation was in the summer (July - mid October), to minimise the confounding effect of seasonal variation in vitamin D levels. This was compared with age, gender and ethnicity matched healthy controls, collected between July and mid October. South Asian donors were excluded from both cohorts in these experiments because of a high background level of vitamin D insufficiency and deficiency in this population. Donor demographics are listed in Table 6.1.

	Sarcoidosis				Control		
	# (#Ex Pulm/SA)	Age (SD)	Male	CAS (0/1/2/3/4)	# (SA)	Age (SD)	Male
Fig 6.2a	46 (3/0)	44 (10.2)	18	(0/21/20/6/1)	39 (0)	42 (8.3)	25
Fig 6.3-6.8	6 (0/0)	46 (11.7)	2	(0/4/2/0/0)	6	38 (4.1)	4
Fig 6.9a-c	3 (0/0)	36 96.7)	1	(0/1/2/0/0)	3 (0)	33 (6.7)	2
Fig 6.10a-d	4 (0/0)	46 (11.3)	2	(0/3/1/0/0)	4 (0)	41 (5.1)	2

Table 6.1. Donor demographics
Number of participants per figure indicated in table, number with extra-pulmonary involvement (Ex Pulm) or South Asian (SA) origin indicated in parenthesis. CAS indicates serum biomarker clinical activity score: Lymphocyte count $<1.0x10^9$/l = 1; $>1.0x10^9$/l = 0. Serum ACE: >100 U/l = 2; 55-100U/l = 1; <55 U/l = 0. IgG >13g/l = 1; ≤ 13g/l = 0. Maximum score = 4.

Serum samples were stored at -80°C prior to batch analysis. Calcidiol [25($OH0D_3$] levels were measured using a chemiluminescence assay on an Immunodiagnostic Systems iSYS (IDS-iSYS) automated analyser by Dr. Tim James, Department of Clinical Biochemistry, Oxford University Hospitals Trust (415). 'Sufficient', 'insufficient' and 'deficient' calcidiol levels refer to the clinical laboratory standards for the assay based on their standardised control measurements.

The monocyte surface phenotype and cytokine profile in response to calcitriol was assessed by flow cytometry (as described in section 2.2.8), after an 18-hour stimulation with calcitriol. Two concentrations of calcitriol were chosen in order to reflect that observed in the clinical setting. Assuming a 1:1 conversion rate for calcidiol to calcitriol, a dose of 10^{-7}M (100nM/L) was used to reflect clinical sufficiency and 10^{-8}M (10nM/L) to reflect deficiency. Monocytes did not survive in culture over this duration of culture with concentrations of 10^{-6}M or greater. Monocyte phagocytosis and fusion response to calcitriol were assessed by conventional flow cytometry and by ImageStream™ and in vitro assay (as previously described in section 2.2.15-16).

6.4. Results

6.4.1. Vitamin D (calcidiol) levels were reduced in sarcoidosis compared with healthy controls.

More than half of healthy controls had insufficient or deficient levels of vitamin D as defined by the laboratory standard (median 75.3ng/ml [IQR 52.0-88.1]), while a greater proportion of individuals with sarcoidosis were vitamin D insufficient or deficient (median 25.35 [18.43-45.05]) (Figure 6.2a).

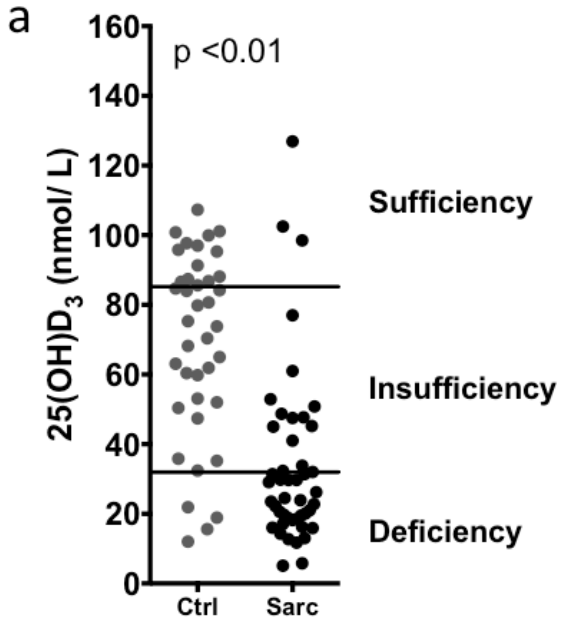


Figure 6.2. Incidence of vitamin D insufficiency and deficiency was increased in sarcoidosis at presentation.
(a) Serum calcidiol levels were on the IDS-iSYS analyser. Samples were collected at first attendance to the Oxford sarcoidosis-ILD service in treatment naïve individuals presenting in the summer (July - mid October). (a) Calcidiol levels were lower in sarcoidosis than healthy controls. D'Agostino and Pearson omnibus normality test and a Mann-Whitney test were used for statistical analysis.

6.4.2. Monocyte surface antigens were altered after co-culture with calcitriol.

The expression of various surface proteins on LPS stimulated monocytes was examined by flow cytometry after 18 hours culture with calcitriol at clinically sufficient (10^{-7}M, 100nm/L) and deficient (10^{-8}M, 10nM/L) levels. Most strikingly, the expression of monocyte CD14 was upregulated when cultured in calcitriol in a dose dependent manner (Figure 6.2a). Baseline CD16 (FcγRIII) expression was 3-4 fold greater on sarcoidosis monocytes than controls as seen previously (Figure 3.1b), but unlike in controls, where calcitriol increased CD16 expression, there was no difference seen in sarcoidosis monocytes (Figure 6.2b). By contrast, CD32 (FcγRII) expression is downregulated after culture with calcitriol in a dose-dependent fashion (Figure 6.2c).

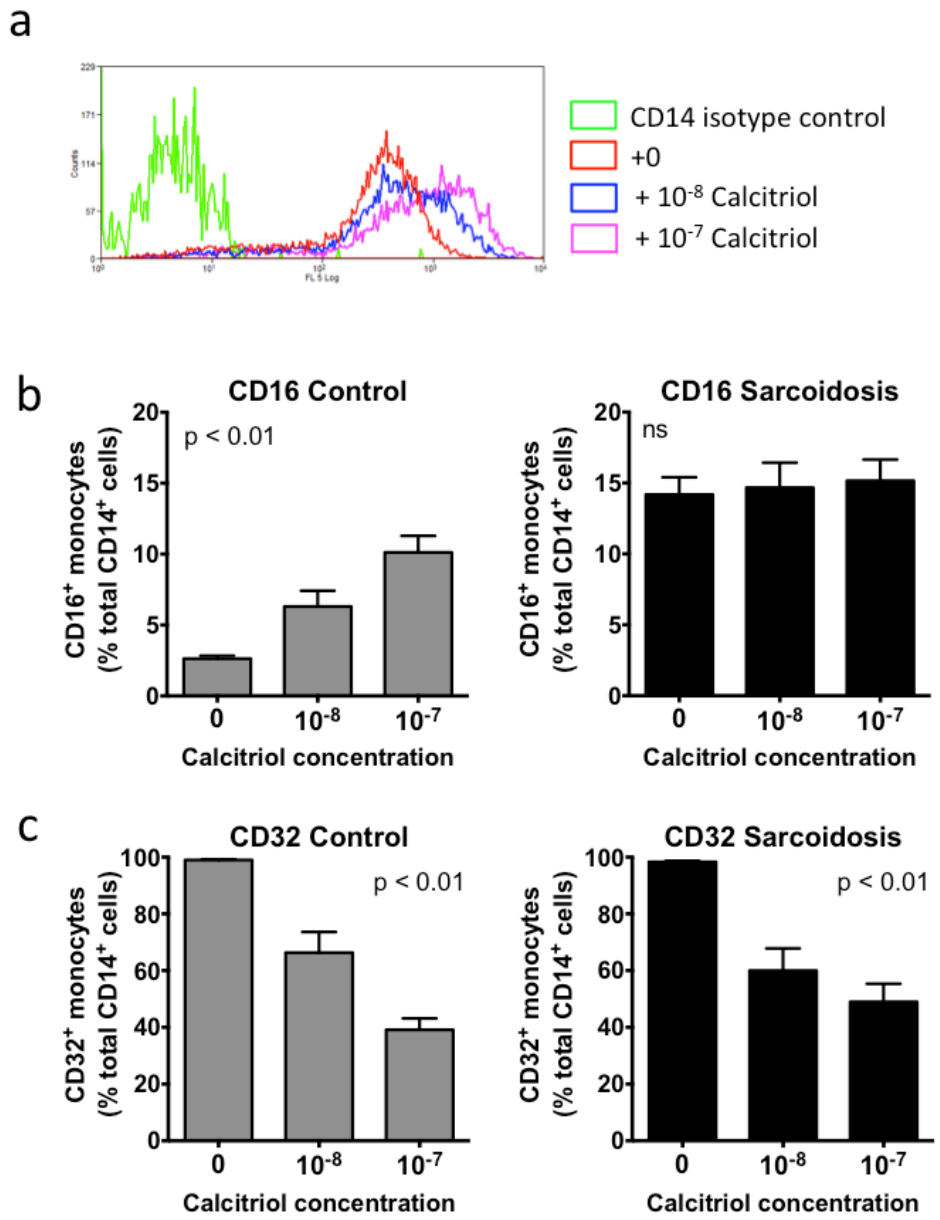


Figure 6.3. Monocyte surface phenotype was altered after calcitriol treatment.

LPS stimulated monocytes were treated with calcitriol (10^{-8}M or 10^{-7}M) for 18 hours. (a) Calcitriol increases monocyte CD14 expression in a dose dependent manner. (b) The proportion of monocytes expressing CD16 is increased after calcitriol treatment in controls (+0: 2.56 [2.18-3.13] [$CD16^+CD14^+$ as %total $CD14^+$], $+10^{-8}$M: 5.75 [4.02-8.93], $+10^{-7}$M: 10.04 [7.80-12.17]) but not sarcoidosis (+0: 14.44 [12.24-16.60], $+10^{-8}$M: 15.20 [10.54-18.92], $+10^{-7}$M: 15.74 [11.20-18.52]). (c) The proportion of monocytes expressing CD32 is reduced after calcitriol treatment in both controls (+0: 99.15 [98.75-99.35] [$CD32^+CD14^+$ as %total $CD14^+$], $+10^{-8}$M: 71.00 [45.00-80.50], $+10^{-7}$M: 0.40 [31.50-46.50]) and sarcoidosis (+0: 98.50 [97.75-99.00], $+10^{-8}$M: 57.50 [45.00-78.75], $+10^{-7}$M: 50.50 [33.25-65.00]). (n=6 per group). Median [IQR]. Kruskal-Wallis with Dunn's multiple comparisons test were used for statistical analysis.

As expected from previously published reports (406), monocyte surface expression of TLR2 and TLR4 were downregulated after culture with calcitriol (Figure 6.3a and b). No difference was seen in surface expression of the activation marker and proliferation marker CD115, the migration and adhesion markers CX3CR1, CCR2, CD62L; or the phagocytosis markers CD15, CD36 or CD163.

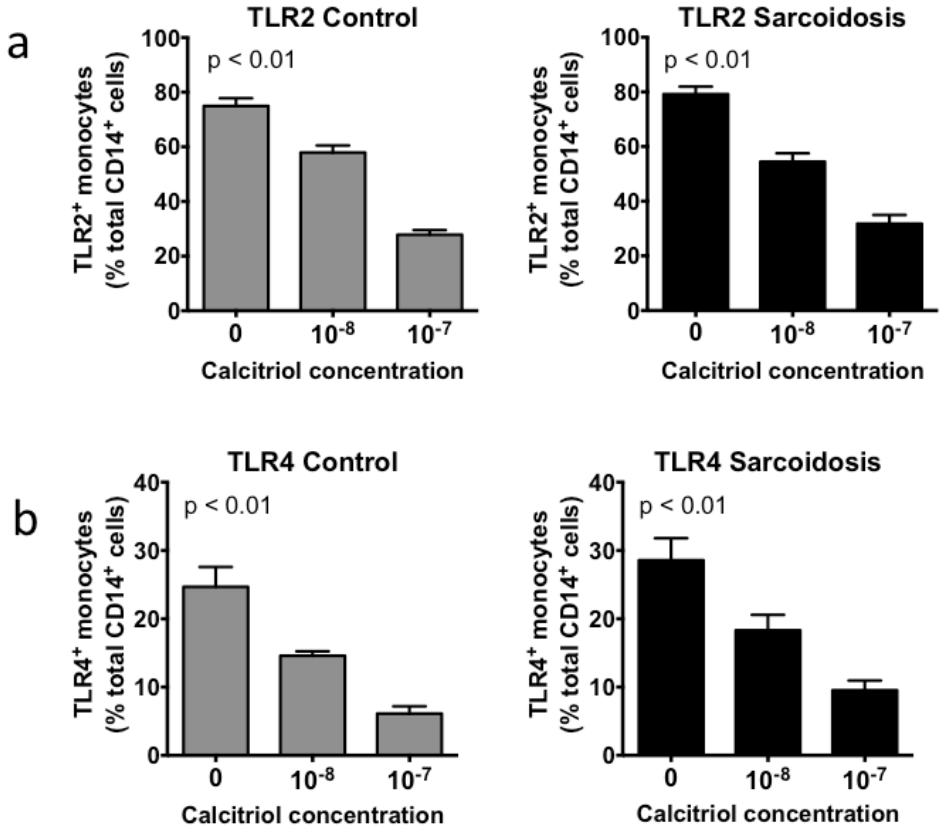


Figure 6.4. Monocyte TLR2 and TLR4 expression was reduced after calcitriol treatment.

LPS stimulated monocytes were treated with calcitriol (10^{-8}M or 10^{-7}M) for 18 hours. (a) The proportion of monocytes expressing TLR2 is decreased after calcitriol treatment in both controls (+0: 74.16 [70.55-78.98] [$TLR2^+CD14^+$ as %total $CD14^+$], $+10^{-8}$M: 57.69 [51.47-63.93], $+10^{-7}$M: 27.50 [24.86-31.15]) sarcoidosis (+0: 79.06 [74.72-85.66], $+10^{-8}$M: 54.74 [47.80-61.84], $+10^{-7}$M: 32.64 [24.65-38.90]). (b) The proportion of monocytes expressing TLR4 is also reduced after calcitriol treatment in both controls (+0: 24.14 [19.90-32.23] [$TLR4^+CD14^+$ as %total $CD14^+$], $+10^{-8}$M: 14.95 [13.02-15.94], $+10^{-7}$M: 6.29 [3.48-8.55]) and sarcoidosis (+0: 24.83 [23.18-35.93], $+10^{-8}$M: 16.60 [15.00-20.89], $+10^{-7}$M: 9.41 [6.25-12.86]). (n=6 per group). Median [IQR]. Kruskal-Wallis with Dunn's multiple comparisons test were used for statistical analysis.

6.4.3. Calcitriol influenced monocyte cytokine production in a dose-dependent manner.

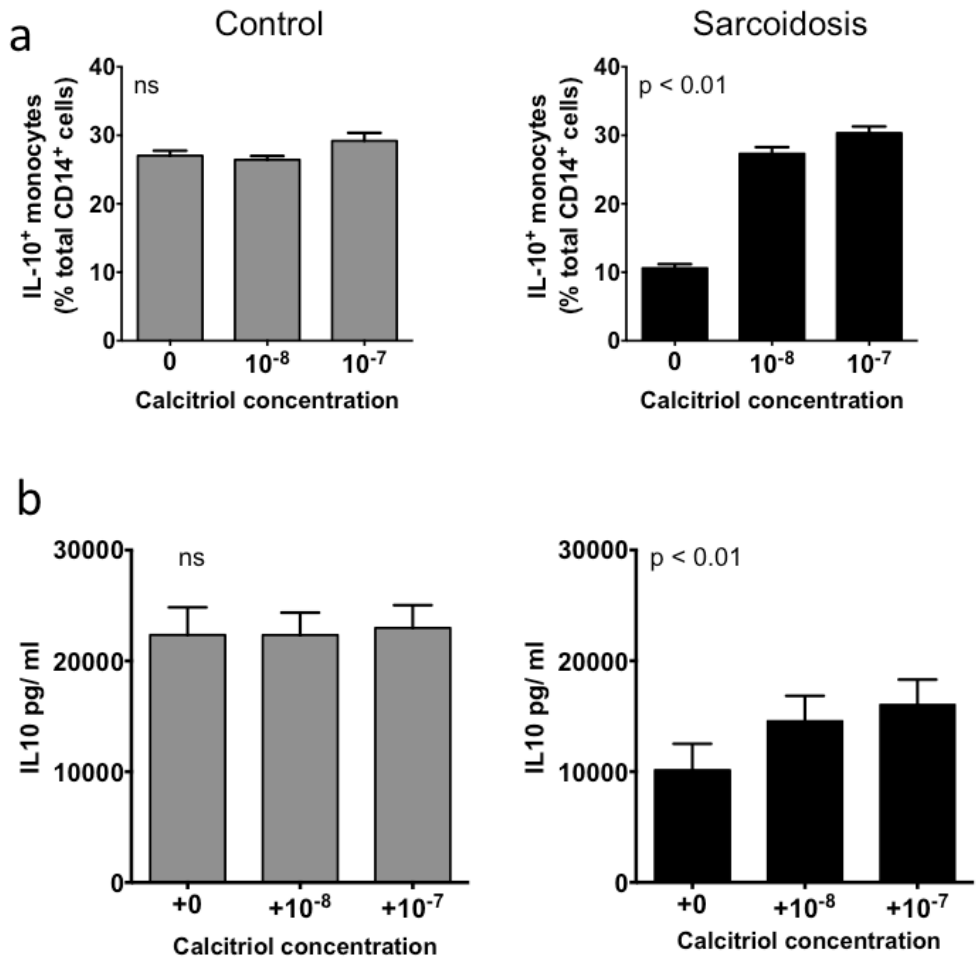


Figure 6.5. Sarcoidosis monocyte IL-10 was increased by calcitriol treatment.
LPS stimulated monocytes were treated with calcitriol (10^{-8}M or 10^{-7}M) for 18 hours. (a) The proportion of monocytes expressing IL-10 after calcitriol treatment is unchanged in controls (+0: 27.15 [25.16-28.64] [IL-$10^{+}$$CD14^{+}$ as %total $CD14^{+}$], +10^{-8}M: 25.90 [25.37-27.66], +10^{-7}M: 29.56 [27.26-31.74]) and increased in sarcoidosis (+0: 10.19 [9.38-12.04], +10^{-8}M: 27.58 [25.64-29.35], +10^{-7}M: 30.76 [28.90-32.60]). (b) The quantity of IL-10 produced is unchanged after calcitriol treatment in controls (+0: 21348 [17750-28095]pg/ml, +10^{-8}M: 22188 [17599-26716], +10^{-7}M: 22942 [18793-29777]) and increased in sarcoidosis (+0: 8035 [5715-15419], +10^{-8}M: 12682 [9864-20456], +10^{-7}M: 15032 [11718-19690]). (n=6 per group). Median [IQR]. Kruskal-Wallis with Dunn's multiple comparisons test were used for statistical analysis.

Calcitriol has been shown to increase IL-10 production by T cells, DCs and monocytic cell lines. Here, in primary sarcoidosis monocytes, I found that the addition of calcitriol (10^{-7}M) did not affect control monocyte IL-10, but that there was a significant, dose dependent increase in both the percentage of IL-10 producing monocytes and the quantity of IL-10 produced in sarcoidosis IL-10 (Figure 6.5a-b).

Examining the production of other cytokines by monocytes, I found that calcitriol did not affect the proportion of control monocytes that express either TNFα or IL-12. However, the proportion of sarcoidosis monocytes expressing both of these cytokines (which I found to be increased in sarcoidosis monocytes in chapter 3 [Figure 3.8]), was significantly reduced (Figure 6.6a-b). For TNFα this was still almost double the control monocyte baseline, whilst for IL-12 this reduction took the proportion down to the control baseline.

Although I found no significant difference between the amount of CCL2 produced by sarcoidosis monocytes compared with controls (Chapter 3 [Figure 3.10]), calcitriol significantly increased CCL2 expression on sarcoidosis but not control monocytes (Figure 6.6c).

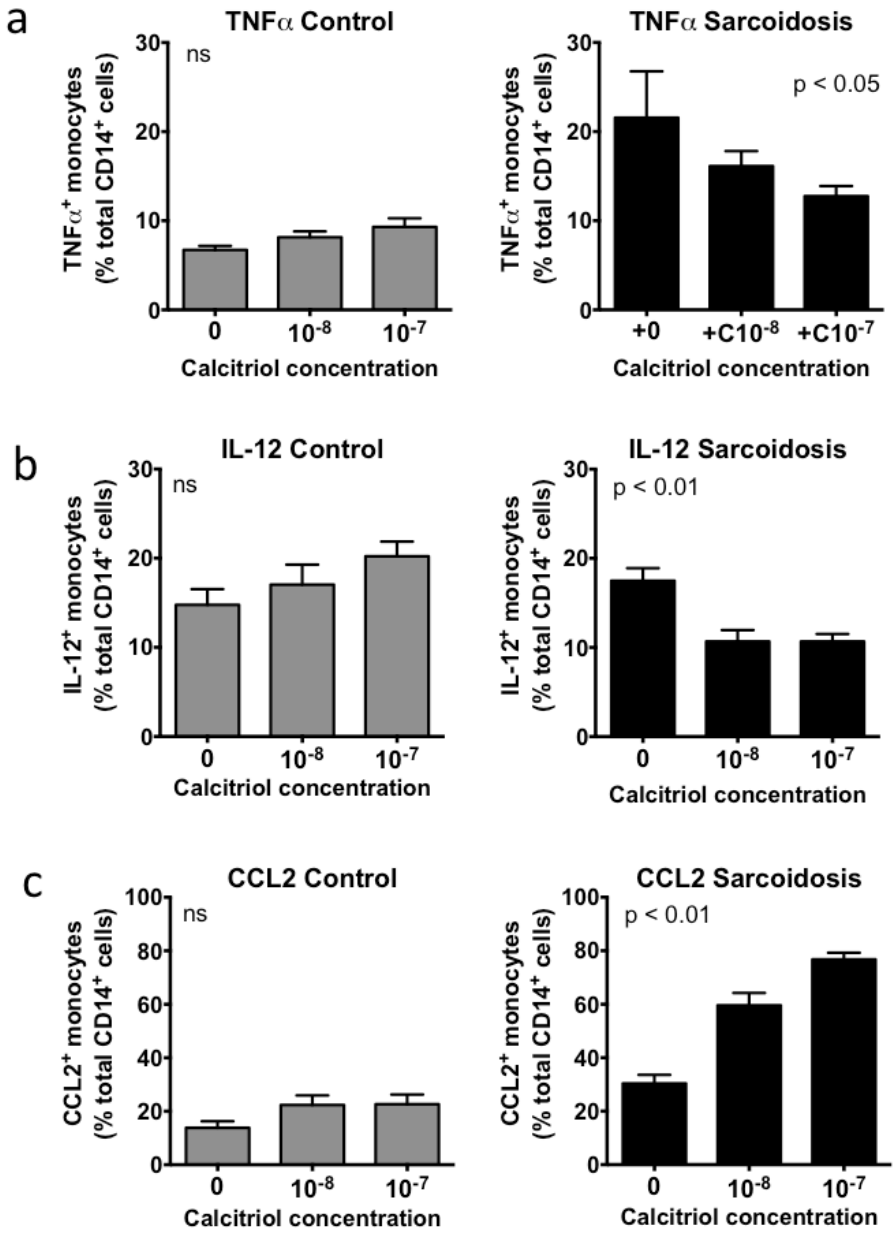


Figure 6.6. Sarcoidosis monocyte TNFα and IL-12 were decreased and CCL2 was increased after calcitriol treatment.
LPS stimulated monocytes were treated with calcitriol (10^{-8}M or 10^{-7}M) for 18 hours. (a) The proportion of monocytes expressing TNFα is unchanged in controls after calcitriol treatment (+0: 6.14 [5.90-8.00] [$TNF\alpha^+CD14^+$ as %total $CD14^+$], $+10^{-8}$M: 8.11 [6.64-9.57], $+10^{-7}$M: 8.86 [7.10-11.80]) and reduced in sarcoidosis (+0: 23.51 [12.98-30.87], $+10^{-8}$M: 15.94 [13.22-19.27], $+10^{-7}$M: 12.38 [9.95-15.91]). (b) The proportion of monocytes expressing IL-12 is also unchanged in controls after calcitriol treatment (+0: 14.04 [10.89-19.29] [$IL\text{-}12^+CD14^+$ as %total $CD14^+$], $+10^{-8}$M: 16.45 [12.09-20.44], $+10^{-7}$M: 19.20 [17.18-22.75]) but reduced in sarcoidosis (+0: 18.19 [15.09-19.84], $+10^{-8}$M: 11.09 [7.69-13.00], $+10^{-7}$M: 10.16 [9.02-12.85]). (c) The proportion of monocytes expressing CCL2 is unchanged in controls after calcitriol treatment (+0: 12.25 [9.58-17.62] [$CCL2^+CD14^+$ as %total $CD14^+$], $+10^{-8}$M: 19.79 [17.80-25.84], $+10^{-7}$M: 19.30 [17.50-26.47]) and increased in sarcoidosis (+0: 32.35 [25.85-36.18], $+10^{-8}$M: 60.20 [52.22-69.83], $+10^{-7}$M: 76.06 [71.62-82.77]). (n=6 per group). Median [IQR]. Kruskal-Wallis with Dunn's multiple comparisons test were used for statistical analysis.

The effect of calcitriol on monocyte expression of IL-4 and IL-13, which influence monocyte fusion and granulomagenesis was also examined. Calcitriol significantly reduced the proportion of monocytes expressing IL-4 in both sarcoidosis and controls (Figure 6.7a). Interestingly, calcitriol significantly increased the proportion of control monocytes expressing IL-13, whilst no difference was seen in sarcoidosis monocytes (Figure 6.7b).

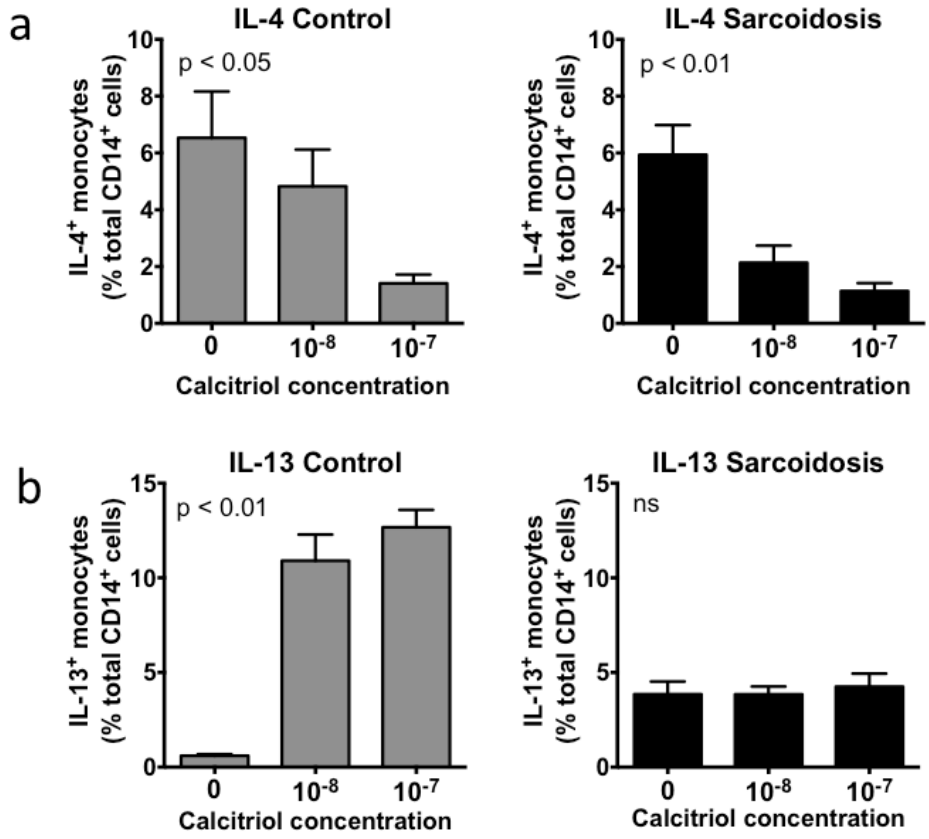


Figure 6.7. Monocyte IL-4 and IL-13 expression were altered by calcitriol treatment.
LPS stimulated monocytes were treated with calcitriol (10^{-8}M or 10^{-7}M) for 18 hours. (a) The proportion of monocytes expressing IL-4 after calcitriol treatment is reduced in both controls (+0: 5.4 [3.15-11.1] [IL-$4^+$$CD14^+$ as %total $CD14^+$], +10^{-8}M: 4.34 [2.25-6.80], +10^{-7}M: 1.19 [0.75-2.20]) and sarcoidosis (+0: 5.45 [3.86-8.60], +10^{-8}M: 1.65 [0.89-3.78], +10^{-7}M: 1.11 [0.44-1.78]). (b) The proportion of monocytes expressing IL-13 is increased after calcitriol treatment in controls (+0: 0.60 [0.40-0.80] [IL-$13^+$$CD14^+$ as %total $CD14^+$], +10^{-8}M: 10.99 [7.81-14.31], +10^{-7}M: 13.38 [10.09-14.36]) but unchanged in sarcoidosis (+0: 3.57 [2.28-5.51], +10^{-8}M: 3.61 [2.92-4.96], +10^{-7}M: 3.94 [2.92-6.21]). (n=6 per group). Median [IQR]. Kruskal-Wallis with Dunn's multiple comparisons test were used for statistical analysis.

I then asked whether the addition of calcitriol might further increase the heightened IL-10 expression induced by iNKT cells, identified and discussed in detail in chapter 4. Whilst the addition of calcitriol (10^{-7}M) or iNKT (ratio 1:1) to LPS stimulated monocytes each increases the monocyte IL-10, there is no additive effect when both are added simultaneously (Figure 6.8a-b) in either sarcoidosis or controls.

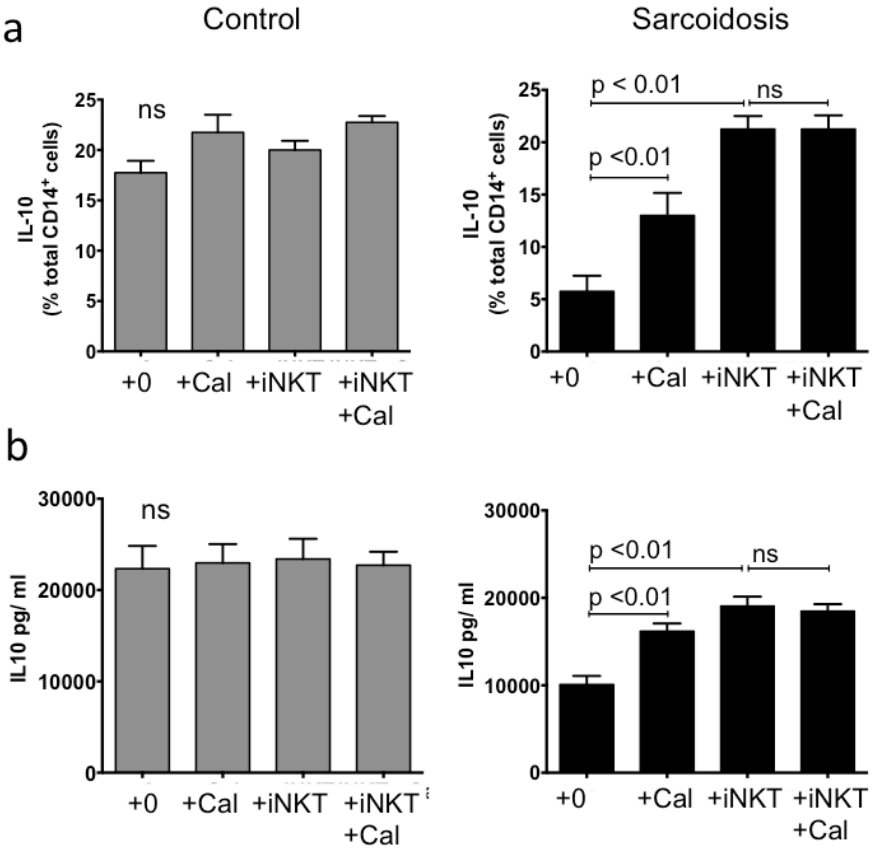


Figure 6.8. Co-culture with iNKT cells did not further augment calcitriol-induced monocyte IL-10.
LPS stimulated monocytes were treated with calcitriol (10^{-7}M) for 18 hours ± iNKT cells (ratio 1:1). (a) The proportion of monocytes expressing IL-10 after iNKT co-culture in addition to calcitriol treatment is unchanged in both controls (+0: 17.00 [16.00-20.25] [IL-10^{+}CD14^{+} as %total CD14^{+}], +calcitriol: 21.50 [18.50-25.25], +iNKT: 20.00 [18.25-21.75], +iNKT+calcitriol: 23.00 [21.50-23.75]) and sarcoidosis (+0: 6.00 [2.75-8.50], +calcitriol: 14.00 [8.50-16.50], +iNKT: 21.50 [18.75-23.50], +iNKT+calcitriol: 20.50 [19.25-24.00]). (b) The quantity of IL-10 produced is unchanged after iNKT co-culture in addition calcitriol treatment in both controls (+0: 21348 [17750-28095]pg/ml, +calcitriol: 22942 [18793-27340], +iNKT: 23843 [18160-27855], +iNKT+calcitriol: 23227 [19246-25955]) and sarcoidosis (+0: 9833 [7981-12810], +calcitriol: 16878 [14260-17414], +iNKT: 19231 [16400-21285], +iNKT+calcitriol: 18883 [16536-20065]). (n=6 per group). Median [IQR]. Kruskal-Wallis with Dunn's multiple comparisons test were used for statistical analysis.

6.4.4. Calcitriol increased monocyte ability to phagocytose latex beads

Having established that treatment with calcitriol affects monocyte surface antigen expression, including some implicated in phagocytic ability, I next examined whether this was translated into altered capacity to phagocytose latex beads. LPS stimulated monocytes were incubated for 2 hours with fluorescent latex beads at a ratio of 1:20 with or without 10^{-7}M calcitriol and data acquired on the imagestream flow cytometer as previously described (sections 2.2.14 and 5.4.2). The mean spot count per cell, indicating phagocytosed beads, was significantly increased in both control and sarcoidosis monocytes after treatment with calcitriol with the sarcoidosis monocytes achieving a similar functional capacity to controls (Figure 6.9a). This was reflected in the both the number of cells containing 1, 2, 3, 4 or 5+ beads (spots), where the number of cells containing multiple and indeed 5+ beads increased with calcitriol treatment (Figure 6.9b-c).

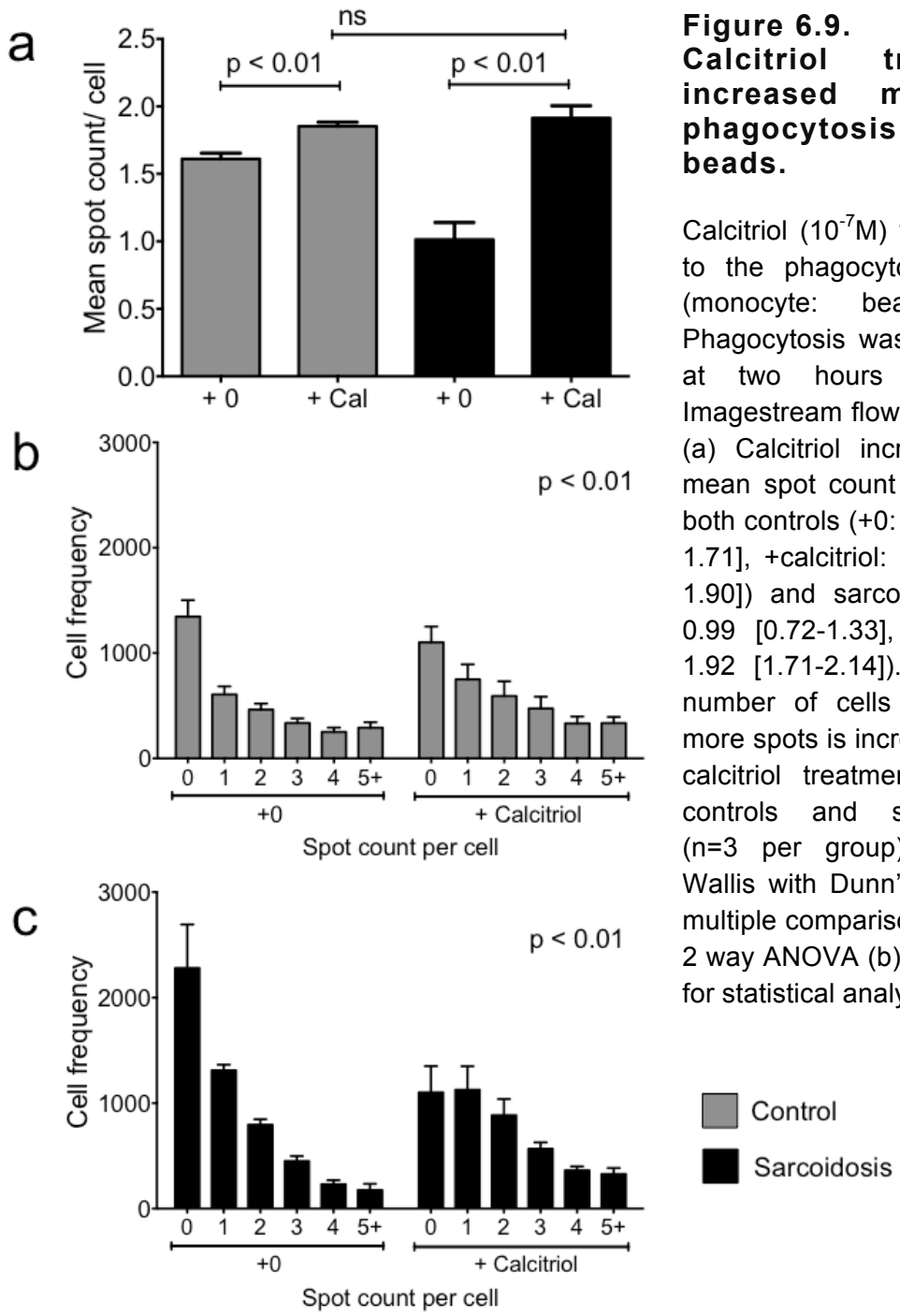


Figure 6.9. Calcitriol treatment increased monocyte phagocytosis of latex beads.

Calcitriol (10^{-7}M) was added to the phagocytosis assay (monocyte: bead 1:20). Phagocytosis was assessed at two hours with the Imagestream flow cytometer. (a) Calcitriol increased the mean spot count per cell in both controls (+0: 1.60 [1.51-1.71], +calcitriol: 1.86 [1.78-1.90]) and sarcoidosis (+0: 0.99 [0.72-1.33], +calcitriol: 1.92 [1.71-2.14]). (b) The number of cells with 2 or more spots is increased after calcitriol treatment in both controls and sarcoidosis. (n=3 per group). Kruskal-Wallis with Dunn's post test multiple comparisons (a) and 2 way ANOVA (b) were used for statistical analysis.

6.4.5. Monocyte fusion capacity was reduced by calcitriol.

To assess the effects of calcitriol on the in vitro model of granulomagenesis (fusion capacity of monocytes), calcitriol at clinically insufficient (10^{-8}M) and clinically sufficient (10^{-7}M) concentrations were added on day 4 to the IL-4

driven monocyte fusion assay (which provided optimum fusion conditions, described in sections 2.2.15 and 5.3). This demonstrated that IL-4 mediated fusion is close to 100%, even in control monocytes at day 7 (i.e. 72 hours after the addition of IL-4 and calcitriol). Addition of calcitriol in this assay had no significant effect on monocyte fusion (Figure 6.10a). The effects of calcitriol on fusion were additionally assessed at day 5 (i.e. 24 hours after the addition of IL-4 and calcitriol). Calcitriol significantly decreased control but not sarcoidosis fusion at 5 days (Figure 6.10b-c). As monocyte fusion in this system is partly abrogated by the addition of IL-10 (Figure 5.5c-d) and calcitriol increased sarcoidosis monocyte IL-10 expression (Figure 6.5b), I hypothesised that the calcitriol mediated decrease in fusion is the result of calcitriol-induced monocyte IL-10. To test this hypothesis, IL-10 blocking antibody (αIL-10, 4μg/ml, Peprotech) was added to the fusion assay at the same time as the IL-4 and calcitriol. This restored the fusion capacity in the control monocytes. No significant change (although the trend was similar) was observed for sarcoidosis monocytes (Figure 6.10c and d).

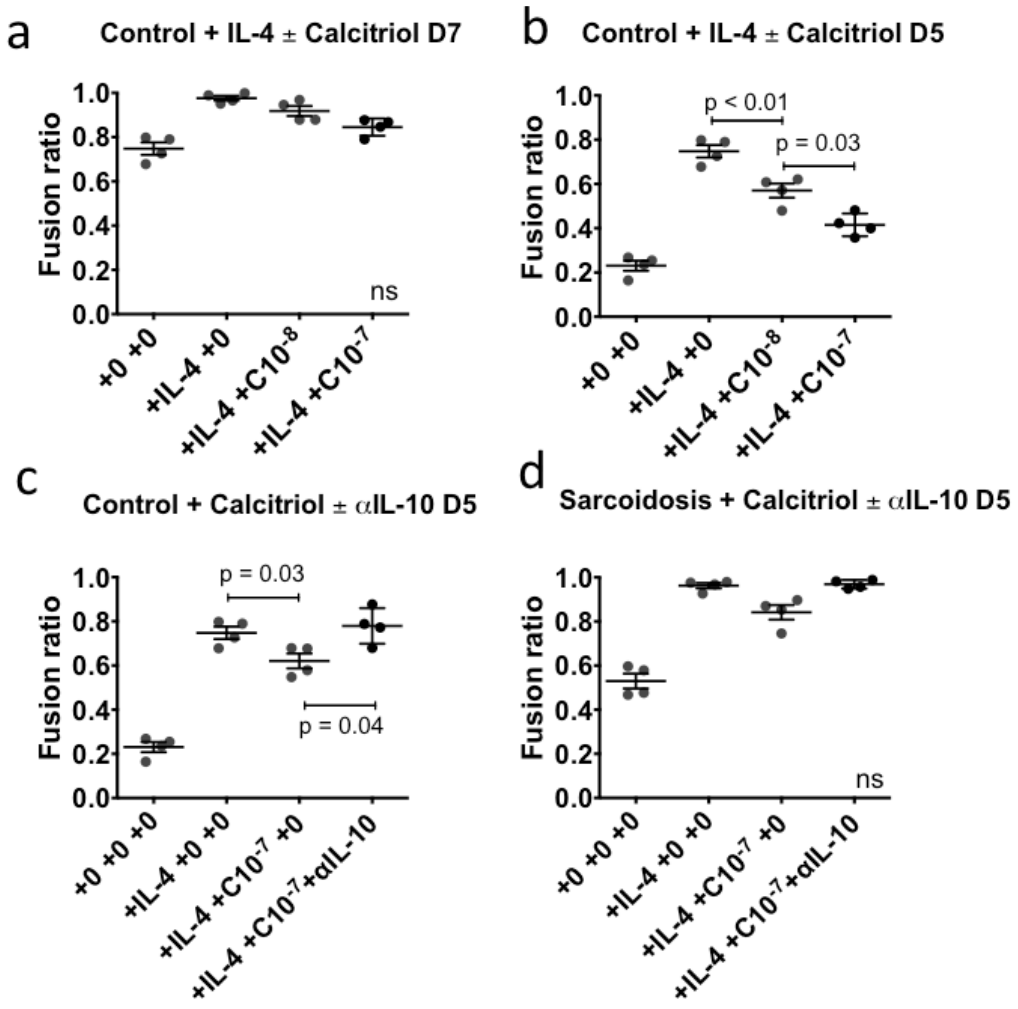


Figure 6.10. Monocyte fusion was reduced by calcitriol in an IL-10 dependent manner.

Calcitriol (10^{-8}M, 10^{-7}M) was added to the in vitro, IL-4 mediated fusion assay on day 4. (a) There was no alteration in control monocyte fusion in response to calcitriol on day 7 (fusion ratio +0: 0.76 [0.69-0.80], +IL-4: 0.98 [0.95-1.00], +IL-4+C10^{-8}M: 0.91 [0.88-0.96], +IL-4+C10^{-7}M: 0.85 [0.80-0.88]), however (b) when the assay was terminated on day 5 (24 hours after the addition of IL-4 and calcitriol), fusion was reduced in a dose-dependent manner (fusion ratio +0: 0.25 [0.18-0.26], +IL-4: 0.76 [0.69-0.80], +IL-4+C10^{-8}M: 0.59 [0.50-0.62], +IL-4+C10^{-7}M: 0.41 [0.37-0.47]). The calcitriol-mediated reduction in fusion was overcome by the addition of 4μg/ml IL-10 blocking antibody in (c) controls (fusion ratio +0: 0.25 [0.18-0.26], +IL-4: 0.75 [0.69-0.80], +IL-4+C10^{-7}M: 0.63 [0.56-0.68], +IL-4+C10^{-7}M+αIL-10: 0.78 [0.70-0.85]), but not (d) sarcoidosis (fusion ratio +0: 0.53 [0.47-0.59]; +IL-4: 0.97 [0.94-0.98], +IL-4+C10^{-7}M: 0.86 [0.77-0.89], +IL-4+C10^{-7}M+αIL-10: 0.97 [0.95-0.99]), although a similar non-significant trend was seen. (n=4 per group). Median [IQR]. Kruskal-Wallis with Dunn's multiple comparisons test were used for statistical analysis.

6.5. Discussion

This chapter shows that vitamin D levels in patients are low at presentation. In vitro, vitamin D increased IL-10 production, reduced monocyte capacity to fuse (in controls but not sarcoidosis), and reduced phagocytosis in sarcoidosis monocytes.

Vitamin D deficiency is common and widespread; up to half of the British population has been found to have low levels of vitamin D during the winter and early spring (416, 417). There is indirect evidence to support hypovitaminosis D as a risk factor for the development of sarcoidosis for example, prevalence roughly increases with distance from the equator with Scandinavian countries reporting the highest worldwide incidence (7). Prevalence is particularly high in populations known to have high rates of vitamin D deficiency such as persons of South Asian origin in the UK (418, 419).

Whilst serum levels of 25-hydroxyvitamin D3 as low as 25 nmol/L will prevent rickets, higher levels, from 75 to 100 nmol/L are required to maintain multiple health outcomes including respiratory health. It has previously been shown that vitamin D status positively correlates with lung function, childhood wheeze, COPD and asthma (420-424). In addition, multiple studies have shown that hypovitaminosis D is a risk factor for the development of active TB (403), a condition that shares many immune pathological features with sarcoidosis.

In this study, care was taken to recruit patients at the same time of the year to avoid seasonal variations in sarcoidosis. The summer months were chosen in order to minimise any confounding signal from otherwise healthy controls, many of whom might be expected to have insufficient or deficient levels, as defined by the laboratory, in the winter. It is unclear whether patients with sarcoidosis have persistently low levels of vitamin D throughout the year. Given that in this study they were sampled at a time of the year when they ought to be highest, it is likely that vitamin deficiency is perennial.

Of note, in this study, the vitamin D precursor calcidiol was measured rather than the biologically active form calcitriol. Calcidiol is the predominant circulating metabolite of vitamin D and reflects vitamin D status (405, 425). The apparent thousand-fold excess of calcidiol over calcitriol in the plasma is not biologically relevant, because of the difference in the binding of these metabolites to D-binding protein, the ratio of free calcidiol to free calcitriol is only about 10 to one (425). Calcidiol is currently measured in clinical laboratories nationally because it has the greatest correlation with endocrine and bone metabolic dysfunction, the two most common indications for the test. Calcidiol [$25(OH)D_3$] is 1α-hydroxylated in the kidney by 25-hydroxyvitamin D_3-1α-hydroxylase (1α-OHase) to form the active calcitriol [$1\alpha,25(OH)_2D_3$] (405). Conversion to calcitriol is tightly controlled by PTH; abnormal calcitriol levels in the context of normal calcidiol levels systemically would be the result of abnormal renal 1α-hydroxylation. In sarcoidosis, AM and macrophages isolated from granulomas have been shown to be capable of extra-renal conversion of calcidiol to calcitriol (398-401). Many cells

including monocytes and alveolar macrophages express CYP27B1 (25-hydroxyvitamin D-1α-hydroxylase), which converts calcidiol to calcitriol, a defect in which may prevent the conversion of calcidiol to calcitriol.

Direct comparisons between primary monocytes and monocytic cell lines have shown that CD14 is significantly up regulated by calcitriol in both primary monocytes and cell lines, but that the effect is much more pronounced in the U937 cell line (406, 409, 426, 427). In my study, I found that CD14 (TLR4 co-receptor) surface expression was strongly upregulated in response to calcitriol in primary monocytes in a dose dependent manner. This might suggest that calcitriol increases monocyte LPS responsiveness. However, contradictory to this hypothesis, both the expression of the pattern recognition receptors TLR4 and TLR2 are widely reported in the literature to be down regulated in response to calcitriol, a finding confirmed in this study. Further work to tease out the implication of calcitriol mediated TLR2 and TLR4 down regulation and CD14 up regulation is required in order to determine the functional consequence on calcitriol on altered monocyte antigen recognition in sarcoidosis.

Surface expression of CD16 increased with calcitriol in healthy controls, suggesting increased activation. However, the baseline levels in sarcoidosis monocytes are already high and no further increase was seen with calcitriol. This might suggest that whilst calcitriol may be required to expand inflammatory ($CD14^{++}CD16^{+}$) monocytes in healthy individuals, other signals are more important for this in sarcoidosis monocytes. There was no alteration

in the expression of the activation marker and proliferation marker CD115 after treatment with calcitriol, which might be expected if the monocytes were more activated. The level of CD32, involved in phagocytosis, was down regulated by calcitriol, although interestingly, none of the other phagocytosis markers examined (CD15, CD36 or CD163) were altered. Interpretation of these changes is probably superseded by findings from the phagocytosis and fusion studies after addition of calcitriol.

The changes in cytokine profile after calcitriol treatment suggest that vitamin D promotes a regulatory monocyte cytokine profile. The pro-inflammatory cytokines TNFα and IL-12 are down regulated whilst the regulatory cytokine IL-10 is increased after monocyte culture with calcitriol in sarcoidosis thereby correcting the major cytokine anomalies found in chapter 3.

IL-4 can regulate the production of IL-10, promoting the alternative activation and fusion of macrophages. I have demonstrated that whilst there is no difference in the proportion of monocytes in sarcoidosis that secrete IL-4, there is an almost thousand fold decrease in the quantity of IL-4 produced. The proportion of monocytes that express IL-4 following culture with calcitriol is reduced in both sarcoidosis and controls but, given that for IL-4 surface expression does not give a good indication of supernatant protein levels, quantification with ELISA is required

Interestingly, it has recently been reported that calcitriol can increase macrophage-mediated suppression of T cell proliferation through induction of

IL-10 (346). This activity was not explored in the context of sarcoidosis monocytes, but would warrant further study in the context of the findings discussed earlier (Chapter 4).

The cytokine studies herein were performed at 18 hours having optimised the system for study in chapter 3. Accepting as in the case of IL-10 where different stimulation times have differing effects on cytokine profiling, it would be important to assesses cytokine functionality at different time points, confirming findings with quantitative measurements of supernatant protein.

Vitamin D has previously been reported to increase monocyte chemotaxis, which was postulated to be IL-8, CXCR2 and the antimicrobial peptide cathelicidin (LL37) dependent (407). This is supported by the finding that CCL2 is upregulated on monocytes after treatment with calcitriol, with a resultant increase in migration capacity (428). Contradictory to this, however, recent studies demonstrate that calcitriol decreases the production of CCL2 reducing monocyte migration (429). In this study of primary human monocytes, although chemotaxis was not directly measured, no alteration was seen in the surface migration and adhesion markers CX3CR1, CCR2, , CD62L nor indeed in CCL2 in response to calcitriol. These contradictory findings may be explained by differences in cell separation, cell culture techniques and the inclusion of cell lines, monocyte-derived macrophages and monocyte-derived DCs as “monocytes.” Given that monocyte numbers are expanded in sarcoidosis, determining how they arrive at the granuloma is mediated may prove to be an interesting line of future inquiry.

I found that phagocytosis of latex beads was increased by treatment with calcitriol in both sarcoidosis and control monocytes, reflected by both the mean number of beads per cell and by the frequency of cells with a higher spot count. Translating this to the pathogenesis of sarcoidosis, low levels of vitamin D may impair efficient phagocytosis through an unknown mechanism. It has been reported that phagocytosis is more efficient in monocytes isolated by CD14 negative rather than positive magnetic beads(430), a finding I too observed during the course of investigation. Phagocytosis of Listeria but not latex beads could be improved by CD14 activation, suggesting that calcitriol, which significantly up regulates CD14 may be used to modulate phagocytosis of bacterial but not inert antigens. In the context of this, the preliminary phagocytosis work could be extended by examining the differential phagocytic capacity of sarcoidosis and control monocytes to various antigens including bacterial antigens such as E Coli, L. monocytogenes, mKatG; 'endogenous' antigens such as senescent neutrophils and SAA; and inert materials such as latex beads, and silica. Examination of differential surface and cytokine phenotype as well as examination of differential gene expression may give clues as to the underlying mechanism.

The effect of calcitriol on monocyte fusion was assessed in the IL-4 driven fusion system previously described. I found that at day 7, calcitriol did not affect fusion capacity but that at day 5 calcitriol reduced monocyte fusion in a dose dependent manner. It may that the optimal fusion conditions in the 7-day assay make regulation more difficult or that calcitriol is merely delaying

the process. IL-10 blocking studies in the fusion assays suggest that the calcitriol related reduction in fusion is, at least in part, mediated by IL-10. Sarcoidosis monocytes fuse so readily in this assay, that it is not clear if the lack of calcitriol activity in this system is because the calcitriol capable of overcoming the fusion process, which would have become established by day 4 when the calcitriol was added, or if sarcoidosis monocyte fusion responses to calcitriol are altered. IL-10 is known to decrease monocyte expression of the protease MMP9, which is required for monocyte fusion to MGC (431, 432). Calcitriol induced increase in IL-10 may be a potential pathway through which monocyte fusion to MGC could be modulated in the treatment of sarcoidosis. Further work is required to model fusion kinetics in primary monocytes including surface antigen and cytokine profiling.

Although not examined here, vitamin D increases human macrophage production of cathelicidin when stimulated by TLR2/1L, as occurs in TB infection enhancing mycobactericidal activity and autophagy (433, 434). Interestingly, the amount of cathelicidin in the BAL of sarcoidosis patients is reduced, with lower levels being associated with more severe disease (435, 436).

Hypercalcaemia in sarcoidosis has led clinicians to be wary of vitamin D supplementation even in the face of severe deficiency (437-440). The incidence of hypercalcaemia is not entirely clear. The literature on true incidence is lacking, with even recent reviews on the subject quoting the same figures from 50-70 year-old small cohort studies (441-443). Hypercalcemia is

routinely screened for in sarcoidosis and the subset of patients in whom vitamin D supplementation may be problematic are easily identified. I have shown that vitamin D levels are low in sarcoidosis at presentation. In addition to altered immune regulation and altered skeletal health, hypovitaminosis D causes extreme fatigue and depression, both of which cause significant morbidity in sarcoidosis; some experts recommend vitamin D replacement in sarcoidosis for these reasons alone (444). A recent randomised control trial of vitamin D supplementation (50 000 IU weekly cholecalciferol for 4 weeks, then 50 000 IU monthly for 11 months or placebo) in sarcoidosis examined change in serum calcium over 12 months as the primary endpoint (445). They found that in patients with sarcoidosis and calcidiol level less than 50 nmol/L, vitamin D supplementation did not alter average serum calcium or urine calcium. Although one case of hypercalcaemia was seen this was asymptomatic and detectable through regular monitoring. Vitamin D supplementation is already very widely available, including over the counter. Provided that patients with sarcoidosis are carefully selected and monitored, vitamin D supplementation may have potential to address the monocyte defect in sarcoidois, through modulation of monocyte IL-10 at the site of immune pathology. Further in vivo work will clarify that what I have observed in vitro can be detected in patients after vitamin D treatment.

6.6. Summary

In summary, in this chapter I have shown that vitamin D is low in sarcoidosis at presentation and that calcitriol, the active metabolite of vitamin D, can alter monocyte surface phenotype, cytokine profile, phagocytic ability and fusion

capacity. It reduces TNFα and IL-12 and increases IL-10 production. I have shown that calcitriol alters monocyte granulomagenic potential by increasing phagocytosis and hence increasing antigen clearance capacity. Vitamin D is inexpensive, widely available and has a good safety profile. Provided that careful monitoring is instituted, this may prove a means of modulating altered monocyte immune activity in sarcoidosis.

Chapter 7: Monocyte gene expression in sarcoidosis

7.1. Introduction

I thus far have established that there are significant perturbations in monocyte biology in sarcoidosis. An increase in monocyte number is seen with a disproportionate increase in the proportion of inflammatory $CD14^{++}CD16^{+}$ subset. They have an altered surface phenotype, expressing higher levels of CD115, CD163 and CX3CR1 than controls. This altered surface phenotype is accompanied by functional changes in terms of altered cytokine production, monocytes in sarcoidosis produce more of the inflammatory cytokines TNFα and IL-12 and less of the regulatory cytokines IL-4 and IL-10; as well as altered phagocytic and fusion potential. Additionally, I have demonstrated that monocyte activity can be modulated by contact with iNKT cells or treatment with calcitriol.

These findings suggest that monocytes are potential key cellular players in the early part of disease genesis. I was therefore interested to understand the genome-wide changes in gene expression in monocytes derived from sarcoidosis patients compared with healthy controls. There is a paucity of array data available on untouched monocytes, i.e. monocytes isolated without CD14 binding. Much of the work to date has focused either on monocytes as they transition to macrophages and DCs or on monocyte cell lines in response to various stimuli; there is relatively little data on ex vivo monocyte profiles. Gene expression profiling studies in sarcoidosis have focused on expression

profiles in PBMCs, whole blood, AM or lung tissue (257, 258, 260, 446, 447) although a recent study has included sarcoidosis monocytes isolated by MACS positive selection (260). These studies have seen upregulation of genes involved in the IFN signaling pathways such as IFNA1, IFNA21, IFGNGR1 IRF1, SOCS1, JAK2, STAT1/2 and TYK2 (257, 260); genes related to antigen presentation, PRR recognition of bacteria and viruses; genes related to T-cell homeostasis and survival such as IL15 and IL7R (CD127) (257, 446); and genes related to clonal expansion of sarcoidosis-antigen primed T-helper-lymphocytes including FABP4 and B-MYB (447).

I was thus interested to compare gene expression in ex vivo, untouched monocytes (derived by negative selection) in sarcoidosis compared with healthy controls.

7.2. Aim

To explore genes, biological pathways and processes that are involved in monocyte abnormalities in sarcoidosis.

7.3. Methods

7.3.1. Donor characteristics

All subjects recruited for the monocyte gene expression study were non-smokers and were not on corticosteroids or other disease modifying agents. All had biopsy confirmed pulmonary sarcoidosis with lymphadenopathy with no evidence of other organ involvement. They were divided into two groups according to their serum clinical activity score (CAS): High activity (SHA) and low activity (SLA). Age and gender matched non-smoking controls with no history of recent medication or any reported pulmonary or atopic conditions were recruited (Table 7.1).

	Age (M/F)	Serum CAS	CXR stage	Ctrl Age (M/F)
Sarcoidosis High activity	32 (M)	3	2	32 (M)
Sarcoidosis High activity	53 (M)	2	3	50 (M)
Sarcoidosis High activity	48 (F)	3	2	44 (F)
Sarcoidosis Low activity	53 (M)	1	2	51 (M)
Sarcoidosis Low activity	34 (F)	0	2	35 (F)
Sarcoidosis Low activity	51 (M)	1	2	47 (M)

Table 7.1. Sarcoidosis donors and matched controls.
Serum CAS: Lymphocyte count $<1.0x10^9$/l = 1; $>1.0x10^9$/l = 0. Serum ACE: >100 U/l = 2; 55-100U/l = 1; <55 U/l = 0. IgG >13g/l = 1; ≤ 13g/l = 0. Maximum score = 4. CXR stage not used in assessment of disease activity.

7.3.2. Experimental design

Blood was collected directly into lithium-heparin tubes and PBMCs isolated by density gradient separation within 3 hours of venesection. Monocytes were then isolated by CD14 negative magnetic selection. The cells were washed to remove any trace of EDTA from the magnetic separation buffers and RNA

extracted using Qiagen RNeasy mini kit, including the optional DNAse treatment, according to manufacturers instructions (section 2.2.18.2). RNA quantity was assessed on a NanoDrop photospectrometer and quality assessed using a high-resolution electrophoresis bioanalyser system (Agilent Technologies). RNA was stored at -80°C.

cRNA synthesis and gene chip hybridisation was performed by Dr. Karene Argoud at the Wellcome Trust Centre for Human Genetics, in a single batch using an Illumina HumanHT-12 v4 Expression BeadChip Kit. RT-qPCR validation was performed using TaqMan® gene expression assay (as described in section 2.2.19).

7.3.3 Data analysis

Prior to statistical analysis, the mean intensity was computed for each probeset. A Bayesian moderated t-test was applied to identify differentially expressed genes between sarcoidosis monocytes control monocytes, using the limma (linear models for microarray analysis) package from BioConductor (266). The Benjamini–Hochberg false discovery rate (FDR) procedure was then applied to correct for multiple testing (267). The inverse log of the absolute log2 fold change (FC) generated by limma was used to determine the FC between groups, with the direction of change indicated by the sign. Further visualisation of the data using heatmaps and hierarchical clustering was performed using the TMeV4 software (448). These analyses were performed in congunjtion with Dr. Steve Taylor at the Computational Biology Research Group, Weatherall Institute of Molecular Medicine and Dr. Helen

Lockstone, Bioinformatics and Statistical Genetics Core, Oxford Genomics Centre, Wellcome Trust Centre for Human Genetics.

Fold change in gene expression and adjusted p values from the microarray data were entered into the web based programme MetaCore™ (Thomson Reuters) in order to identify biological networks and Gene Ontology (GO) processes differentially represented in the dataset: (https://portal.gengo.com/cgi/data_manager.cgi#). MetaCore creates a list of possible networks, ranked according to how many objects in the network correspond to the user's list of genes, how many nodes are in the network and how many nodes are in each smaller network. Network size varies widely (from less than 10 to over 2000). In MetaCore, a pathway is equivalent to a canonical map that has multiple sequential steps of interactions, defining a signaling mechanism and can be thought of as a ‘snapshot’ of a section of a network. Each step is well defined and experientially validated and accepted by the field.

To further analyse the biological meaning of the differentially expressed genes, Gene-Set Enrichment Analysis (GSEA) was employed. GSEA is a powerful method of testing the biological meaning of differentially expressed genes (268) and was performed using web-based software (www.broad.mit.edu/gsea/). GSEA assesses a ranked list of all genes, ordered such that genes showing higher expression in one phenotype (for example, the sarcoidosis group) are at the top, and those showing higher expression in the other phenotype (the healthy control group) are at the

bottom, and unchanged genes are in the middle. Pre-defined gene-sets are then tested for enrichment in one of the phenotypes, and the significance of the enrichment is assessed by a permutation method. In this study, GSEA was used in Pre-Ranked mode, where the list of genes was ranked according to the moderated t-statistic computed by limma. Gene-sets comprising all biological process categories defined by the Gene Ontology Consortium (449) were tested, together with a further 9 additional custom gene-sets. The latter were derived through keyword searches via the NCBI (National Center for Biotechnology Information) GEO (Gene expression omnibus) Datasets and EBI (European Bioinformatics Institute) ArrayExpress platforms. Abstracts for the top 100 hits per search term were screened and full articles repository entries accessed to ensure relevance. Data sets were then analysed with GEO2R to determine the required gene lists, for example genes differentially expressed in monocytes with and without exogenous IL-10 treatment (GSE43700) (450). Deposited data that did not pass satisfactory QC criteria were excluded (260). A complete list of GSE accession details with experimental derivation is given in Appendix 3.

7.3.4. Data interpretation

The enrichment score (ES) reflects the degree to which a gene set is overrepresented at the top or bottom of a ranked list of genes. A positive ES indicates gene set enrichment at the top of the ranked list; a negative ES indicates gene set enrichment at the bottom of the ranked list. The normalised enrichment score (NES) is the primary statistic for examining gene set

enrichment results. It normalises the enrichment score for gene set size and correlations between gene sets and the expression dataset.

The nominal p value estimates the statistical significance of the enrichment score for a single gene set. The false discovery rate (FDR) is the estimated probability that a gene set with a given NES represents a false positive finding. For example, an FDR of 0.25 indicates that the result is likely to be valid 3 out of 4 times. I have used a stringent DFR cut off of 0.05 due to the relatively smaller number of samples I have for analysis.

The “leading-edge subset” in a gene set are those genes that accounts for the gene set enrichment signal.

7.4. Results

7.4.1. Quality control of microarray samples

RNA quality was assessed through the “RNA integrity number” (RIN) of the RNA samples. RIN is an algorithm, produced by the Agilent Bioanalyser, which evaluates the entire electrophoretic trace of RNA and determines the RNA integrity. The algorithm assigns a score of 1 to 10, where 10 represents completely intact RNA. The RIN values verified high RNA integrity indicating sample suitability for hybridisation (Table 7.2).

Sample	RIN	Sample	RIN
Ctrl 1	9.6	**Sarc 1 (SLA)**	9.8
Ctrl 2	9.8	**Sarc 2 (SHA)**	9.4
Ctrl 3	8.2	**Sarc 3 (SHA)**	10.0
Ctrl 4	8.2	**Sarc 4 (SHA)**	8.6
Ctrl 5	8.0	**Sarc 5 (SLA)**	10.0
Ctrl 6	8.2	**Sarc 6 (SLA)**	10.0

Table 7.2. RIN values prior to chip hybridization.

More than 13 500 genes were detected in each sample (Figure 7.1a). The data was then preprocessed by Dr. Steve Taylor and Dr. Helen Lockstone. The VSN (variance-stabilisation and normalisation) algorithm was used to process raw data from Illumina gene expression arrays in order to: (i) stabilise the variance to better meet the assumptions of the statistical test for differential expression and to (ii) normalise signal intensities from different samples so that they are comparable. The mean variance demonstrated constant variance across the entire intensity range, indicating that the VSN processed data are suitable for further analysis (Figure 7.1b).

To assess unintended bias that may occur due to outlier samples in terms of quality, the intensity patterns of the genes were assessed using histograms, hierarchical clustering and principal component analysis of samples according to intensity. The density histograms and box plots of intensity patterns demonstrated comparable expression across all samples (Figure 7.1c-f). These indicate high quality data and consistent profiles across the samples in both the unfiltered and filtered data sets.

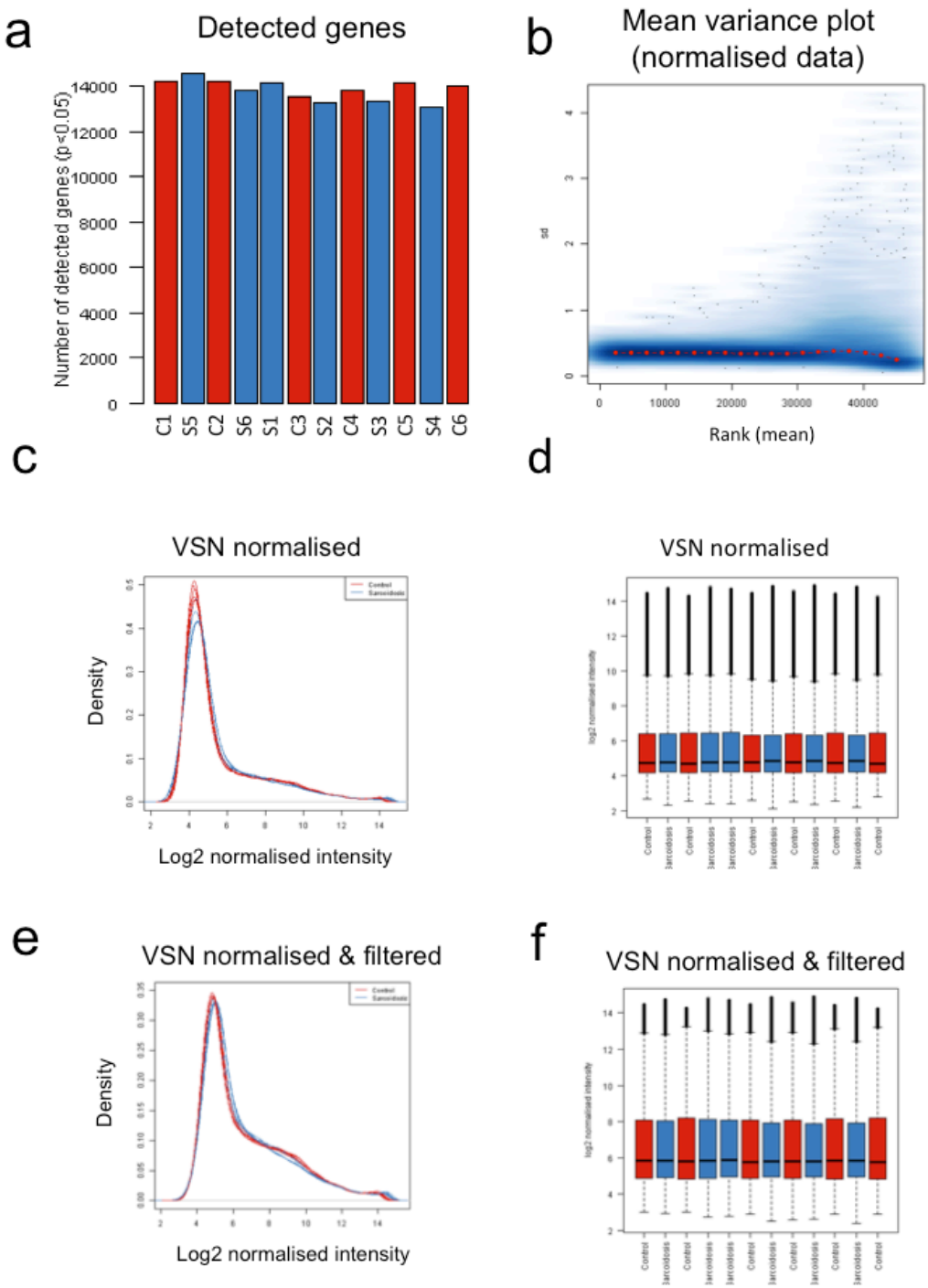
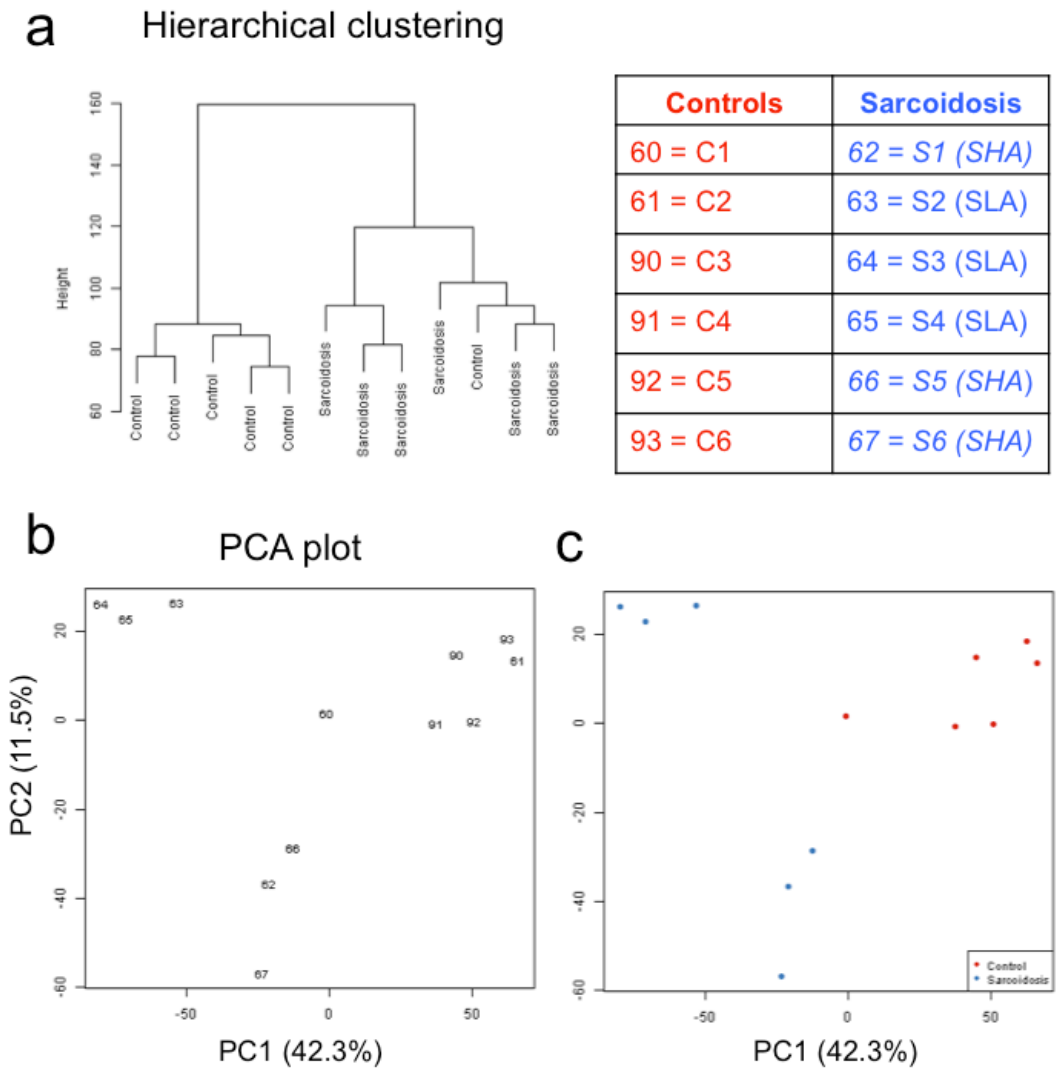


Figure 7.1. Microarray sample quality control.
(a) The number of genes detected by the microarray was high and consistent across samples. Samples ordered by chip position and coloured by experimental group (control – red, sarcoidosis – blue). Expression intensities after variance-stabilisation and normalisation (VSN) are illustrated as density plots and box plots and demonstrate that there was constant variance across the intensity range. Probe intensity distribution for (c) VSN-normalised data including all 47,231 probes and (b) VSN-normalised filtered data (filtered to exclude undetected probes with a detection score of <0.95, leaving 28,039 probes) indicated that the sample profiles were consistent across all samples.

Hierarchical clustering and principal component analysis were then used to examine the natural grouping of similarities and dissimilarities of the samples. This identifies unintentional bias that may occur due to experimental condition or technical factors such as unintentional sample batching or chip anomalies. Unsupervised hierarchical clustering using “complete linkage” method, which computes the maximum distance between any two data points in neighbouring sets (i.e. the minimum of similarity), was employed (Figure 7.2a). As expected, the statistical algorithm found two main clusters of samples, which almost completely segregate by disease status. However, one of the controls samples clusters with the disease samples, the reason for which is unclear. A similar picture is observed with PCA plots where samples are identified by (i) sample ID (Figure 7.2b) and (ii) coloured by disease group (Figure 7.2c). This more clearly shows the relationship between the samples. The sarcoidosis group clearly segregates into two subsets according to disease activity, comprising S1/62, S2/66 and S3/67 and S2/63, S3/64 and S5/65 and the latter are more similar to controls than the 3 patients with active sarcoidosis. One of the controls (C1/60) is slightly distant to the rest and in fact is more similar to 3 of the low activity sarcoidosis group as they are all in the mid-point of PC1. These data show that there is no unexpected bias with the caveat of C1, and that the groupings fall into the expected clusters. It confers technical reassurance on the data to be analysed.



Controls	Sarcoidosis
60 = C1	*62 = S1 (SHA)*
61 = C2	63 = S2 (SLA)
90 = C3	64 = S3 (SLA)
91 = C4	65 = S4 (SLA)
92 = C5	*66 = S5 (SHA)*
93 = C6	*67 = S6 (SHA)*

Figure 7.2. Sarcoidosis and controls segregate as expected using hierarchical clustering and principal components analysis.

Hierarchical clustering and principal components analysis (PCA) was used to identify outlier samples and assess the factors varying most between samples. (a) The clustering plot based on filtered data shows two main clusters of samples, which almost completely segregate by disease status. However, one of the controls clusters with the disease samples. This is also observed in the PCA plots where samples are identified by (b) sample ID and (c) coloured by disease group. Sample ID coding is illustrated in the table where red represents controls, blue sarcoidosis, SHA sarcoidosis high activity and SLA sarcoidosis low activity.

7.4.2. Comparison of monocyte gene expression profiles between sarcoidosis and controls

After statistical analysis, a total of 3437 genes with an adjusted p value of less than 0.05 were differentially expressed in sarcoidosis monocytes compared with controls. Filtering by Log2 fold change of 1.5 gives 151 genes with an adjusted p value of less than 0.05 (listed in Appendix 4), the top 25 upregulated (excluding additional transcript variants) and all downregulated genes are listed in Table 7.3.

To visualise the differential expression between sarcoidosis and control monocytes, TMeV4 was used to construct a heat map (Figure7.3a) and further cluster the genes by hierarchical clustering using “complete linkage” (Pearson Correlation). This showed as expected, a clear difference in gene expression between sarcoidosis and control monocytes, illustrates that there are two sarcoidosis subsets and that the C1 profile shares a number of similarities with the SLA subset.

Alias	Log2 FC	Adjusted p value	Description
IL6	4.723	0.001	Interleukin 6 (interferon, beta 2) (IL6).
CCL4L1	4.616	0.001	Chemokine ligand 4-like 1 (CCL4L1).
CCL4L2	4.549	0.002	Chemokine ligand 4-like 2 (CCL4L2).
LOC728835	4.449	0.001	PREDICTED: Similar to cytokine, transcript variant 3.
HBB	4.370	0.001	Hemoglobin, beta (HBB).
HBA2	4.269	0.000	Hemoglobin, alpha 2 (HBA2).
CCL20	4.212	0.002	Chemokine ligand 20 (CCL20).
CCL3L1	4.186	0.002	Chemokine ligand 3-like 1 (CCL3L1).
C15ORF48	4.107	0.000	Chromosome 15 ORF 48 (C15orf48).
CXCL2	4.057	0.000	Chemokine ligand 2 (CXCL2).
HBA1	3.965	0.000	Hemoglobin, alpha 1 (HBA1).
CCL3L1	3.699	0.003	Chemokine ligand 3-like 1 (CCL3L1).
CCL3	3.470	0.003	Chemokine ligand 3 (CCL3).
CD83	3.373	0.001	CD83 molecule (CD83).
G0S2	3.333	0.001	G0/G1switch 2 (G0S2).
SOD2	3.184	0.000	Superoxide dismutase 2, mitochondrial nuclear gene
MIR155HG	3.117	0.002	MIR155 host gene, non-coding RNA.
EBI2	3.068	0.001	Epstein-Barr virus induced gene 2 (lymphocyte-specific G protein-coupled receptor).
IL1A	2.987	0.001	Interleukin 1, alpha (IL1A).
CCL3L3	2.983	0.003	Chemokine ligand 3-like 3 (CCL3L3).
LRRC50	2.981	0.001	Leucine rich repeat containing 50 (LRRC50).
IL1B	2.952	0.001	Interleukin 1, beta (IL1B).
PHLDA1	2.880	0.000	Pleckstrin homology-like domain, family A, member 1.
IL8	2.872	0.008	Interleukin 8 (IL8).
PTGS2	2.775	0.002	Prostaglandin-endoperoxide synthase 2
TXNIP	-1.521	0.003	Homo sapiens thioredoxin interacting protein.
TNFRSF1A	-1.540	0.000	TNF receptor superfamily member 1A.
HPS6	-1.712	0.000	Hermansky-Pudlak syndrome 6 (HPS6).
RAB11FIP1	-1.765	0.000	RAB11 family interacting protein 1 (class I)
P2RY13	-1.771	0.001	Purinergic receptor P2Y, G-protein coupled, 13.
HSPA1A	-1.884	0.000	Homo sapiens heat shock 70kDa protein 1A.
GIMAP4	-2.032	0.001	Homo sapiens GTPase, IMAP family member 4.
FRAT2	-2.116	0.000	Frequently rearranged in advanced T-cell lymphomas 2
GIMAP8	-2.333	0.000	GTPase, IMAP family member 8.
CCR2	-2.471	0.001	Chemokine receptor 2 (CCR2).
GIMAP7	-2.712	0.001	GTPase, IMAP family member 7.

Table 7.3. Top 25 genes upregulated and all downregulated in sarcoidosis monocytes, adjusted p <0.05 and Log2 Fc > 1.5.

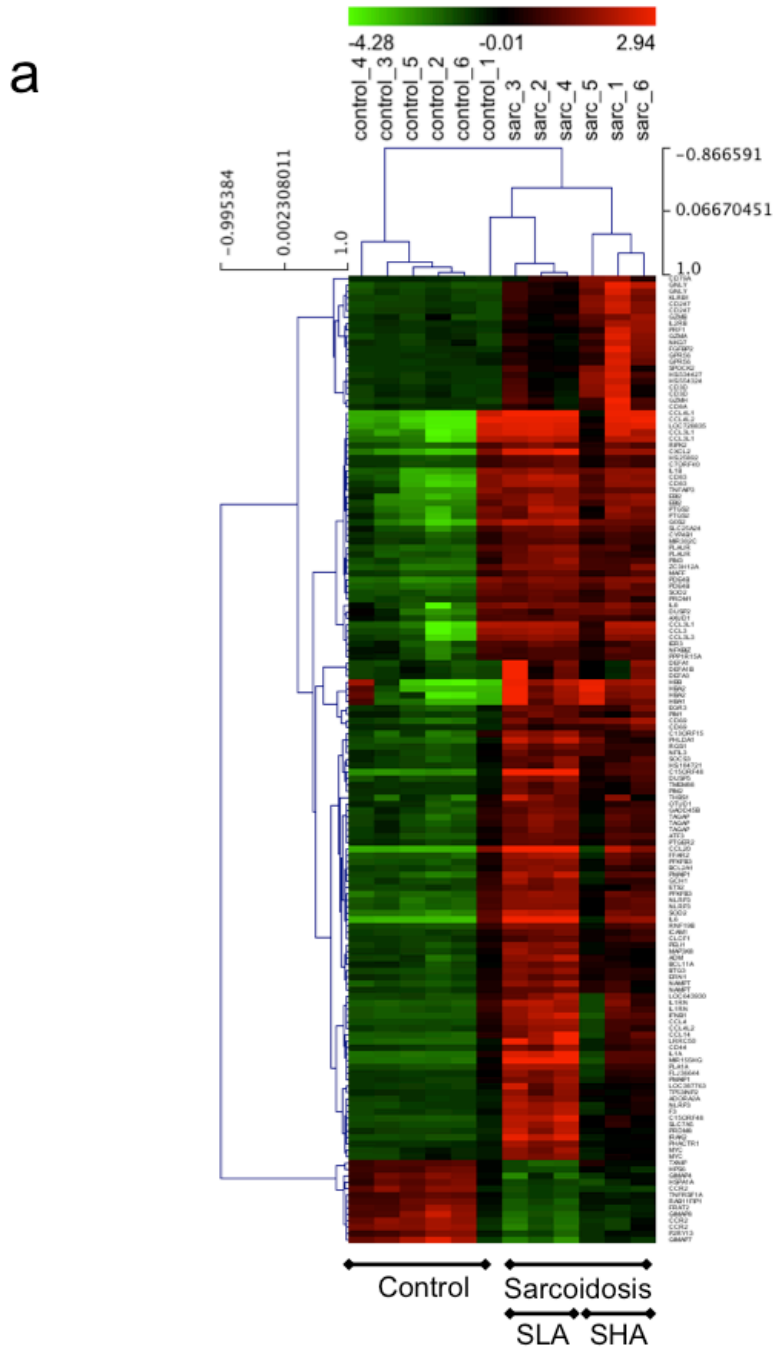


Figure 7.3. Heat map illustrating segregation of sarcoidosis and controls and of disease high activity versus low activity groups. (a) Heat map drawn using complete linkage hierarchical clustering, the signal intensities for the 151 genes differentially expressed with a p value of less than 0.05 and a log2 fold change of greater than 1.5. Expression levels are coloured red for high and green for low intensities colour bar values represent the Log2 of intensities. Illustrates segregation of sarcoidosis and control and additionally the sarcoidosis high activity and low activity subjects.

7.4.3. Functional relevance of sarcoidosis monocyte gene expression profiles.

In order to understand the functional relevance of these differences in monocyte gene expression, data were analysed using MetaCore. This identified a number of biological pathways and networks that are differentially represented in the dataset.

Pathway Maps	P value	FDR	Ratio
Immune response: IL-18 signaling	3.929e-11	3.041e-8	24/60
Immune response: HSP60 and HSP70/ TLR signaling pathway	1.227e-9	4.750e-7	21/54
Transcription: NF-kB activation pathways	2.568e-9	6.625e-7	20/51
Immune response: TLR2 and TLR4 signaling pathways	3.913e-9	7.571e-7	21/57
Transport: Clathrin-coated vesicle cycle	1.256e-8	1.945e-6	23/71
Apoptosis and survival: Role of PKR in stress-induced apoptosis	3.643e-8	3.585e-6	19/53
Immune response: HMGB1/RAGE signaling pathway	3.643e-8	3.585e-6	19/53
Immune response: TLR5, TLR7, TLR8 and TLR9 signaling pathways	3.706e-8	3.585e-6	18/48
Immune response: HMGB1/TLR signaling pathway	9.895e-8	8.510e-6	15/36
Immune response: ICOS pathway in T-helper cell	1.143e-7	8.848e-6	17/46

Table 7.4. Top 10 differentially expressed pathways in sarcoidosis monocytes.
The ratio given indicates the number of pathway objects differentially expressed in the data set.

Pathways indicate the sequential links between the identified gene products. The top ten differentially expressed pathways are listed in table 7.4 (for a full list see Appendix 5). Seven of the top ten differential expressed pathways identified in the monocyte gene profile relate to monocyte-relevant immune signaling, indicating activation compared with control monocytes. Two further pathways involved in apoptosis, cell survival and cytoskeletal remodeling are

also highlighted which is of interest in the context of increased monocyte number and abnormal phagocytic and fusion capacity. Of note, the ICOS pathway, specific to T cell co-stimulation was highlighted, suggesting possible contamination with T cells, the implication of this is addressed in the discussion at the end of the chapter.

The IL-18 signaling pathway was the most differentially expressed pathway with significant upregulation seen in the protein kinases IRAK1, TAK1 (MAP3K7), MEK3 (MAP2k3) and p38MAPK as well as the lipid phosphatase PI3K and the binding proteins p47-phox, p67-phox and I-κB. There is associated upregulation of the transcription factor NF-κB and, downstream to this, ICAM1, IL-1β, IL-1α, IL-6 and IL-8.

The NF-κB pathway itself is significantly upregulated in sarcoidosis with increased expression of numerous pathway kinases such as IRAK1&2, RIPK1 and TAK1 as well as various downstream binding proteins and NF-κB itself.

As may be expected there is overlap in the expressed genes contributing to the prominence of the top pathways (Figure 7.4a). The increased prominence of the TLR signaling pathways can be attributed to the increased expression in IRAK1&2, TAK1, MEK3&4 and p38MAPK.

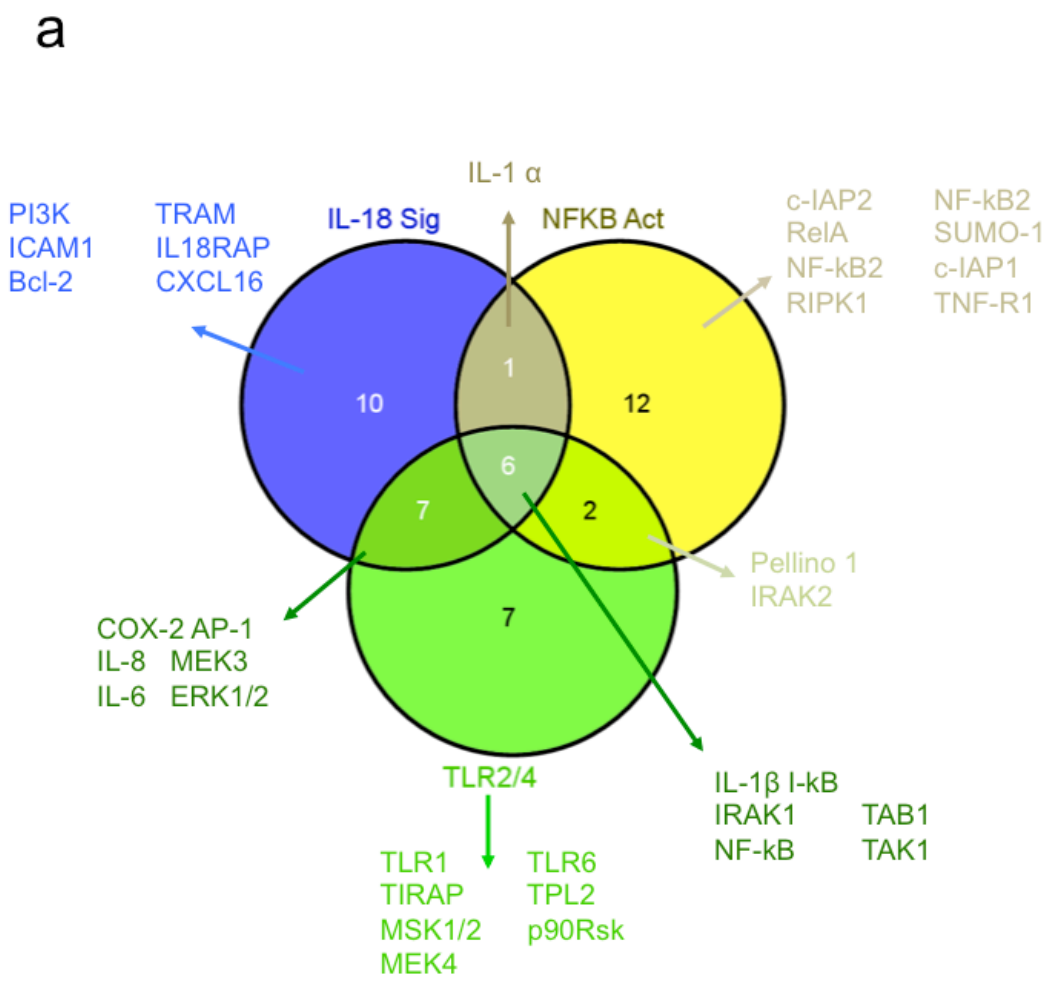


Figure 7.4. Venn diagram representation of gene overlap in the top 3 pathways identified by MetaCore.
(a) The most significantly differentially expressed genes in each group are illustrated. IL-18 sig: IL-18 signaling pathway, NFκB Act: NFκB activation pathway, TLR2/4: TLR2 and TLR4 signaling pathways. Created using VENNY: *http://bioinfogp.cnb.csic.es/tools/venny/index.html.*

Pathway analysis highlights single sequential links between genes and may be thought of as a two-dimensional representation of the data. It is clear that any one gene may be involved in a number of pathways and as such, a process network, which takes this into account, provides a more complete, "three-dimensional" visualization of these interactions. The top ten

differentially expressed process networks identified by MetaCore are listed in Table 7.5 (For a full list see Appendix 6).

Process Networks	P value	FDR	Ratio
Inflammation: IL-10 anti-inflammatory response.	1.673e-8	1.480e-6	34/87
Transcription: mRNA processing	1.862e-8	1.480e-6	51/160
Inflammation: Inflammasome	3.103e-7	1.645e-5	39/118
Transcription: Nuclear receptors transcriptional regulation	4.054e-6	1.611e-4	53/197
Immune response: TCR signaling	5.480e-6	1.743e-4	48/174
Cardiac development: Role of NADPH oxidase and ROS	2.188e-5	4.324e-4	38/133
Transcription: Transcription by RNA polymerase II	1.231e-5	3.262e-4	44/159
Cell cycle: G1-S Interleukin regulation	2.107e-5	4.324e-4	37/128
DNA damage: Checkpoint	2.448e-5	4.324e-4	36/124
Cell adhesion - Leucocyte chemotaxis	1.250e-4	1.175e-3	50/205

Table 7.5. Top 10 differentially expressed process networks in sarcoidosis monocytes.

Inflammatory process networks such as Inflammasome and TCR signaling were amongst the most differentially expressed networks, the latter again suggests potential T cell contamination. Transcriptional networks such as mRNA processing and nuclear receptors transcriptional regulation are differentially expressed. Upregulation of certain binding proteins such as CARD11, BCl-2, GRB2 and SKAP55 is seen with downregulation of others such as calmodulin, and NCK1; kinases such as ERK1/2, p38 MAP and PI3K are downregulated. These modifications are associated with a downregulation in NF-κB.

The chemokines CCL20 (MIP-3a), CXCL2 (MIP-2a), CXCL3 (MIP-2b), CCL3 (MIP-1α), IL-8, CCL14 and CCL4 (MIP-1β) and the adhesion molecule ICAM1

are all upregulated, and reflect the presence of the chemotaxis and cell adhesion network in the top ten differentially expressed networks.

Interestingly, the IL-10 signaling process network was the most significantly differentially altered, with significant alterations in pathway kinases, binding proteins and transcription factors (Table 7.6 and Figure 7.5a). Again, increased expression of IRF8, NF-κB, MIP1α (CCL3), IL-6, IL-8, IL-1α, IL-1β all contribute to the prominence of this network and there is overlap with the IL-18 pathway map (Figure 7.5b).

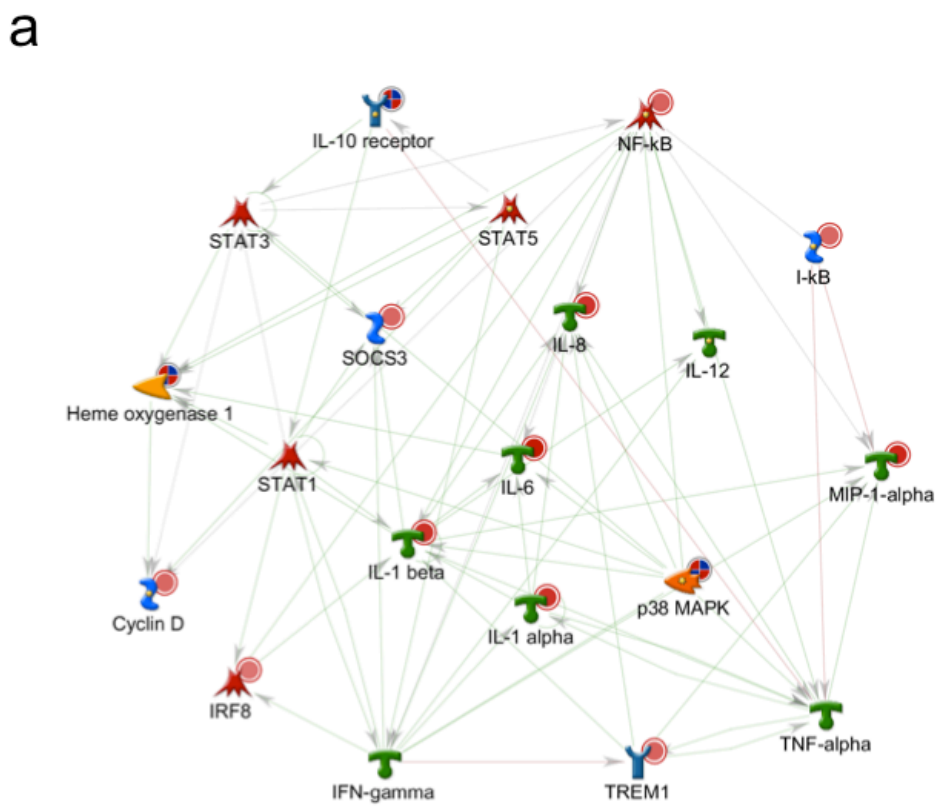


Figure 7.5. IL-10 process network.
(a) Network map illustrating the most differentially expressed genes contributing to the prominence of the IL-10 process network and their network links. Red/pink circle next to gene indicates up regulation (red greatest intensity), chequerboard indicates up- and downregulated components.

Again, increased expression of IRF8, NF-κB, MIP1α (CCL3), IL-6, IL-8, IL-1α, IL-1β all contribute to the prominence of this network and there is overlap with the IL-18 pathway map (Figure 7.6a).

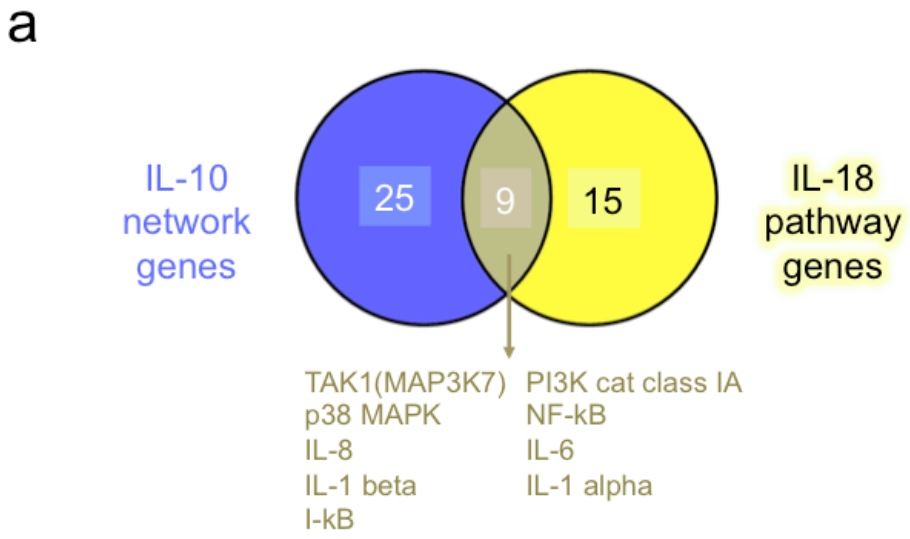


Figure 7.6. Gene overlap between IL-10 network and IL-18 pathway.
(a) Venn diagram illustrating gene overlap between the IL-10 network and IL-18 pathway. The most significantly differentially expressed genes the overlap groupare illustrated.

Because of the prominence of the IL-10 network in my data set and the relevance of this in the context of the earlier findings in this thesis, I identified three genes that were significantly upregulated in this network for validation with RT-qPCR: IL6 (Log2 Fc 3.42), IL1B (Log2 Fc 3.16) and IL-8 (Log2 Fc 2.872), all of which were in the top 20 most upregulated genes overall. This confirmed the relative expression of these genes to be increased in sarcoidosis monocytes compared with controls in six additional donors (3 sarcoidosis, 3 controls) (Table 7.7 and Figure 7.7a).

Alias	Description	Log2FC
IL-6	Interleukin 6 (interferon, beta 2).	4.723
CCL3	MIP-1-alpha Chemokine ligand 3.	3.470
IL-1 alpha	Interleukin 1, alpha (IL-1A).	2.987
IL-1 beta	Interleukin 1, beta (IL1B).	2.952
IL-8	Interleukin 8 (IL8), mRNA.	2.872
IRAK1/2	Interleukin-1 receptor-associated kinase 2.	2.420
SOCS3	Suppressor of cytokine signaling 3.	1.884
Pim-1	Pim-1 oncogene (PIM1), mRNA.	1.626
c-Myc	Myc proto-oncogene protein	1.514
NF-kB2 p52	NF-kB2 p52/ RelB	1.469
NF-kB2	Nuclear factor NF-kappa-B p100 subunit	1.469
TREM1	Triggering receptor expressed on myeloid cells 1.	1.067
Cyclin D2	Cyclin D2 (CCND2), mRNA.	0.906
NKKBIE	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon (IκB).	0.903
MAPKAP2	Mitogen-activated protein kinase-activated protein kinase 2.	0.900
NF-kB p52		0.884
NFKBIB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta.	0.753
IL-10RA	Interleukin 10 receptor, alpha.	0.736
IRF8	Interferon regulatory factor 8.	0.450
NF-kB p65	NF-kB p65/p65.	0.402
IRAK1/2	Interleukin-1 receptor-associated kinase 1.	0.261
Nucleolysin	Nucleolysin TIAR	-0.271
TAK1	Mitogen-activated protein kinase 7 (MAP3K7)	-0.286
PI3K cat (p110 beta)	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit beta isoform	-0.320
TIA-1		-0.330
P38 alpha	Mitogen-activated protein kinase 14 (MAPK14).	-0.410
IL-10R	Interleukin 10 receptor	-0.414
IL10RB	Interleukin 10 receptor, beta (IL10RB), mRNA.	-0.414
PIAS1	Protein inhibitor of activated STAT, 1, mRNA.	-0.754
H.oxygenase1	Haem oxygenase1	

Table 7.6. IL-10 network genes differentially expressed in sarcoidosis monocytes.

	Age (M/F)	Serum CAS	Ctrl Age (M/F)
Sarcoidosis high activity	35 (F)	3	33 (F)
Sarcoidosis high activity	40 (F)	3	38 (F)
Sarcoidosis high activity	39 (M)	2	37 (M)

Table 7.7. Sarcoidosis donors and matched controls for RT-qPCR validation.

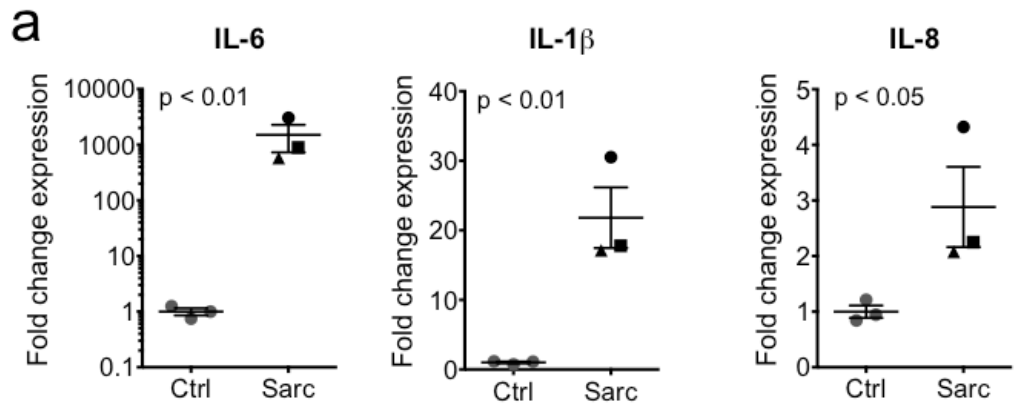


Figure 7.7. Sarcoidosis monocytes are enriched for IL-6, IL1B and IL-8.
(a) RT-qPCR analysis of negatively selected monocyte RNA from six additional donors. Relative expression normalised to GAPDH illustrated. Fold change calculated from the average of control samples. Mean ± SEM illustrated. Data analysed using the ΔΔCT method. Statistical analysis performed using Student's T test.

7.4.4. Functional differences between disease subgroups and controls

Having established that there is a clear difference in monocyte gene expression between sarcoidosis and controls, I next examined the relationship between the different sarcoidosis subgroups (high activity [SHA] and low activity [SLA]) and controls to ascertain whether there was an active disease signature. I analysed the difference in gene expression between SHA and SLA in order to identify the processes that are upregulated when sarcoidosis is active. There were 75 genes that were differentially expressed in SHA compared with SLA with a $p<0.05$ and a Log fold change of greater than 1.5 (Table 7.8 lists the top 20 upregulated and all those downregulated, for a full list, see Appendix 7).

Alias	Log2 FC	Adjusted p value	Description
SERPINB2	2.993	3.593E-04	Serpin peptidase inhibitor, clade B
IFIT3	2.864	1.009E-02	Interferon-induced protein with tetratricopeptide repeats 3
MIR155HG	2.809	1.564E-02	MIR155 host gene (MIR155HG), non-coding RNA.
IRAK2	2.797	1.713E-04	Interleukin-1 receptor-associated kinase 2.
LOC730249	2.659	3.211E-05	PREDICTED: Similar to Immune-responsive protein 1.
C15ORF48	2.649	5.363E-03	Chromosome 15 open reading frame 48.
TNFAIP6	2.594	6.694E-04	Tumor necrosis factor, alpha-induced protein 6 (TNFAIP6), mRNA.
UPB1	2.520	1.713E-04	Ureidopropionase, beta (UPB1), mRNA.
IL1A	2.391	3.943E-02	Interleukin 1, alpha (IL1A).
MSC	2.388	2.067E-04	Musculin (activated B-cell factor-1).
CXCL1	2.320	1.584E-02	Chemokine ligand 1 (melanoma growth stimulating activity, alpha).
PRDM8	2.231	2.401E-04	PR domain containing 8 (PRDM8).
F3	2.221	1.083E-03	Coagulation factor III (thromboplastin, tissue factor) (F3), mRNA.
NLRP3	2.210	1.133E-03	NLR family, pyrin domain containing 3.
OASL	2.128	8.120E-03	2'-5'-oligoadenylate synthetase-like.
KCNJ2	2.082	2.186E-02	Potassium inwardly-rectifying channel, subfamily J, member 2.
F3	2.079	1.033E-04	Coagulation factor III (thromboplastin, tissue factor).
SAMSN1	2.001	9.853E-04	SAM domain, SH3 domain and nuclear localization signals 1.
PLA1A	1.926	2.541E-02	Phospholipase A1 member A.
CCL2	1.867	7.531E-03	Chemokine ligand 2.
IL2RB	-1.507	2.706E-03	Interleukin 2 receptor, beta (IL2RB).
PRF1	-1.532	2.415E-02	Perforin 1 (pore forming protein).
GZMA	-1.538	2.582E-02	Granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3).
TXNIP	-1.544	8.372E-03	Thioredoxin interacting protein.
CD247	-1.608	1.611E-03	CD247 molecule.
HS.534427	-1.654	1.564E-02	Human mRNA for T-cell specific protein
FGFBP2	-1.662	1.759E-02	Fibroblast growth factor binding protein 2.
GNLY	-1.789	2.706E-03	Granulysin (GNLY).

Table 7.8. Top 20 upregulated and all downregulated genes in high activity versus low activity sarcoidosis monocytes.

The significantly upregulated genes include those suggestive of a general increased pro-inflammatory activity such as TNFα, IL-6, IL-1α as well those

for the chemotactic cytokines CCL13, CCL2, CXCL2 (MIP-2a), CXCL3 (MIP-2b).

Pathway Maps	P value	FDR	Ratio
Transcription - NF-kB activation pathways	1.105e-14	6.851e-12	17/51
HSP60 and HSP70/ TLR signaling pathway	1.779e-8	5.515e-6	12/54
Immune response - TLR2 and TLR4 signaling pathways	3.409e-8	7.045e-6	12/57
Apoptosis and survival - role of PKR in stress-induced apoptosis	1.430e-6	2.217e-4	10/53
Immune response - TNF-R2 signaling pathways	2.918e-6	3.619e-4	9/45
Cytoskeleton remodeling	4.015e-6	3.810e-4	13/102
Bacterial infections in CF airways	5.139e-6	3.810e-4	9/48
TLR5, TLR7, TLR8 and TLR9 signaling pathways	5.139e-6	3.810e-4	9/48
Development - PEDF signaling	6.146e-6	3.810e-4	9/49
Th17 cells in CF (mouse model)	6.146e-6	3.810e-4	9/49

Table 7.9. Top 10 differentially expressed pathway maps in high activity versus low activity sarcoidosis monocytes.

Process Networks	P value	FDR	Ratio
Immune response - TCR signaling	2.995e-15	4.762e-13	49/174
Inflammation - IL-10 anti-inflammatory response	9.908e-11	7.877e-9	28/87
Inflammation - MIF signaling	4.470e-9	2.369e-7	34/140
Cell cycle: G1-S Interleukin regulation	5.991e-9	2.381e-7	32/128
Cell cycle: G1-S Growth factor regulation	1.065e-7	3.326e-6	39/195
Immune response - BCR pathway	1.255e-7	3.326e-6	31/137
Translation - regulation of initiation	2.614e-7	5.938e-6	29/127
Development - Haemopoiesis, Erythropoietin pathway	3.630e-7	7.215e-6	30/136
Inflammation - IL-6 signaling	7.769e-7	1.373e-5	27/119
Inflammation - Protein C signaling	1.345e-6	2.138e-5	25/108

Table 7.10. Top 10 differentially expressed pathway maps in high activity versus low activity sarcoidosis monocytes.

I then used MetaCore to identify functionally relevant pathways and networks that differ according to disease activity (Tables 7.9 and 7.10). This reveals that the IL-10 signaling pathway remains one of the most differentially expressed processes, as does TCR signaling. Additionally, immune processes such as MIF, IL-6 and protein C signaling and the B cell receptor pathway are all differentially expressed. This suggests, as may be expected,

that there is a heightened pro-inflammatory response in sarcoidosis monocytes, compared with controls.

7.4.5. Gene set enrichment analysis

GSEA was performed in order to ascertain whether previously identified gene signatures or expression patterns are seen in the monocyte gene array data set from this study. The published 'biological process' available via services such as Gene Ontology (GO), MetaCore and the Broad Institute's MSigDB are largely derived from T cell studies, therefore in addition to using the GO biological processes datasets, gene sets derived from similar studies in the literature were used in the GSEA analysis. This has the advantage of encompassing broader expression patterns, rather than focusing on genes from a short gene list with an arbitrary inclusion cut off. 298 of the 476 gene sets were positively enriched; 132 gene sets were significant at FDR < 0.25; 67 gene sets were significantly enriched at nominal p value < 0.01%; and 97 gene sets were significantly enriched at nominal p value < 0.05. The top 10 significantly ($p < 0.05$) enriched genes sets plus the positively sarcoidosis gene PBMC gene signature are listed in Table 7.11 (for a complete list see Appendix 8). A plot of p values versus normalised enrichment scores, which provides a quick, visual way to identify the number of enriched gene sets that are significant and example enrichment plots are illustrated in Figure 7.8a-d.

178 of the 476 gene sets were negatively enriched; 26 gene sets were significant at FDR < 0.25; 17 gene sets were significantly enriched at nominal

p value < 0.01; 32 gene sets are significantly enriched at nominal p value < 0.05. The top 10 significantly negatively enriched gene sets are listed in Table 7.12.

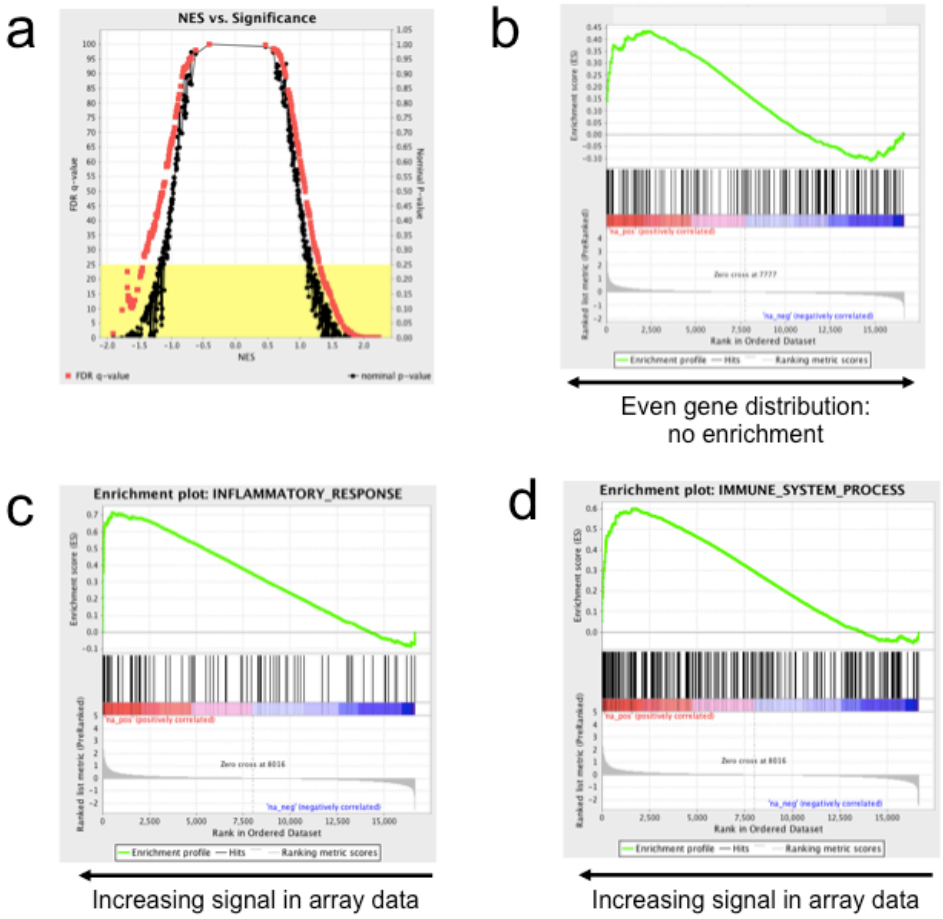


Figure 7.8. Biological process enrichment plots in sarcoidosis. (a) Plot of p values versus normalised enrichment scores, which gives a visual representation of the number of significantly enriched gene sets. (b) Enrichment plot illustrating even gene distribution and therefore no enrichment. (c-d) Examples of enrichment plots for four biological processes enriched sarcoidosis compared with healthy control monocytes illustrating increasing signal with a left shift indicating enrichment. The red-blue strip represents the 16032 genes expressed in the samples ranked according to differential expression between sarcoidosis and controls. Each line in the 'bar code' represents presence of a gene from the GO-defined gene-set (e.g. inflammatory response gene set) within the ranked genes. The enrichment score (ES; on plotted on the ordinate) is a cumulative statistic, which increases when the gene in that particular gene-set is found in the ranked gene list and decreases otherwise. The changes are weighted such that the magnitude is greater if there is a strong correlation of the gene from the defined gene-set with phenotype (i.e. found towards the extreme ends of the ranked list). The leading-edge subset is defined as genes appearing at or before the cumulative sum reaches maximum deviation from zero.

#	Name	ES	NES	NOM p-val	FDR q-val
1	Inflammatory response	0.716	2.224	0	0
2	Response to wounding	0.673	2.198	0	0
3	Upregulated after monocyte IL-10 treatment	0.618	2.122	0	0
4	Response to external stimulus	0.612	2.107	0	0
5	Immune system process	0.601	2.106	0	0
6	Behavior	0.695	2.100	0	0
7	Negative regulation of programmed cell death	0.642	2.087	0	0
8	Locomotory behavior	0.707	2.082	0	0
9	Immune response	0.613	2.076	0	0
10	Negative regulation of developmental process	0.623	2.069	0	0
43	Sarcoidosis PBMC gene signature	0.707	1.757	0	0.021

Table 7.11. Top 10 significantly positively enriched gene sets plus custom gene set of interest in sarcoidosis monocytes. General GO sets with less relevance to this study in grey.

#	Name	ES	NES	NOM p-val	FDR q-val
1	Cofactor Metabolic Process	-0.645	-1.904	0.000	0.013
2	mRNA Metabolic Process	-0.559	-1.772	0.000	0.096
3	Chromosome Organization And Biogenesis	-0.497	-1.677	0.000	0.145
4	RNA Processing	-0.465	-1.648	0.000	0.125
5	Golgi Vesicle Transport	-0.546	-1.626	0.000	0.115
6	M2 Genes	-0.395	-1.532	0.000	0.165
7	mRNA Processing	-0.537	-1.641	0.002	0.109
8	Protein Folding	-0.563	-1.681	0.002	0.172
9	Transcription Initiation	-0.580	-1.590	0.004	0.116
10	Carbohydrate Metabolic Process	-0.427	-1.489	0.005	0.208

Table 7.12. Top 10 significantly downregulated gene sets in sarcoidosis monocytes.

The inflammatory process GO gene set was the most significantly enriched gene set in the gene expression profiling data, with immune processes overall showing high levels of enrichment. The custom set of genes downregulated in response to IL-10 and the 31-gene sarcoidosis PBMC signature (Gene

signature listed in Appendix 9) were significantly enriched in the gene expression profiling data thereby strengthening my findings (257, 258).

7.5. Discussion

In this chapter I have demonstrated that there is a significant difference in the monocyte gene profile between sarcoidosis and controls, and between active and non-active disease. Previous immune profiling in sarcoidosis have predominantly focused on whole blood or PBMCs and have highlighted IFN-dominant and TCR/JAK-STAT signaling/cytokine-cytokine signaling pathways (257, 258, 260, 446, 447). This study adds to the current literature by having evaluated monocyte gene expression in negatively selected, untouched monocytes. This has the advantage over whole blood or PBMC study in giving a more pure monocyte signal. In addition, having established that monocyte activity is rapidly altered by isolation method, I extracted RNA from monocytes within 4 hours of venesection was felt to be crucial in minimising signal artifact.

That the TCR signaling pathways was prominently differentially expressed in sarcoidosis monocytes is perhaps unexpected, since overall, this pathway includes genes such as CD3 zeta, CD8 and the kinase ITK, not usually expected in monocytes. Although purity of monocytes isolated by negative selection was greater 98% (Appendix 1), it is possible that this signal originates from the few remaining T cells. These genes however do not feature highly in the data, however, much of this ‘TCR’ signal actually comes

from genes that are expected to be expressed in monocytes such as the adhesion molecule ICAM; the kinases TPL2 (MAP3K8) and MEK3 (MAP2K3); the nuclear factor I-kB; and the NF-kB family of transcription factors.

The IL-10 anti-inflammatory response was highlighted by MetaCore analysis as a significantly differentially expressed network in this study. Whilst MetaCore is a widely used to interpret gene expression profiling data, it is important to consider a few factors when utilising this, and other similar, tool(s). Firstly, the tool merely looks at the differentially expressed genes and tries to fit them into known pathways and processes. Closer inspection may reveal that only certain genes within pathways are actually differentially expressed without the intermediaries required to comprise a biologically meaningful pathway. Whilst experienced scientists in the field reviewed the evidence for these pathways and processes manually, much of this comes from studies of T cell biology and hence the relevance to myeloid cell activity is unclear. Despite the IL-10 network being highlighted, it is important to note that this reflects the expression of only 37 of the 87 genes attributed to the IL-10 network by MetaCore. Additionally, a number of these genes are not specific to IL-10 signaling or activity or indeed myeloid cell function, such as the transcription factor NF-κB and subunits, and molecules such as the MAP kinases. These factors (which relate to all the processes and pathways highlighted) notwithstanding, MetaCore is a useful tool in microarray data interpretation, provided that the relevance of the output “signatures” are carefully considered.

The β subunit of the IL-10 receptor, IL10RB, and the kinases PI3K, p38 MAPK and MAP3K7 with putative roles in the upstream regulation of IL-10 signaling are all downregulated (321). TLR2 and TLR4 induced IL-10 production is by a number of factors such as ERK1/2, MEK and p38, which are all downregulated in sarcoidosis monocytes, suggesting decreased IL-10 responsiveness to pathogenic stimuli. Additionally the TLR5 and TLR6 pathways are also downregulated in sarcoidosis monocytes. STAT1 has been reported to be a negative regulator of IL-10 production in monocytes (451), the downregulation of PIAS, a negative regulator of STAT1 may result in increased STAT1 activity in monocytes and therefore a brake on IL-10 production.

Interestingly, in sarcoidosis monocytes, a number of genes required for IL-10 production and signaling are increased, including: IL-6, IL-1α, IL-1β, NF-kB, SOCS3, I-kB and, perhaps most interesting of all, the α subunit of the IL-10R. For example, IL-6, which induces STAT3 mediated IL-10 production in monocytes (452), is amongst the most significantly upregulated genes in sarcoidosis in the whole array dataset, a finding validated by RT-qPCR. Additionally, the NF-κB signaling pathway is significantly upregulated in sarcoidosis monocytes, as are numerous NF-κB subunits, binding of which at a κB site located 4.5 kb upstream of the *Il10* start site enhancing macrophage IL-10 (453).

IL-10 has autocrine functions and it may be proposed that, in sarcoidosis, decreased IL-10 production by monocytes results in decreased availability at

the site of pathology. Monocytes themselves, therefore receive a decreased IL-10 signal, and in response upregulate a number of genes in order to facilitate IL-10 sensing and downstream signaling and, additionally to increase IL-10 production capacity. Overall, these findings support the suggestion from earlier studies in this thesis that the fine balance of IL-10 regulation is disturbed in sarcoidosis, and that further elucidation of the involved mechanisms, particularly in view of this microarray data, may lead to potentially modifiable disease targets.

As might be expected, a number of pro-inflammatory markers and processes are prominently upregulated in monocytes sarcoidosis such as CD69 and CD83; the cytokines IL-6 and IL1β; and the IL-18 signaling pathway. The activation marker CD69, a member of the natural killer cell gene complex family of signal transducing receptors, is expressed on human monocytes and is has been previously reported to increased on sarcoidosis monocytes (188, 189). Crosslinking of CD69 receptors on monocytes induces extracellular calcium influx, the secretion of inflammatory mediators, such as prostaglandins E2 and F1 and leukotriene B4, and increased production of nitric oxide, all of which are reportedly released by AM in sarcoidosis, suggesting they may play a part in modulating granulomatous lung inflammation (189, 454-456). It has been suggested in a murine model of lung fibrosis that CD69 may be important in controlling the immune response by limiting TGF-β1 expression and collagen deposition (457).

IL6 was the most significantly upregulated genes in sarcoidosis monocytes. It is a pleiotropic cytokine that bridges innate and acquired immunity. It induces the differentiation of activated B cells into immunoglobulin- producing plasma cells and the continuous over-expression of IL-6 results in hypergammaglobulinemia, seen commonly in sarcoidosis, and autoantibody production. Although reports about the role of IL-6 in sarcoidosis have been inconsistent, it is reportedly increased in the serum, sputum, BAL and urine of sarcoidosis patients as well as in the supernatant of cultured monocytes and AM (458-461) and may be involved in the initiation and maintenance of alveolitis by activating and causing the proliferation of T cells (462, 463).

IL-6 promotes specific differentiation of naïve CD4-positive T cells into effector T-cell subsets and the differentiation of CD8-positive T cells into cytotoxic T cells (464). It has been proposed that IL-6 directed predominance of T_h17 cells over T_{regs} may be responsible for the disruption of immunological tolerance and is thus pathologically involved in the development of inflammatory autoimmune diseases (465-467), supported by IL-6 blockade studies in animal models autoimmune disease (468, 469). IL-6 further contributes to the inflammatory response by rapidly inducing the production of acute phase reactants such as C-reactive protein (CRP), serum amyloid A (SAA), fibrinogen by hepatocytes (470). The humanised anti-IL-6R antibody, Tocilizumab, has been recently licenced for the treatment of RA and has shown early promise in the treatment of a number of other immune conditions such as systemic sclerosis (clinical trial number: NCT01532869), giant cell arteritis (NCT01450137 and NCT01791153), amyloidosis, Crohn's, SLE and

IgG4 related disease (463, 471, 472). Functional characterisation of the role of IL-6 in the pathogenesis of sarcoidosis is therefore an exciting prospect.

IL-1β, another highly significantly upregulated gene in this study, is a pro-inflammatory cytokine that is produced spontaneously in vitro by cultured AM at greater levels in sarcoidosis than controls (473-475) and has been associated with granuloma formation (476, 477). In the context of sarcoidosis being very rare amongst smokers, it is interesting that the levels of IL-1β are reportedly less in current smokers (473).

The IL-18 pathway is the most significantly differentially expressed pathway in sarcoidosis monocytes. IL-18 is IL-1 superfamily cytokine. It acts, together with IL-12, to induce cytotoxic T cell activity and IFNγ production; activation of iNKT cells; and reduction in IL-4 production. A number of the key pathway components are upregulated including the IL-18 receptor accessory protein. IL-18 itself was not upregulated. It was seen in chapter 4, that sarcoidosis monocytes have a reduced capacity for IL-18 production in response to iNKT stimulation. The role of IL-18 in sarcoidosis warrants further consideration, particularly in view of its iNKT stimulatory capacity. Overall, the prominence of this pathway supports a pro-inflammatory role for sarcoidosis monocytes.

Mediators involved in the regulation of leucocyte chemotaxis and adhesion were prominently upregulated in sarcoidosis monocytes, these include: CCL20 (MIP-3α), CXCL2 (MIP-2α), CXCL3 (MIP-2β), CCL3 (MIP-1α), IL-8, CCL14 and CCL4 (MIP-1β) and the adhesion molecule ICAM1. This supports

the role of monocytes as key early activators in sarcoidosis, capable of rapidly migrating to and remaining at the site of pathology from where they are able to recruit other cell types by influencing the cytokine milieu. CCL20 is the only known chemokine ligand for CCR6 (478) and is expressed by airway epithelial cells and AM (479-481). Increased CCL20 is expressed by AM found in the BAL, granuloma macrophages and epithelioid cells on lung biopsy samples in sarcoidosis (92). CCL20 recruits $CD4^+CCR6^+IL\text{-}17^+$ T_H17 cells reportedly contributing to alveolitis and granuloma formation (52, 92, 482). CCL20 expression is controlled directly by the transcriptional factor NF-κB, which is in turn upregulated by TNF-α and IL-1β (483, 484). The CCL20 receptor, CCR6, is expressed by IL-10-producing Tregs ($CCR6^+CD25^+CD4^+$) at inflammatory sites (485); it may be helpful to determine if it is expressed on the IL-10 producing monocyte subset explored in this study. Interestingly, in psoriasis, the use of soluble TNF receptors reduces CCL20 expression and is thought to account for the reduction of infiltrating T cells and DC in psoriatic plaques (486) and may hint at another mechanism through which successful anti-TNF therapy may be mediated in sarcoidosis.

The data in this chapter suggest that the altered inflammatory profile in sarcoidosis is heightened in high activity disease compared with low activity disease rather than being substantially different. Interestingly IL-6 signaling becomes even more prominent with increased disease activity, although the significance of this is unclear. It could be acting as a pro-inflammatory agent, or conversely, could be being upregulated by cells in an attempt to increase the amount of IL-10 available. The pro-inflammatory mediators TNFα,

TNFSF8 (TNF receptor), IL1A and CXCL1, are all upregulated, which may be expected given the inflammatory functional phenotype of sarcoidosis monocytes.

The gene for the monocyte chemotactic cytokine CCL2 is upregulated in active sarcoidosis compared with non-active disease, although this did not translate to altered levels in the functional studies in chapter 3. This might be because CCL2 is produced by monocytes with different kinetics to those of the in vitro study, or perhaps that increased gene expression is not translated into altered protein levels. Interestingly however, CCR2, the receptor for CCL2, is the most downregulated gene in sarcoidosis monocytes compared with controls. Reduction in CCR2 levels are reportedly induced by CCL2 (487, 488), which is required for monocyte release from the bone marrow such that decreased levels may be expected to result in monocytopenia (132, 133). Furthermore, CCL2 is rapidly downregulated upon monocyte differentiation to macrophages (262, 489). It might be that monocytes are producing more CCL2 in order to over come decreased responsiveness, or that the reduction in CCL2 expression is an expected consequence of auto-regulation, i.e. that it is downregulated in the face of excess CCL2 in order to prevent further recruitment. CCR2 polymorphisms have controversially been associated with disease severity in sarcoidosis (490-493).

Macrophage inhibitory factor (MIF) signaling is also significantly upregulated in active sarcoidosis. MIF has been implicated in pro-inflammatory activity and neutrophil homing to the lung in inflammatory pathology (494).

Therapeutic manipulation of MIF has been shown to increase steroid responsiveness (495). This would be a potential avenue for further investigation is steroid resistant sarcoidosis.

GSEA suggested enrichment of genes associated with immune and inflammatory processes, as might be expected. In addition, there was perturbation of the IL-10 associated genes, further supporting my earlier findings. A gene signature from a recent study in sarcoidosis PBMCs was found to be concordant with this array data set (258). It is important, particularly given the heterogeneity of sarcoidosis that, having created this 'training cohort' that the signature seen is validated in a larger cohort. This should include patients, not only with high disease activity and low disease activity disease, but also with the different disease phenotypes including those with non-active self-limiting and progressive disease. An evaluation of monocyte activity pre and post treatment would also be extremely valuable in determining potentially modifiable disease targets. Another important question is whether the sarcoidosis monocyte profile is enriched in other published data sets from sarcoidosis BAL or lung tissue. Many of these have just looked at all cells present, which is likely to include T cells, and AM. Enrichment of monocyte gene signatures here would further validate the role of monocytes at these sites.

Once a validation cohort has been established and a sarcoidosis monocyte specific signature determined, it may be possible to narrow the gene signature profile to a more manageable number of genes relevant to myeloid cell

processes. The advantage of this is that it would make the signature more accessible and facilitate further validation with additional data sets, the disadvantage being that some of the lower order genes contributing to the signature would be ignored. Whilst the 'leading edge' genes contribute most to an enrichment pattern a more complete approach is valuable when exploring such heterogeneous a condition as sarcoidosis.

As an alternative approach to assess gene expression patterns in sarcoidosis would be to use the "gene module" strategy which examines groups of genes that are coordinately expressed, such as genes related to myeloid linage, and T cells, as well as other functional categories (496, 497). For example, I could treat monocytes with IL-10, calcitriol, glucocorticoid or anti-TNFα and assess the difference in monocyte gene expression, building up signatures for monocytes under the specific conditions of study (i.e. ex vivo, negatively selected monocytes). I could then see if these gene signatures were enriched in my array data set to give an indication of some of the potential influencing factors on monocytes in sarcoidosis. This is particularly of value in studies such as this where T cells are not the focus of study because of the aforementioned bias in the gene database literature towards T cell studies.

7.6. Summary

The data show that there is a significant difference in the gene expression profile of sarcoidosis monocytes, providing further evidence and elaboration of their role in the immune pathology of sarcoidosis. The profile in high activity

sarcoidosis is a more exaggerated signature of that seen in low activity disease. The IL-10 network was the most significantly altered process supporting my earlier findings that IL-10 activity is important in the immune pathology of sarcoidosis. Inflammatory processes are upregulated, as is chemotaxis. IL-6 appears to be central to many of the differential networks and requires further consideration in sarcoidosis. Teasing out critical pathways, including those affected by glucocorticoid or TNFα blockade may help determine pathways for further analysis and drug targeting.

Chapter 8: Final discussion and future directions

8.1. Main findings

The overall aim of this project was to characterise the phenotype and functional profile of monocytes in sarcoidosis, and to explore the cause and potential implications of any anomalies found in the pathogenesis of sarcoidosis. In order to achieve this, I first characterised the phenotypic and functional profile of monocytes derived from sarcoidosis compared with those from healthy controls. I then explored the relationship between abnormalities in monocyte function and the known deficiency in iNKT cell numbers in sarcoidosis before examining methods for modulating altered monocyte activity. Lastly, I interrogated the gene expression profile of monocytes derived from treatment-naïve sarcoidosis patients in comparison with those derived from healthy control subjects. My main findings were as follows:

i. Monocyte frequency is increased in sarcoidosis and is associated with a pro inflammatory surface antigen phenotype.
ii. Sarcoidosis monocytes are functionally abnormal with altered (a) cytokine profiles, (b) T cell suppressive capability, (c) fusogenic and (d) phagocytic competence.
iii. Sarcoidosis monocytes exhibit a distinct gene expression profile characterised by high inflammatory and pattern recognition receptor signaling pathways, with monocytes from active disease segregating from monocytes from less active disease.

The most notable finding from this work is the role of decreased sarcoidosis monocyte IL-10 production in disease pathogenesis. Sarcoidosis monocytes display defective suppression of T cell activity in an IL-10 dependent manner, suggesting a regulatory role for these cells. These could come under the cell grouping of myeloid derived suppressor cells (MDSCs). Cells of the myeloid lineage are known to suppress T cell proliferation both under resting and disease conditions, where they become sub-specialised and are referred to as myeloid-derived suppressor cells (352-354). In humans, MDSC subsets have been characterised by the co-expression of $CD11b^{+}CD33^{+}CD14^{-}HLA\text{-}DR^{-}$ or $Lin^{-}CD11b^{+}CD33^{+}HLA\text{-}DR^{-}$ (498-500). MDSCs accumulate in models of several autoimmune diseases, including multiple sclerosis (501-504), type 1 diabetes (505), inflammatory arthritis (506, 507) and inflammatory bowel disease (508) and persist in chronic inflammation suggesting that their function may be compromised in the dysfunctional immune response (508). The ability of MDSCs to suppress T cell responses is dependent on two enzymes that are involved in the metabolism of L-arginine: arginase 1 and inducible NO synthase (iNOS) (509, 510). Additionally, exogenous IL-10 has been shown to suppress T cell proliferation (345) and IL-10-deficient macrophages reportedly fail to reproduce the characteristic repression on inflammatory mediators or T cell proliferation (309, 346) supporting the finding that monocytic MDSC activity may be contextually IL-10 dependent. Despite the growing evidence that MDSCs accumulate and possess the potential to suppress T cell immune responses, the precise roles of MDSCs in non-cancer immune diseases remains controversial and have not previously been described in the T cell dominant sarcoidosis. The loss of IL-10 producing

capability in sarcoidosis monocytes is therefore proposed to be deleterious in the context of T cell hyper-activity and excess proliferation in sarcoidosis.

This study also proposes an additional role for the pathogenic role of deficient monocyte IL-10 in sarcoidosis, that of inadequate regulation of monocyte fusion capacity leading to excess multinucleate giant cell (MGC) formation and granulomagenesis. Monocytes in sarcoidosis have a greater fusion capacity than control monocytes. Fusion activity can be reduced by the addition of IL-10 and restored by concomitant addition of an IL-10 blocking antibody. This is supported by previous work demonstrating that IL-10 curtails the inflammatory granulomatous response animal in vivo and human TB in vitro systems (322, 395, 511). The mechanism for IL-10 regulation of fusion is not known, however it may occur via IL-10 mediated decreased phosphorylation of the mitogen-activated protein kinase (MAPK) c-Jun N-terminal kinase (JNK) (511, 512).

The granuloma in sarcoidosis is characterised by a core of monocyte-derived epithelioid histiocytes, MGCs and $CD4^+$ T cells with a predominantly T_H1 bias, interspersed with a small number of $CD8^+$ T cells. IL-10 deficient monocytes and T cells may interact at the site of pathology, the granuloma, where the overall consequence of insufficient IL-10 is decreased immune regulation leading to excess T cell proliferation, increased monocyte fusion and promotion of granuloma persistence. Once established, this dynamic, cytokine-producing structure is likely to contribute to maintenance of the inflammatory response.

Notwithstanding the lack of mechanism for interaction between iNKT cells and monocytes, the finding that iNKT cells have the capacity to restore defective monocyte IL-10 production in sarcoidosis suggested a potentially modifiable pathway in sarcoidosis immune pathogenesis. This is supported by a growing body of evidence for monocyte-iNKT cross talk, including chemokine receptor expression patterns; spatial and temporal association in disease; and in vitro iNKT directed maturation of myeloid cells (138, 140, 226, 254-256, 347).

iNKT therapy still in early stages and focuses on activating, rather than replacing deficient cells. Pre clinical trials, mostly in the cancer field, have been promising but have not shown consistent therapeutic benefit (305-307, 513-515). The discrepancy between the results obtained in tumour-bearing mice and human cancer patients treated with αGalCer may be related to the differences between human and mouse CD1d molecules and the much greater abundance of iNKT cells in mice compared with humans. The establishment of a human CD1d (hCD1d) knock-in mouse model for in vivo study of human CD1d presentation of lipids to iNKT cells is promising (513). The development of potential iNKT therapy in sarcoidosis is fraught with challenges. iNKT cells are almost completely absent in sarcoidosis, and even where they are present, the frequency is extremely low potentially limiting the benefit of activation. Even if we were able to activate iNKT cells in sarcoidosis, their actions are multifarious and targeting the appropriate subset may provide additional challenges. Additionally, the lack of a suitable mouse

model of sarcoidosis may further hinder study of iNKT therapeutic activity in the disease.

Whilst modulation of monocyte IL-10 deficiency with iNKT cells may be a long way in the future, the use of vitamin D seems more immediately promising. Vitamin D is available over the counter without prescription as a health supplement and is already widely used in clinical settings and indeed has been shown to increase $CD4^+$ T cell IL-10 mRNA in patients with asthma (414). I established that defective monocyte IL-10 production could be rescued by treatment with calcitriol, the active form of vitamin D, a finding supported by other studies using monocytic cell lines (413). This opens up the therapeutic possibility of up regulating monocyte IL-10 production in sarcoidosis with the intended benefit of boosting monocyte suppression of T cell proliferation and regulating excess fusion. That vitamin D supplementation may help restore monocyte regulation of T cell activity is supported by a recent report that calcitriol can increase macrophage-mediated suppression of T cell proliferation through induction of IL-10 (346). Additionally, I confirmed that 25-hydroxyvitamin D3 is low in individuals with sarcoidosis at presentation to the clinic, which may be a contributory factor in the reduction in monocyte IL-10 seen in the disease. Replacement can improve some of the more non-specific features of sarcoidosis such as lethargy and fatigue which cause significant morbidity in sarcoidosis and as such, vitamin D replacement could readily be justified for this reason alone (444).

In addition to defective IL-10 production, I confirmed that monocytes in sarcoidosis produce greater levels of the pro inflammatory cytokines TNFα and IL-12. Furthermore, although not measured in the functional studies, the gene expression profile study suggests that monocyte IL-6, IL-8, IL-1α and IL1β are all significantly up regulated in sarcoidosis monocytes, contributing to the pro inflammatory phenotype. The role of IL-6 is particularly fascinating as it is reportedly increased in sarcoidosis in response to ESAT-6/ KatG peptide stimulation (516). Perhaps most striking however is the inability of sarcoidosis monocytes to produce IL-4 (as measured by both ICS and ELISA), a phenomenon previously noted in sarcoidosis T cells (334). This suggests the possibility that the level of IL-4 in the micro-environment surrounding the sarcoidosis monocyte may be decreased, which, in combination with decreased IL-10 levels may result in an alteration of monocyte-macrophage biasing in the disease (331, 517). In monocytes, IL-4 signals through the IL-4 receptor, which comprises IL-4Rα and IL-2Rγc; these receptor components are phosphorylated upon exposure of to IL-4. Downstream signaling includes STAT3 and STAT6, via the kinases JAK1 and TYK2 (518). Upon activation STAT3 and STAT6 form dimmers and, translocate into the nucleus, bind DNA and facilitate the induction of 15-LO, MAO-A and CD36 gene expression leading to an alternatively activated phenotype. Interrogation of this signaling pathway in sarcoidosis may help determine the specific defect in sarcoidosis and is the next step in the continuation of this work.

Altered phagocytic capacity might be expected in sarcoidosis, but that monocyte phagocytic ability was reduced in sarcoidosis was notable. It may

be hypothesised that altered antigen degradation and elimination by monocytes results in a prolonged or heightened immune response (519-522). Phagocytosis, degradation and clearance of antigens are dependent on Fc receptors including: Fc γ RI (CD64), Fc γ RII (CD32), Fc γ RIII (CD16) and/or complement receptors: CR1 (CD35), CR3 (CD11b), CR4 (CD11c). A previous study reports increased phagocytosis of FITC labeled, opsonised *E. coli* by sarcoidosis monocytes, and attributed this to increased expression of CD64, CD32 and CD16 (302). Whilst I also found increased CD16 expression on sarcoidosis monocytes, I found no difference in the amount of CD32 and did not examine CD64 expression. The reason for this difference may be due to experimental method, in this study, phagocytosis was examined by ImageStream™and gave the capability to directly visualise cells. Monocytes, particularly with enhanced Fc receptor expression are extremely adherent and may bind fluorescent particles on their exterior without internalising them. This would not be detected by conventional flow cytometry and could explain an increase in phagocytosis seen in the other study. Alternatively, it may be more likely that monocytes possess different phagocytic ability depending on the antigen. The latex bead model used in this thesis is likely to be closer than the use of opsonised bacteria, to the biological reality in sarcoidosis where a non-specific, non-degradable antigen is thought to be the trigger for granulomagenesis.

Having established the importance of monocyte isolation techniques, care was taken to ensure that primary monocyte RNA was isolated under optimal conditions Each method of monocyte isolation has advantages and

disadvantages. FACS sorting and CD14 positive selection yield very high purities, but risk activation of the cells. CD14 negative selection has slightly lower purity (98% purity rather than 99%), but has the advantage of less monocyte activation. The contamination of monocyte RNA with T cell RNA may be problematic and strategies for how this maybe overcome are discussed in the next section. It is worth considering that current gene databases are heavily biased towards T cell work, and as such, many genes shared between T cells and monocytes maybe upregulated and result in the highlighting of 'T cell activity' pathways and networks, when in reality this may simply reflect the general cell activation status.

There was a clear difference in the genetic signature between sarcoidosis and control monocytes, with the active and non-active sarcoidosis subsets also clearly segregating. Initial analysis suggested that the biggest difference was between sarcoidosis and control monocytes, rather than between different disease activity levels. As may be expected, the differentially regulated genes were predominantly immune specific with a bias towards an inflammatory profile. The most significantly up regulated genes, IL-6, IL-1α, IL-1β, IL-8, and the MAPK and NFκB family are all involved in numerous pro-inflammatory processes. An important next step would be to further validate these findings with RT-qPCR on a larger sample population, before examining cytokine activity, or signaling activity in the case of MAPK and NFκB, in sarcoidosis. The identification of the IL-10 inflammatory response as the most statistically significant biological process network in the differentially expressed genes, comparing sarcoidosis to controls, supports the perturbation of IL-10 secretory

function from these cells although the non-specificity, in terms of the IL-10 process network, of the implicated genes must be carefully considered. The significant upregulation of a number of the NFκB complex genes is potentially relevant in the context of decreased monocyte IL-10. A lack of IL-10 leads to higher NFκB activation in macrophages in mice (523) and it is not clear which is cause and effect with regard to the relationship between IL-10 and NFκB. Additionally, the monocyte response to TLR2, TLR4, TLR5 and TLR6 are all downregulated, suggesting a decreased ability of sarcoidosis monocytes to sense and process antigenic stimulation by these TLRs.

In conclusion, my findings show that IL-10 production and T cell regulation capabilities by monocytes are defective in patients with sarcoidosis. The in vitro data suggest this could be secondary to loss of iNKT cells, which under normal circumstances enhanced monocytic IL-10 production. I found that monocyte IL-10 production could be augmented by treatment with vitamin D in vitro, which had the additional benefits of reducing monocyte fusion in an IL-10 dependent manner and increasing phagocytosis through and undetermined mechanism. These findings offer new insights into the cause for T cell over-activity in sarcoidosis, identify monocyte anomalies as a key and early defect in disease pathogenesis for sarcoidosis, and provide fresh platforms for therapeutic exploration for this condition.

8.2. Future work

This study has raised some interesting questions for further study. I observed a previously unreported increase in the frequency of the patrolling $CD14^{+}16^{++}$ monocyte subset in sarcoidosis. Their role in sarcoidosis immune pathology is unknown and it may be, by virtue of their location on the vascular endothelium that they are ideally placed to influence early stages of sarcoidosis immune pathology. I hypothesise that they are involved in abnormal antigen sensing in sarcoidosis, an idea supported by the phagocytic studies in this thesis. It would be theoretically possible to use the advancing technique of intravital microscopy to study the kinetics of human monocytes adoptively transferred into a humanised mouse model (129, 382). For example, intravital microscopy of the ear dermis from Cx3cr $^{gfp/+}$ $Rag2^{-/-}Il2rg^{-/-}$ mice has been used to examine the activity of human FACS sorted patrolling monocytes, labeled with Vibrant-DiD (Life Technologies) (129). In this model, blood vessels were labeled by intravenous injection of fluorescent 70 kDa dextran, and murine monocytes expressed gfp (green). This model could be used to establish if there were a difference in sarcoidosis and control $CD14^{+}CD16^{++}$ (also $Slan^{+}Cx3CR1^{hi}CD163^{-}CD11b^{lo}CD11c^{hi}CD62L^{lo}$) monocyte ability to sensing of antigen such as SAA and mKatG.

The gene expression profile studies suggested that monocyte chemotaxis and adhesion is dysregulated in sarcoidosis, which might be expected given the influx of cells to the site of pathology. What was most surprising however was that CCR2, the receptor for the monocyte chemotactic cytokine CCL2 was so prominently down regulated. Multiple probes detected both CCR2 transcript

variants as being amongst the most significantly down regulated genes in sarcoidosis. It is unclear what this could mean in sarcoidosis and teasing out this pathway could give insight into the kinetics of monocyte trafficking into the granuloma. For example, sarcoidosis and control monocyte migration in response to varying CCL2 gradients with or without CCR2 blockade could be assessed using a standard transwell migration (Boyden chamber) assay (524, 525). Differences in FACS sorted monocyte subset migration and the influence of perturbations in other chemotactic mediators found to be altered in sarcoidois such as CX_3CR1 could be determined in this way.

Sarcoidosis is likely to result from an undetermined immunological insult in a genetically susceptible host. It is clear that the gene expression profile in monocytes is very different in sarcoidois and controls and I would like to concentrate further on delineating this genetic signature and any potentially modifiable pathways identified. The numbers in this study are small and so initial work would focus on examining a larger validation cohort, determining a sarcoidosis monocyte genetic signature, and validating the findings with functional studies. In addition, the potential issue of T cell contamination will need to be addressed in order to strengthen my findings. I order to do this, I will first repeat the study by using FACS sorted monocytes or MACS bead isolation with an addition CD3 positive selection step after the first round of negative selection. I will then repeat the gene expression analysis and compare the two outputs.

Further understanding of the data might involve the use of the “gene module” strategy (496, 497) in order to determine the alterations in genetic signature resulting from controlled modulation of monocytes and then using the resulting genetic signatures to interrogate the sarcoidosis monocyte data. For example the response to IL-10, IL-4 and calcitriol, under the specific conditions of study would help delineate the monocyte response to these factors more clearly (i.e. ex vivo, negatively selected monocytes). In addition, the response to established treatments such as glucocorticoids or anti-TNFα could illuminate the functionally aberrant pathways in sarcoidosis. Ideally these studies would be on monocytes isolated from sarcoidosis patients treated with glucocorticoids or anti-TNFα, but could be performed on in vitro manipulated monocytes in lieu of this. We still know remarkably little about the actual mechanism of activity of these drugs in sarcoidois and their pre treatment efficacy. With the increasing availability and scope of biologic therapies, careful elucidation of such pathways may be crucial in identifying new treatment strategies. Biologic therapies are increasingly available and targeting the right system is key.

This study provided evidence for vitamin D deficiency in sarcoidosis. Vitamin D has a range of modulatory activities on both innate and adaptive immunity. Vitamin D deficiency affects bacterial clearance (526, 527), can alter DC function and morphology inducing a more tolerogenic, immature state (528-530), which leads to reduced antigen presentation, reduced IL-12 and increased IL-10 production. Calcitriol is reported to inhibit T cell IL-2 and IL-17 (531), and, as confirmed in this study, downregulates monocyte TLRs.

Additionally, high-dose calcitriol supplementation in healthy humans (1µg twice daily for 7 days) has been shown to lead to a significant reduction in peripheral mononuclear cell IL-6 production (532). Calcitriol is involved in the regulation of T cell proliferation, suppression of T cell proliferation, Treg induction, and the inhibition of TH1 (IL-2, IFNγ, TNFα) cytokines and promotion of Th2 cytokines (IL-4, IL-5, IL-10) (533-535). I would therefore like to examine the effect on of vitamin D supplementation on monocyte activity (445). Such a study would include assays of serum calcitriol in addition to calcidiol, assessment of monocyte cytokine, fusion and phagocytic function as well as T cell suppressive capacity pre and post vitamin D supplementation in healthy human subjects.

8.3. Final conclusions

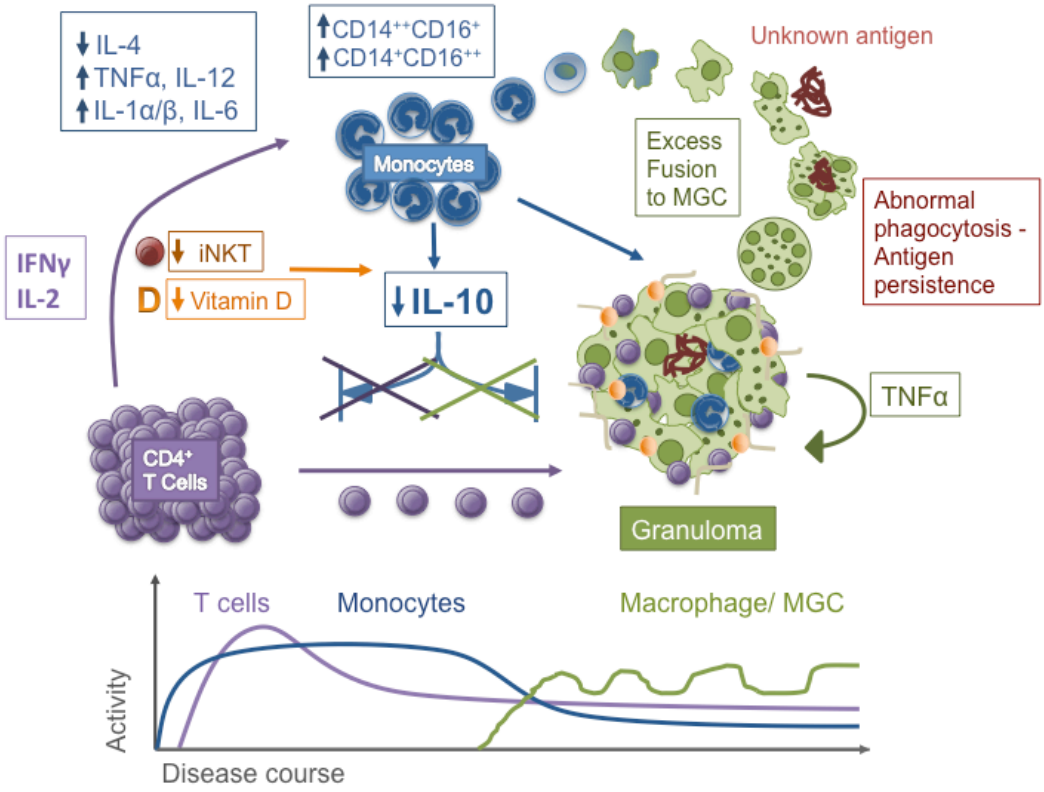


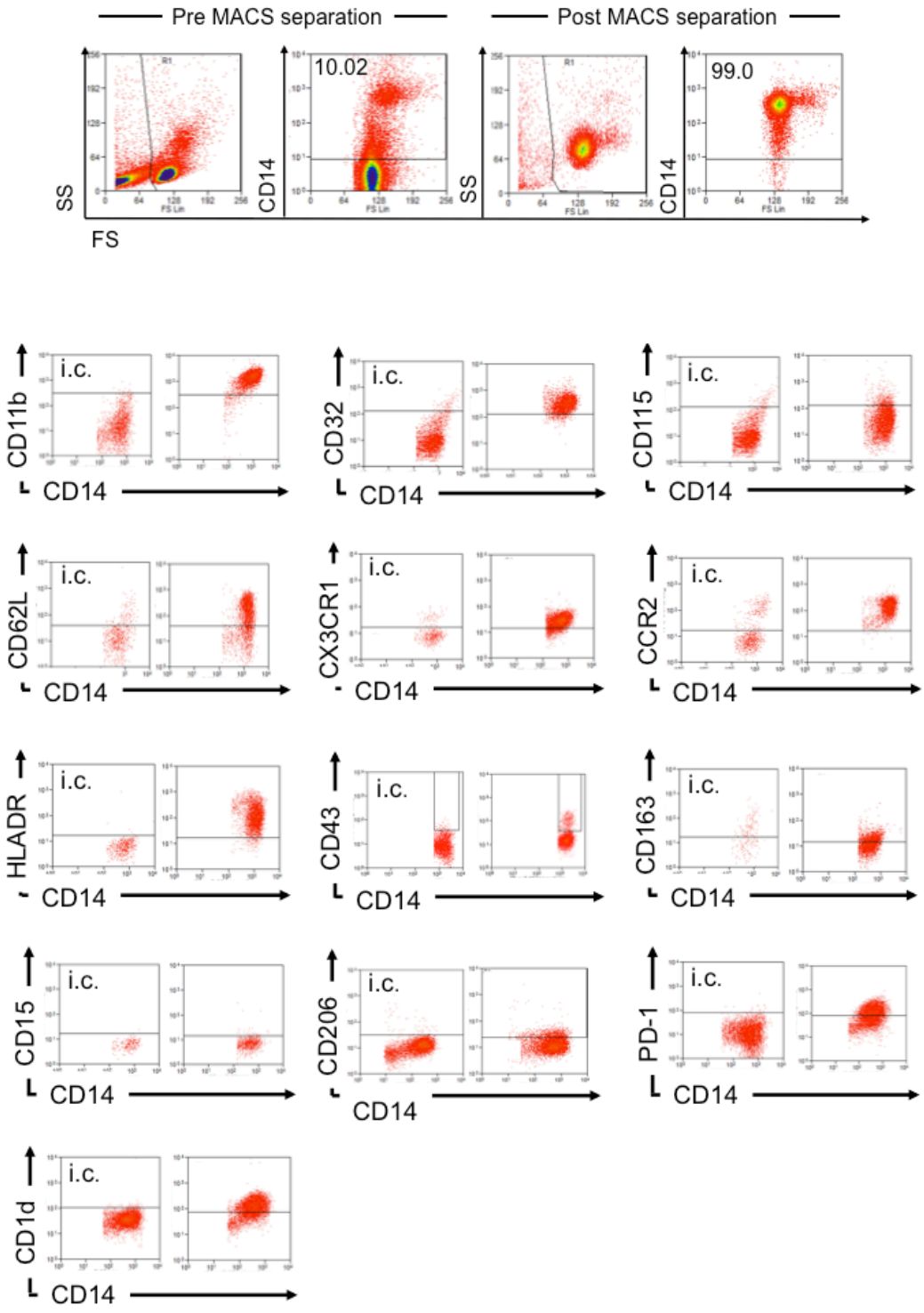
Figure 8.1. Aberrant monocyte activity in sarcoidosis.
Proposed model of aberrant monocyte activity in sarcoidosis: Monocytes are recruited to the site of pathology where they may remain as monocytes, fuse to form MGC or mature to macrophages. Altered monocyte cytokine activity is heightened by deficiencies in iNKT and vitamin D status, resulting in deficient monocyte IL-10 production. There is a consequent loss of monocyte T cell suppressive capacity, increased fusion to MGC, which contribute to granulomagenesis. Granuloma formation is heightened by abnormal antigen clearance, an expanded T cell compartment and increased TNFα. Monocyte activity is crucial, alongside T_H1 cells, in early disease pathogenesis, later giving way to the immune granuloma. Exogenous vitamin D can rescue the monocyte IL-10 deficit in sarcoidosis.

In summary, this thesis provides evidence for abnormal monocyte function in sarcoidosis centering on defective IL-10 production (Figure 8.1). Restoring monocyte IL-10 capacity has the potential to ameliorate aberrant monocyte MDSC function and regulate excess fusion to MGC. iNKT cells and vitamin D were shown to rescue monocyte IL-10 restoring monocyte control of T cell

proliferation and reducing fusion respectively; and the use of vitamin D has the potential to be useful in monocyte manipulation in the clinical setting.

Appendices

Appendix 1. FACS plots illustrating representative purity after MACS selection and staining with isotype controls (figures 3.4- 3.7)



Appendix 2: Kendrick Y, Helm E, Benamore R, Ho LP. **A Combination Score Of Raised sACE, Lymphopenia And Hypergammaglobulinemia Correlates With Active Disease On Thoracic CT Scan Of Patients With Pulmonary Sarcoidosis.** Am J Respir Crit Care Med 187;2013:A1391.

Rationale. Clinical decisions about treatment in pulmonary sarcoidosis, and phenotyping research studies are hampered by inability to objectively measure disease activity. We first reviewed 160 consecutive patients from our Sarcoidosis Clinic and observed that raised sACE, immunoglobulin and/or presence of lymphopenia, and CXR changes were associated with clinical decisions to change treatment. We question if these markers could be combined with extent (rather tham stage) of disease on CXR to form an objective and quantifiable measure of disease activity in pulmonary sarcoidosis. **Methods.** As there is no 'gold standard' for disease activity in sarcoidosis, we used high-resolution thoracic CT scan to quantify disease activity on the basis that ground glass opacity, nodularity, consolidation, interlobular septal thickening and conglomeration reflect T cell alveolitis, cellular infiltrate and granulomatous deposits1-2. Using Fleischner Society definition of terms3, we first designed a scoring system to quantify this ("CT activity score"; CTAS). Then we defined a composite clinical score ("clinical activity score"; CAS incorporating the following - IgG>13 g/l= 1, Lymphocytes <1x10^9/l = 1, sACE <55 U/l = 0, 56-100 U/l = 1, >100 U/l = 2, number of CXR 'zones' affected by specific radiographic abnormalities. We collected data from 100 consecutive patients diagnosed according to WASOG/ATS criteria (with histological confirmation), who had thoracic CT scan, CXR and blood parameters within three months of each other. Two radiologists scored the CT scans blindly; and the analysis were done by two blinded investigators. We report here, results for the first 70 cases. **Results.** CTAS score for the cases (maximum possible - 81) ranged from 0 to 61; CAS from 1 to 12 (maximum 12). We found a significant correlation between CAS and CTAS with ($r^2=0.4$; $p<0.0001$). No correlation was observed between the CTAS and FVC, TLCO or KCO, supporting the premise that losses in lung function ('severe disease') can co-exist with inactive disease, and vice versa. **Conclusion.** A combination score incorporating lymphopenia, sACE levels, and hypergammaglobulinemia with extent of abnormalities on CXR is strongly correlated with CT quantification of disease activity in pulmonary sarcoidosis. This score could be used as an indicator of disease activity to aid clinical decisions on treatment and responses to drugs, and paves the way for collation of larger numbers and longitudinal studies to further validate the tool.

1. Nishimura K Radiology 1993
2. Oberstein A Sarc Vasc Diffuse Lung Dis 1997
3. Hansell DM Radiology 2008

Appendix 3. GSE accession details and experiments for gene data sets

Gene set	GEOID/ Platform	Experiment design
Upregulated after IL-10 treatment Downregulated after IL-10	GSE35683/ Affymetrix	LPS stimulated human monocytes isolated from cord blood ± IL-10 10^{-8}M for 4 hours.
Upregulated after Vitamin D treatment Downregulated after Vitamin D treatment	GSE26268/ Affymetrix	Monocytes from 4 healthy control donors ± calcitriol 10^{-8}M for 18 hours
M1 polarisation M2 polarisation	GSE5099/ Affymetrix	Monocytes treated with M-CSF ± either LPS & IFNγ or IL-4.
Upregulated in sarcoidosis whole blood Down regulated in sarcoidosis whole blood	GSE19314/ Affymetrix	Whole blood RNA from 37 patients with sarcoidosis and 20 healthy controls.
Sarcoidosis PBMC signature	GSE397912/ Affymetrix	RNA isolated from PBMCs of 37 sarcoidosis donors with both complicated and 37 healthy controls.

Appendix 4. Full differentially expressed gene list at p<0.05 and Log2Fc >1.5 in sarcoidosis compared with controls

Alias	Log2 FC	Adjusted P Value	Description
IL6	4.723	0.001	Homo sapiens interleukin 6 (interferon, beta 2) (IL6), mRNA.
CCL4L1	4.616	0.001	Homo sapiens chemokine (C-C motif) ligand 4-like 1 (CCL4L1), mRNA.
CCL4L2	4.549	0.002	Homo sapiens chemokine (C-C motif) ligand 4-like 2 (CCL4L2), mRNA.
LOC728835	4.449	0.001	PREDICTED: Homo sapiens similar to cytokine, transcript variant 3 (LOC728835), mRNA.
HBB	4.370	0.001	Homo sapiens hemoglobin, beta (HBB), mRNA.
HBA2	4.269	0.000	Homo sapiens hemoglobin, alpha 2 (HBA2), mRNA.
CCL20	4.212	0.002	Homo sapiens chemokine (C-C motif) ligand 20 (CCL20), mRNA.
CCL3L1	4.186	0.002	Homo sapiens chemokine (C-C motif) ligand 3-like 1 (CCL3L1), mRNA.
C15ORF48	4.107	0.000	Homo sapiens chromosome 15 open reading frame 48 (C15orf48), transcript variant 2, mRNA.
HBA2	4.080	0.000	Homo sapiens hemoglobin, alpha 2 (HBA2), mRNA.
CXCL2	4.057	0.000	Homo sapiens chemokine (C-X-C motif) ligand 2 (CXCL2), mRNA.
HBA1	3.965	0.000	Homo sapiens hemoglobin, alpha 1 (HBA1), mRNA.
CCL3L1	3.699	0.003	Homo sapiens chemokine (C-C motif) ligand 3-like 1 (CCL3L1), mRNA.
CCL3L1	3.687	0.002	Homo sapiens chemokine (C-C motif) ligand 3-like 1 (CCL3L1), mRNA.
CCL3	3.470	0.003	Homo sapiens chemokine (C-C motif) ligand 3 (CCL3), mRNA.
CD83	3.373	0.001	Homo sapiens CD83 molecule (CD83), transcript variant 1, mRNA.
G0S2	3.333	0.001	Homo sapiens G0/G1switch 2 (G0S2), mRNA.
CD83	3.310	0.001	Homo sapiens CD83 molecule (CD83), transcript variant 2, mRNA.
SOD2	3.184	0.000	Homo sapiens superoxide dismutase 2, mitochondrial (SOD2), nuclear gene encoding mitochondrial protein, transcript variant 3, mRNA.
MIR155HG	3.117	0.002	Homo sapiens MIR155 host gene (non-protein coding) (MIR155HG), non-coding RNA.
EBI2	3.068	0.001	Homo sapiens Epstein-Barr virus induced gene 2 (lymphocyte-specific G protein-coupled receptor) (EBI2), mRNA.
IL1A	2.987	0.001	Homo sapiens interleukin 1, alpha (IL1A), mRNA.
CCL3L3	2.983	0.003	Homo sapiens chemokine (C-C motif) ligand 3-like 3 (CCL3L3), mRNA.
LRRC50	2.981	0.001	Homo sapiens leucine rich repeat containing 50 (LRRC50), mRNA.
IL1B	2.952	0.001	Homo sapiens interleukin 1, beta (IL1B), mRNA.
EBI2	2.904	0.000	Homo sapiens Epstein-Barr virus induced gene 2 (lymphocyte-specific G protein-coupled receptor) (EBI2), mRNA.
PHLDA1	2.880	0.000	Homo sapiens pleckstrin homology-like domain, family A, member 1 (PHLDA1), mRNA.
IL8	2.872	0.008	Homo sapiens interleukin 8 (IL8), mRNA.
PTGS2	2.775	0.002	Homo sapiens prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2), mRNA.
PDE4B	2.769	0.001	Homo sapiens phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila) (PDE4B), transcript variant a, mRNA.

CD69	2.763	0.000	Homo sapiens CD69 molecule (CD69), mRNA.
GNLY	2.676	0.000	Homo sapiens granulysin (GNLY), transcript variant 519, mRNA.
ZC3H12A	2.554	0.000	Homo sapiens zinc finger CCCH-type containing 12A (ZC3H12A), mRNA.
C15ORF48	2.543	0.000	Homo sapiens chromosome 15 open reading frame 48 (C15orf48), transcript variant 2, mRNA.
PTGS2	2.508	0.003	Homo sapiens prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2), mRNA.
PMAIP1	2.488	0.001	Homo sapiens phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1), mRNA.
PFKFB3	2.488	0.000	Homo sapiens 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), mRNA.
FFAR2	2.467	0.001	Homo sapiens free fatty acid receptor 2 (FFAR2), mRNA.
C13ORF15	2.447	0.000	Homo sapiens chromosome 13 open reading frame 15 (C13orf15), mRNA.
CCL14	2.431	0.004	Homo sapiens chemokine (C-C motif) ligand 14 (CCL14), transcript variant 2, mRNA.
IRAK2	2.420	0.003	Homo sapiens interleukin-1 receptor-associated kinase 2 (IRAK2), mRNA.
THBS1	2.403	0.001	Homo sapiens thrombospondin 1 (THBS1), mRNA.
DEFA1	2.384	0.003	Homo sapiens defensin, alpha 1 (DEFA1), mRNA.
IFNB1	2.329	0.004	Homo sapiens interferon, beta 1, fibroblast (IFNB1), mRNA.
IL1RN	2.325	0.004	Homo sapiens interleukin 1 receptor antagonist (IL1RN), transcript variant 1, mRNA.
TNFAIP3	2.302	0.004	Homo sapiens tumor necrosis factor, alpha-induced protein 3 (TNFAIP3), mRNA.
PIM3	2.298	0.000	PREDICTED: Homo sapiens pim-3 oncogene (PIM3), mRNA.
NLRP3	2.290	0.000	Homo sapiens NLR family, pyrin domain containing 3 (NLRP3), transcript variant 1, mRNA.
PDE4B	2.289	0.001	Homo sapiens phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila) (PDE4B), transcript variant a, mRNA.
SLC7A5	2.280	0.000	Homo sapiens solute carrier family 7 (cationic amino acid transporter, y+ system), member 5 (SLC7A5), mRNA.
GNLY	2.274	0.000	Homo sapiens granulysin (GNLY), transcript variant NKG5, mRNA.
CD247	2.229	0.000	Homo sapiens CD247 molecule (CD247), transcript variant 1, mRNA.
NLRP3	2.207	0.001	Homo sapiens NLR family, pyrin domain containing 3 (NLRP3), transcript variant 3, mRNA.
EGR3	2.204	0.000	Homo sapiens early growth response 3 (EGR3), mRNA.
KLRB1	2.201	0.000	Homo sapiens killer cell lectin-like receptor subfamily B, member 1 (KLRB1), mRNA.
SLC25A24	2.187	0.001	Homo sapiens solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 24 (SLC25A24), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA.
ADM	2.182	0.001	Homo sapiens adrenomedullin (ADM), mRNA.
PFKFB3	2.171	0.000	Homo sapiens 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), mRNA.
NAMPT	2.161	0.000	Homo sapiens nicotinamide phosphoribosyltransferase (NAMPT), mRNA.
RIPK2	2.137	0.000	Homo sapiens receptor-interacting serine-threonine kinase 2 (RIPK2), mRNA.
PLAUR	2.126	0.001	Homo sapiens plasminogen activator, urokinase receptor (PLAUR), transcript variant 1, mRNA.
RGS1	2.109	0.000	Homo sapiens regulator of G-protein signaling 1 (RGS1), mRNA.
IL1RN	2.109	0.004	Homo sapiens interleukin 1 receptor antagonist (IL1RN), transcript variant 4, mRNA.
PLA1A	2.103	0.002	Homo sapiens phospholipase A1 member A (PLA1A), mRNA.
CD247	2.101	0.000	Homo sapiens CD247 molecule (CD247), transcript variant 2, mRNA.

DEFA1B	2.096	0.005	Homo sapiens defensin, alpha 1B (DEFA1B), mRNA.
DUSP5	2.078	0.000	Homo sapiens dual specificity phosphatase 5 (DUSP5), mRNA.
DEFA3	2.067	0.006	Homo sapiens defensin, alpha 3, neutrophil-specific (DEFA3), mRNA.
GZMH	2.031	0.003	Homo sapiens granzyme H (cathepsin G-like 2, protein h-CCPX) (GZMH), mRNA.
RNF19B	2.029	0.001	Homo sapiens ring finger protein 19B (RNF19B), mRNA.
CCL4	2.012	0.003	Homo sapiens chemokine (C-C motif) ligand 4 (CCL4), mRNA.
BTG3	1.988	0.000	Homo sapiens BTG family, member 3 (BTG3), mRNA.
TAGAP	1.986	0.001	Homo sapiens T-cell activation RhoGTPase activating protein (TAGAP), transcript variant 3, mRNA.
PLAUR	1.975	0.000	Homo sapiens plasminogen activator, urokinase receptor (PLAUR), transcript variant 2, mRNA.
GZMA	1.968	0.001	Homo sapiens granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3) (GZMA), mRNA.
SOD2	1.961	0.001	Homo sapiens superoxide dismutase 2, mitochondrial (SOD2), nuclear gene encoding mitochondrial protein, transcript variant 2, mRNA.
ATF3	1.941	0.000	Homo sapiens activating transcription factor 3 (ATF3), transcript variant 4, mRNA.
NLRP3	1.933	0.003	Homo sapiens NLR family, pyrin domain containing 3 (NLRP3), transcript variant 1, mRNA.
SPOCK2	1.899	0.000	Homo sapiens sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2 (SPOCK2), mRNA.
PRDM8	1.887	0.003	Homo sapiens PR domain containing 8 (PRDM8), transcript variant 1, mRNA.
DUSP2	1.886	0.006	Homo sapiens dual specificity phosphatase 2 (DUSP2), mRNA.
SOCS3	1.884	0.000	Homo sapiens suppressor of cytokine signaling 3 (SOCS3), mRNA.
LOC643930	1.882	0.005	PREDICTED: Homo sapiens similar to Small inducible cytokine A3-like 1 precursor (Tonsillar lymphocyte LD78 beta protein) (LD78-beta(1-70)) (G0/G1 switch regulatory protein 19-2) (G0S19-2 protein) (PAT 464.2), transcript variant 2 (LOC643930), mRNA.
HS.534427	1.865	0.001	Human mRNA for T-cell specific protein
HS.184721	1.854	0.000	EST366269 MAGE resequences, MAGC Homo sapiens cDNA, mRNA sequence
ADORA2A	1.854	0.000	Homo sapiens adenosine A2a receptor (ADORA2A), mRNA.
BCL2A1	1.845	0.001	Homo sapiens BCL2-related protein A1 (BCL2A1), mRNA.
CYP4B1	1.832	0.000	Homo sapiens cytochrome P450, family 4, subfamily B, polypeptide 1 (CYP4B1), mRNA.
F3	1.825	0.005	Homo sapiens coagulation factor III (thromboplastin, tissue factor) (F3), mRNA.
GADD45B	1.814	0.001	Homo sapiens growth arrest and DNA-damage-inducible, beta (GADD45B), mRNA.
FGFBP2	1.813	0.002	Homo sapiens fibroblast growth factor binding protein 2 (FGFBP2), mRNA.
PELI1	1.802	0.002	Homo sapiens pellino homolog 1 (Drosophila) (PELI1), mRNA.
GZMB	1.800	0.000	Homo sapiens granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1) (GZMB), mRNA.
MAFF	1.799	0.000	Homo sapiens v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian) (MAFF), transcript variant 1, mRNA.
HS.554324	1.765	0.001	full-length cDNA clone CS0DI056YK21 of Placenta Cot 25-normalized of Homo sapiens (human)
LOC387763	1.753	0.002	PREDICTED: Homo sapiens hypothetical LOC387763 (LOC387763), mRNA.
GCH1	1.739	0.003	Homo sapiens GTP cyclohydrolase 1 (GCH1), transcript variant 3, mRNA.
TAGAP	1.725	0.001	Homo sapiens T-cell activation RhoGTPase activating protein (TAGAP), transcript variant 2, mRNA.
IL2RB	1.718	0.001	Homo sapiens interleukin 2 receptor, beta (IL2RB), mRNA.
CD3D	1.714	0.003	Homo sapiens CD3d molecule, delta (CD3-TCR complex) (CD3D), transcript variant 1, mRNA.

CCL4L2	1.702	0.005	Homo sapiens chemokine (C-C motif) ligand 4-like 2 (CCL4L2), mRNA.
PRF1	1.702	0.002	Homo sapiens perforin 1 (pore forming protein) (PRF1), transcript variant 1, mRNA.
CD3D	1.697	0.003	Homo sapiens CD3d molecule, delta (CD3-TCR complex) (CD3D), transcript variant 2, mRNA.
CD44	1.688	0.001	Homo sapiens CD44 molecule (Indian blood group) (CD44), transcript variant 1, mRNA.
NFIL3	1.664	0.000	Homo sapiens nuclear factor, interleukin 3 regulated (NFIL3), mRNA.
HS.25892	1.662	0.000	Homo sapiens cDNA FLJ38860 fis, clone MESAN2011977
OTUD1	1.653	0.001	PREDICTED: Homo sapiens OTU domain containing 1 (OTUD1), mRNA.
ICAM1	1.635	0.000	Homo sapiens intercellular adhesion molecule 1 (CD54), human rhinovirus receptor (ICAM1), mRNA.
PIM1	1.626	0.000	Homo sapiens pim-1 oncogene (PIM1), mRNA.
TAGAP	1.621	0.000	Homo sapiens T-cell activation RhoGTPase activating protein (TAGAP), transcript variant 3, mRNA.
AXUD1	1.619	0.004	Homo sapiens AXIN1 up-regulated 1 (AXUD1), mRNA.
GPR56	1.605	0.002	Homo sapiens G protein-coupled receptor 56 (GPR56), transcript variant 3, mRNA.
C7ORF40	1.602	0.001	Homo sapiens chromosome 7 open reading frame 40 (C7orf40), non-coding RNA.
NKG7	1.596	0.003	Homo sapiens natural killer cell group 7 sequence (NKG7), mRNA.
MIR302C	1.589	0.000	Homo sapiens microRNA 302c (MIR302C), microRNA.
GPR56	1.581	0.002	Homo sapiens G protein-coupled receptor 56 (GPR56), transcript variant 2, mRNA.
IER3	1.579	0.004	Homo sapiens immediate early response 3 (IER3), mRNA.
MAP3K8	1.575	0.002	Homo sapiens mitogen-activated protein kinase kinase kinase 8 (MAP3K8), mRNA.
PRDM1	1.575	0.001	Homo sapiens PR domain containing 1, with ZNF domain (PRDM1), transcript variant 1, mRNA.
CD79A	1.575	0.001	Homo sapiens CD79a molecule, immunoglobulin-associated alpha (CD79A), transcript variant 2, mRNA.
BCL11A	1.574	0.000	Homo sapiens B-cell CLL/lymphoma 11A (zinc finger protein) (BCL11A), transcript variant 5, mRNA.
TMEM88	1.573	0.000	Homo sapiens transmembrane protein 88 (TMEM88), mRNA.
ETS2	1.569	0.003	Homo sapiens v-ets erythroblastosis virus E26 oncogene homolog 2 (avian) (ETS2), mRNA.
FLJ36644	1.565	0.005	PREDICTED: Homo sapiens hypothetical gene supported by AK093963 (FLJ36644), mRNA.
PIM2	1.561	0.000	Homo sapiens pim-2 oncogene (PIM2), mRNA.
PTGER2	1.553	0.000	Homo sapiens prostaglandin E receptor 2 (subtype EP2), 53kDa (PTGER2), mRNA.
CD69	1.549	0.000	Homo sapiens CD69 molecule (CD69), mRNA.
NFKBIZ	1.546	0.006	Homo sapiens nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta (NFKBIZ), transcript variant 2, mRNA.
TP53INP2	1.540	0.001	Homo sapiens tumor protein p53 inducible nuclear protein 2 (TP53INP2), mRNA.
PMAIP1	1.532	0.005	Homo sapiens phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1), mRNA.
ERN1	1.531	0.000	Homo sapiens endoplasmic reticulum to nucleus signalling 1 (ERN1), transcript variant 2, mRNA.
CD8A	1.515	0.006	Homo sapiens CD8a molecule (CD8A), transcript variant 2, mRNA.
MYC	1.514	0.005	Homo sapiens v-myc myelocytomatosis viral oncogene homolog (avian) (MYC), mRNA.
CLCF1	1.511	0.000	Homo sapiens cardiotrophin-like cytokine factor 1 (CLCF1), transcript variant 1, mRNA.
NAMPT	1.508	0.000	Homo sapiens nicotinamide phosphoribosyltransferase (NAMPT), mRNA.

PPP1R15A	1.504	0.003	Homo sapiens protein phosphatase 1, regulatory (inhibitor) subunit 15A (PPP1R15A), mRNA.
PHACTR1	1.503	0.004	Homo sapiens phosphatase and actin regulator 1 (PHACTR1), mRNA.
MYC	1.503	0.004	Homo sapiens v-myc myelocytomatosis viral oncogene homolog (avian) (MYC), mRNA.
TXNIP	-1.521	0.003	Homo sapiens thioredoxin interacting protein (TXNIP), mRNA.
TNFRSF1A	-1.540	0.000	Homo sapiens tumor necrosis factor receptor superfamily, member 1A (TNFRSF1A), mRNA.
HPS6	-1.712	0.000	Homo sapiens Hermansky-Pudlak syndrome 6 (HPS6), mRNA.
RAB11FIP1	-1.765	0.000	Homo sapiens RAB11 family interacting protein 1 (class I) (RAB11FIP1), transcript variant 3, mRNA.
P2RY13	-1.771	0.001	Homo sapiens purinergic receptor P2Y, G-protein coupled, 13 (P2RY13), transcript variant 1, mRNA.
HSPA1A	-1.884	0.000	Homo sapiens heat shock 70kDa protein 1A (HSPA1A), mRNA.
GIMAP4	-2.032	0.001	Homo sapiens GTPase, IMAP family member 4 (GIMAP4), mRNA.
FRAT2	-2.116	0.000	Homo sapiens frequently rearranged in advanced T-cell lymphomas 2 (FRAT2), mRNA.
CCR2	-2.242	0.000	Homo sapiens chemokine (C-C motif) receptor 2 (CCR2), transcript variant B, mRNA.
GIMAP8	-2.333	0.000	Homo sapiens GTPase, IMAP family member 8 (GIMAP8), mRNA.
CCR2	-2.414	0.000	Homo sapiens chemokine (C-C motif) receptor 2 (CCR2), transcript variant A, mRNA.
CCR2	-2.471	0.001	Homo sapiens chemokine (C-C motif) receptor 2 (CCR2), transcript variant A, mRNA.
GIMAP7	-2.712	0.001	Homo sapiens GTPase, IMAP family member 7 (GIMAP7), mRNA.

Appendix 5. Top 50 differentially expressed pathways in sarcoidosis compared with controls.

#	Pathways	p-value	FDR	In Data	Total
1	Immune response_IL-18 signaling	3.929e-11	3.041e-8	24/60	60
2	Immune response_HSP60 and HSP70/ TLR signaling pathway	1.227e-9	4.750e-7	21/54	51
3	Transcription_NF-kB activation pathways	2.568e-9	6.625e-7	20/51	57
4	Immune response_TLR2 and TLR4 signaling pathways	3.913e-9	7.571e-7	21/57	54
5	Transport_Clathrin-coated vesicle cycle	1.256e-8	1.945e-6	23/71	71
6	Apoptosis and survival_Role of PKR in stress-induced apoptosis	3.643e-8	3.585e-6	19/53	53
7	Immune response_HMGB1/RAGE signaling pathway	3.643e-8	3.585e-6	19/53	48
8	Immune response_TLR5, TLR7, TLR8 and TLR9 signaling pathways	3.706e-8	3.585e-6	18/48	53
9	Immune response_HMGB1/TLR signaling pathway	9.895e-8	8.510e-6	15/36	111
10	Immune response_ICOS pathway in T-helper cell	1.143e-7	8.848e-6	17/46	36
11	Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling	1.512e-7	1.000e-5	28/111	46
12	Signal transduction_Activation of PKC via G-Protein coupled receptor	1.551e-7	1.000e-5	18/52	57
13	Mucin expression in CF airways	1.815e-7	1.081e-5	21/69	52
14	Immune response_IL-1 signaling pathway	3.507e-7	1.939e-5	16/44	69
15	Immune response_TNF-R2 signaling pathways	5.009e-7	2.585e-5	16/45	53
16	Immune response_CD28 signaling	5.534e-7	2.677e-5	18/56	39
17	Immune response_Role of PKR in stress-induced antiviral cell response	7.444e-7	3.389e-5	18/57	44
18	Neuroprotective action of lithium	8.159e-7	3.395e-5	19/63	55
19	Transcription_Role of Akt in hypoxia induced HIF1 activation	8.335e-7	3.395e-5	12/27	45
20	Ovarian cancer (main signaling cascades)	1.068e-6	3.940e-5	19/64	84
21	Apoptosis and survival_Anti-apoptotic TNFs/NF-kB/Bcl-2 pathway	1.069e-6	3.940e-5	15/42	56
22	Immune response_T cell receptor signaling pathway	1.181e-6	3.975e-5	17/53	27
23	Development_WNT signaling pathway. Part 2	1.181e-6	3.975e-5	17/53	63
24	Immune response _IFN gamma signaling pathway	1.587e-6	5.082e-5	17/54	42
25	Development_VEGF signaling via VEGFR2 - generic cascades	1.641e-6	5.082e-5	22/84	64
26	Development_Flt3 signaling	2.112e-6	5.643e-5	15/44	53
27	K-RAS signaling in pancreatic cancer	2.112e-6	5.643e-5	15/44	53
28	Apoptosis and survival_Endoplasmic reticulum stress response pathway	2.114e-6	5.643e-5	17/55	54
29	Immune response_Fc epsilon RI pathway	2.114e-6	5.643e-5	17/55	71
30	Transcription_P53 signaling pathway	2.283e-6	5.889e-5	14/39	49
31	Transcription_Transcription regulation of aminoacid metabolism	2.735e-6	6.827e-5	11/25	49
32	Immune response_IL-33 signaling pathway	3.666e-6	8.599e-5	17/57	44
33	Transcription_Epigenetic regulation of gene expression	3.666e-6	8.599e-5	17/57	44
34	Development_EGFR signaling pathway	5.932e-6	1.313e-4	19/71	25

35	Development_Differentiation of white adipocytes	5.938e-6	1.313e-4	16/53	55
36	Immune response_TREM1 signaling pathway	6.174e-6	1.327e-4	17/59	57
37	Immune response_IL-17 signaling pathways	7.932e-6	1.659e-4	17/60	57
38	Apoptosis and survival_TNFR1 signaling pathway	8.543e-6	1.740e-4	14/43	31
39	Immune response_IL-2 activation and signaling pathway	9.592e-6	1.856e-4	15/49	36
40	Development_PEDF signaling	9.592e-6	1.856e-4	15/49	64
41	Transcription_Sin3 and NuRD in transcription regulation	1.154e-5	2.179e-4	14/44	42
42	Immune response_C5a signaling	1.262e-5	2.272e-4	15/50	42
43	Development_TGF-beta receptor signaling	1.262e-5	2.272e-4	15/50	59
44	CFTR folding and maturation (normal and CF)	1.420e-5	2.497e-4	10/24	43
45	Cytoskeleton remodeling_Cytoskeleton remodeling	1.473e-5	2.503e-4	23/102	60
46	Immune response_Inflammasome in inflammatory response	1.488e-5	2.503e-4	12/34	60
47	Immune response_IL-15 signaling	2.030e-5	3.279e-4	17/64	49
48	G-protein signaling_RhoB regulation pathway	2.085e-5	3.279e-4	8/16	55
49	Development_Angiopoietin - Tie2 signaling	2.087e-5	3.279e-4	12/35	44
50	Development_Prolactin receptor signaling	2.118e-5	3.279e-4	16/58	44

Appendix 6. Top 50 differentially expressed process networks in sarcoidosis compared with controls.

#	Networks	P - value	FDR	In data
1	Inflammation: IL-10 anti-inflammatory response	1.673e-8	1.480e-6	34/87
2	Transcription: mRNA processing	1.862e-8	1.480e-6	51/160
3	Inflammation: Inflammasome	3.103e-7	1.645e-5	39/118
4	Transcription: Nuclear receptors transcriptional regulation	4.054e-6	1.611e-4	53/197
5	Immune response: TCR signaling	5.480e-6	1.743e-4	48/174
6	Cardiac development: Role of NADPH oxidase and ROS	2.188e-5	4.324e-4	38/133
7	Transcription: Transcription by RNA polymerase II	1.231e-5	3.262e-4	44/159
8	Cell cycle: G1-S Interleukin regulation	2.107e-5	4.324e-4	37/128
9	DNA damage: Checkpoint	2.448e-5	4.324e-4	36/124
10	Cell adhesion: Leucocyte chemotaxis	1.250e-4	1.175e-3	50/205
11	Cell cycle: Mitosis	2.816e-5	4.478e-4	47/179
12	Apoptosis: Anti-apoptosis mediated by external signals via NF-kB	3.117e-5	4.505e-4	33/111
13	Inflammation: IL-2 signaling	1.300e-4	1.175e-3	30/104
14	Development: Hemopoiesis, Erythropoietin pathway	2.003e-4	1.274e-3	36/136
15	Development: Blood vessel morphogenesis	1.550e-4	1.175e-3	54/228
16	Apoptosis: Death Domain receptors & caspases in apoptosis	5.025e-5	6.322e-4	35/123
17	Transcription: Chromatin modification	5.169e-5	6.322e-4	36/128
18	Apoptosis: Apoptotic mitochondria	1.645e-4	1.175e-3	24/77
19	Inflammation_MIF signaling	1.700e-4	1.175e-3	37/140
20	Inflammation_NK cell cytotoxicity	1.367e-4	1.175e-3	42/164
21	DNA damage_BER-NER repair	1.618e-4	1.175e-3	31/110
22	Inflammation_IFN-gamma signaling	1.618e-4	1.175e-3	31/110
23	Development_Regulation of angiogenesis	1.619e-4	1.175e-3	53/223
24	Inflammation_Innate inflammatory response	1.684e-4	1.175e-3	45/181
25	Signal Transduction_Cholecystokinin signaling	1.890e-4	1.252e-3	30/106
26	Translation_Regulation of initiation	5.231e-4	2.327e-3	33/127
27	Protein folding_Response to unfolded proteins	2.117e-4	1.295e-3	22/69
28	Signal transduction_ESR1-nuclear pathway	2.541e-4	1.443e-3	51/216
29	Immune response_BCR pathway	2.343e-4	1.380e-3	36/137
30	Apoptosis_Endoplasmic reticulum stress pathway	4.925e-4	2.303e-3	25/87
31	Protein folding_Folding in normal condition	3.275e-4	1.796e-3	32/119
32	Inflammation_TREM1 signaling	3.628e-4	1.897e-3	37/145
33	Immune response_T helper cell differentiation	3.698e-4	1.897e-3	36/140
34	Proliferation_Lymphocyte proliferation	3.978e-4	1.977e-3	49/209
35	Cell cycle_G2-M	5.270e-4	2.327e-3	48/206
36	Signal transduction_Androgen receptor nuclear signaling	4.487e-4	2.162e-3	33/126
37	Inflammation_IL-6 signaling	1.556e-3	5.621e-3	30/119
38	Apoptosis_Apoptotic nucleus	5.985e-4	2.572e-3	39/159

39	Proteolysis_Proteolysis in cell cycle and apoptosis	8.334e-4	3.313e-3	32/125
40	Immune response_Innate immune response to RNA viral infection	6.940e-4	2.885e-3	24/84
41	Signal transduction_NOTCH signaling	7.075e-4	2.885e-3	53/236
42	Translation_Translation in mitochondria	2.309e-3	8.158e-3	41/181
43	Chemotaxis	4.079e-3	1.272e-2	32/137
44	Cell cycle_G1-S Growth factor regulation	9.653e-4	3.744e-3	45/195
45	Signal transduction_WNT signaling	2.714e-3	9.235e-3	40/177
46	Signal Transduction_TGF-beta, GDF and Activin signaling	1.231e-3	4.661e-3	37/154
47	Signal transduction_ESR2 pathway	2.755e-3	9.235e-3	21/77
48	Cytoskeleton_Cytoplasmic microtubules	3.769e-3	1.198e-2	28/115
49	Proteolysis_Ubiquitin-proteasomal proteolysis	1.444e-3	5.340e-3	39/166
50	DNA damage_DBS repair	4.294e-3	1.288e-2	28/116

Appendix 7. Genes differentially expressed in high activity compared with low activity sarcoidosis.

Alias	Adjusted P Value	logFC	Description
SERPINB2	3.593E-04	2.993E+00	Homo sapiens serpin peptidase inhibitor, clade B (ovalbumin), member 2 (SERPINB2), mRNA.
SERPINB2	2.835E-04	2.916E+00	Homo sapiens serpin peptidase inhibitor, clade B (ovalbumin), member 2 (SERPINB2), mRNA.
IFIT3	1.009E-02	2.864E+00	Homo sapiens interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), mRNA.
MIR155HG	1.564E-02	2.809E+00	Homo sapiens MIR155 host gene (non-protein coding) (MIR155HG), non-coding RNA.
IRAK2	1.713E-04	2.797E+00	Homo sapiens interleukin-1 receptor-associated kinase 2 (IRAK2), mRNA.
LOC730249	3.211E-05	2.659E+00	PREDICTED: Homo sapiens similar to Immune-responsive protein 1 (LOC730249), mRNA.
C15ORF48	5.363E-03	2.649E+00	Homo sapiens chromosome 15 open reading frame 48 (C15orf48), transcript variant 2, mRNA.
TNFAIP6	6.694E-04	2.594E+00	Homo sapiens tumor necrosis factor, alpha-induced protein 6 (TNFAIP6), mRNA.
IFIT3	2.254E-02	2.584E+00	Homo sapiens interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), mRNA.
UPB1	1.713E-04	2.520E+00	Homo sapiens ureidopropionase, beta (UPB1), mRNA.
LRRC50	9.576E-03	2.395E+00	Homo sapiens leucine rich repeat containing 50 (LRRC50), mRNA.
IL1A	3.943E-02	2.391E+00	Homo sapiens interleukin 1, alpha (IL1A), mRNA.
MSC	2.067E-04	2.388E+00	Homo sapiens musculin (activated B-cell factor-1) (MSC), mRNA.
CXCL1	1.584E-02	2.320E+00	Homo sapiens chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha) (CXCL1), mRNA.
IFIT3	1.490E-02	2.294E+00	Homo sapiens interferon-induced protein with tetratricopeptide repeats 3 (IFIT3), mRNA.
C15ORF48	6.041E-04	2.276E+00	Homo sapiens chromosome 15 open reading frame 48 (C15orf48), transcript variant 2, mRNA.
PRDM8	2.401E-04	2.231E+00	Homo sapiens PR domain containing 8 (PRDM8), transcript variant 1, mRNA.
F3	1.083E-03	2.221E+00	Homo sapiens coagulation factor III (thromboplastin, tissue factor) (F3), mRNA.
NLRP3	1.133E-03	2.210E+00	Homo sapiens NLR family, pyrin domain containing 3 (NLRP3), transcript variant 1, mRNA.
OASL	8.120E-03	2.128E+00	Homo sapiens 2'-5'-oligoadenylate synthetase-like (OASL), transcript variant 2, mRNA.
KCNJ2	2.186E-02	2.082E+00	Homo sapiens potassium inwardly-rectifying channel, subfamily J, member 2 (KCNJ2), mRNA.
F3	1.033E-04	2.079E+00	Homo sapiens coagulation factor III (thromboplastin, tissue factor) (F3), mRNA.
SAMSN1	9.853E-04	2.001E+00	Homo sapiens SAM domain, SH3 domain and nuclear localization signals 1 (SAMSN1), mRNA.
PLA1A	2.541E-02	1.926E+00	Homo sapiens phospholipase A1 member A (PLA1A), mRNA.
CCL2	7.531E-03	1.867E+00	Homo sapiens chemokine (C-C motif) ligand 2 (CCL2), mRNA.
SLC7A5	6.507E-04	1.862E+00	Homo sapiens solute carrier family 7 (cationic amino acid transporter, y+ system), member 5 (SLC7A5), mRNA.
PHACTR1	1.713E-04	1.859E+00	Homo sapiens phosphatase and actin regulator 1 (PHACTR1), mRNA.
TNFAIP8	1.425E-03	1.859E+00	Homo sapiens tumor necrosis factor, alpha-induced protein 8 (TNFAIP8), transcript variant 2, mRNA.
ACSL1	8.208E-04	1.858E+00	Homo sapiens acyl-CoA synthetase long-chain family

			member 1 (ACSL1), mRNA.
CH25H	1.665E-02	1.837E+00	Homo sapiens cholesterol 25-hydroxylase (CH25H), mRNA.
MYC	1.234E-03	1.835E+00	Homo sapiens v-myc myelocytomatosis viral oncogene homolog (avian) (MYC), mRNA.
TRAF3IP2	3.274E-03	1.833E+00	Homo sapiens TRAF3 interacting protein 2 (TRAF3IP2), transcript variant 2, mRNA.
OLR1	1.234E-03	1.811E+00	Homo sapiens oxidized low density lipoprotein (lectin-like) receptor 1 (OLR1), mRNA.
LOC387763	5.919E-03	1.789E+00	PREDICTED: Homo sapiens hypothetical LOC387763 (LOC387763), mRNA.
MN1	3.838E-04	1.769E+00	Homo sapiens meningioma (disrupted in balanced translocation) 1 (MN1), mRNA.
MYC	2.553E-03	1.736E+00	Homo sapiens v-myc myelocytomatosis viral oncogene homolog (avian) (MYC), mRNA.
ADM	1.724E-02	1.711E+00	Homo sapiens adrenomedullin (ADM), mRNA.
SDC4	6.507E-04	1.710E+00	Homo sapiens syndecan 4 (SDC4), mRNA.
TNFSF8	5.289E-04	1.706E+00	Homo sapiens tumor necrosis factor (ligand) superfamily, member 8 (TNFSF8), mRNA.
AQP9	1.546E-03	1.702E+00	Homo sapiens aquaporin 9 (AQP9), mRNA.
IRG1	1.015E-04	1.687E+00	PREDICTED: Homo sapiens immunoresponsive 1 homolog (mouse) (IRG1), mRNA.
WTAP	8.357E-04	1.681E+00	Homo sapiens Wilms tumor 1 associated protein (WTAP), transcript variant 1, mRNA.
SERPINB9	6.805E-04	1.662E+00	Homo sapiens serpin peptidase inhibitor, clade B (ovalbumin), member 9 (SERPINB9), mRNA.
GPR18	1.713E-04	1.656E+00	Homo sapiens G protein-coupled receptor 18 (GPR18), transcript variant 2, mRNA.
DRAM1	6.805E-04	1.650E+00	Homo sapiens DNA-damage regulated autophagy modulator 1 (DRAM1), mRNA.
PMAIP1	1.678E-02	1.638E+00	Homo sapiens phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1), mRNA.
SAMSN1	3.639E-04	1.638E+00	Homo sapiens SAM domain, SH3 domain and nuclear localization signals 1 (SAMSN1), mRNA.
FLJ36644	1.937E-02	1.635E+00	PREDICTED: Homo sapiens hypothetical gene supported by AK093963 (FLJ36644), mRNA.
AK3L1	6.805E-04	1.630E+00	Homo sapiens adenylate kinase 3-like 1 (AK3L1), nuclear gene encoding mitochondrial protein, transcript variant 6, mRNA.
MAP1LC3A	9.568E-04	1.623E+00	Homo sapiens microtubule-associated protein 1 light chain 3 alpha (MAP1LC3A), transcript variant 2, mRNA.
MAP3K8	7.129E-03	1.604E+00	Homo sapiens mitogen-activated protein kinase kinase kinase 8 (MAP3K8), mRNA.
ADORA2A	6.204E-04	1.603E+00	Homo sapiens adenosine A2a receptor (ADORA2A), mRNA.
FPR2	3.426E-04	1.585E+00	Homo sapiens formyl peptide receptor 2 (FPR2), transcript variant 1, mRNA.
PELI1	2.330E-02	1.582E+00	Homo sapiens pellino homolog 1 (Drosophila) (PELI1), mRNA.
MGC39372	6.335E-04	1.581E+00	PREDICTED: Homo sapiens hypothetical protein MGC39372 (MGC39372), mRNA.
LOC652226	1.780E-03	1.581E+00	PREDICTED: Homo sapiens hypothetical protein LOC652226 (LOC652226), mRNA.
AHR	6.335E-04	1.568E+00	Homo sapiens aryl hydrocarbon receptor (AHR), mRNA.
WTAP	2.386E-03	1.566E+00	Homo sapiens Wilms tumor 1 associated protein (WTAP), transcript variant 3, mRNA.
CCRL2	5.069E-03	1.559E+00	Homo sapiens chemokine (C-C motif) receptor-like 2 (CCRL2), transcript variant 2, mRNA.
B4GALT5	8.105E-04	1.559E+00	Homo sapiens UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 5 (B4GALT5), mRNA.
HIF1A	1.544E-02	1.541E+00	Homo sapiens hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)

			(HIF1A), transcript variant 1, mRNA.
PHLDA1	3.223E-02	1.529E+00	Homo sapiens pleckstrin homology-like domain, family A, member 1 (PHLDA1), mRNA.
STEAP4	8.208E-04	1.529E+00	Homo sapiens STEAP family member 4 (STEAP4), mRNA.
LAMB3	6.694E-04	1.522E+00	Homo sapiens laminin, beta 3 (LAMB3), transcript variant 1, mRNA.
CLIC4	3.287E-03	1.518E+00	Homo sapiens chloride intracellular channel 4 (CLIC4), nuclear gene encoding mitochondrial protein, mRNA.
IL2RB	2.706E-03	-1.507E+00	Homo sapiens interleukin 2 receptor, beta (IL2RB), mRNA.
PRF1	2.415E-02	-1.532E+00	Homo sapiens perforin 1 (pore forming protein) (PRF1), transcript variant 1, mRNA.
GZMA	2.582E-02	-1.538E+00	Homo sapiens granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3) (GZMA), mRNA.
TXNIP	8.372E-03	-1.544E+00	Homo sapiens thioredoxin interacting protein (TXNIP), mRNA.
CD247	1.611E-03	-1.608E+00	Homo sapiens CD247 molecule (CD247), transcript variant 2, mRNA.
CD247	1.644E-03	-1.639E+00	Homo sapiens CD247 molecule (CD247), transcript variant 1, mRNA.
HS.534427	1.564E-02	-1.654E+00	Human mRNA for T-cell specific protein
FGFBP2	1.759E-02	-1.662E+00	Homo sapiens fibroblast growth factor binding protein 2 (FGFBP2), mRNA.
GNLY	2.706E-03	-1.789E+00	Homo sapiens granulysin (GNLY), transcript variant 519, mRNA.

Appendix 8. All significantly positively enriched gene sets with p<0.05 in sarcoidosis compared with controls.

#	Name	ES	NES	NOM p-val	FDR q-val
1	Inflammatory response	0.716	2.224	0.000	0.000
2	Response to wounding	0.673	2.198	0.000	0.000
3	Mono upreg after il_10	0.618	2.122	0.000	0.000
4	Response to external stimulus	0.612	2.107	0.000	0.000
5	Immune system process	0.601	2.106	0.000	0.000
6	Behavior	0.695	2.100	0.000	0.000
7	Negative regulation of programmed cell death	0.642	2.087	0.000	0.000
8	Locomotory behavior	0.707	2.082	0.000	0.000
9	Immune response	0.613	2.076	0.000	0.000
10	Negative regulation of developmental process	0.623	2.069	0.000	0.000
11	Downreg sarc wb	0.608	2.066	0.000	0.000
12	Negative regulation of apoptosis	0.642	2.050	0.000	0.000
13	Viral infectious cycle	0.832	2.027	0.000	0.000
14	Viral reproduction	0.773	2.016	0.000	0.000
15	Negative regulation of cell proliferation	0.623	1.988	0.000	0.001
16	Viral genome replication	0.884	1.953	0.000	0.002
17	Cell cell signaling	0.579	1.944	0.000	0.003
18	Defence response	0.566	1.943	0.000	0.003
19	Anti apoptosis	0.615	1.925	0.000	0.003
20	Viral reproductive process	0.788	1.924	0.002	0.003
21	Regulation of cell proliferation	0.552	1.909	0.000	0.004
22	Homeostatic process	0.578	1.886	0.000	0.006
23	Humoral immune response	0.766	1.870	0.000	0.007
24	Negative regulation of biological process	0.500	1.864	0.000	0.007
25	Negative regulation of cellular process	0.497	1.845	0.000	0.009
26	Regulation of nucleocytoplasmic transport	0.824	1.843	0.004	0.009
27	Homeostasis of number of cells	0.777	1.834	0.004	0.010
28	Regulation of intracellular transport	0.796	1.795	0.004	0.019
29	Regulation of dna binding	0.672	1.793	0.000	0.019
30	Chemical homeostasis	0.577	1.792	0.003	0.019
31	Positive regulation of cell proliferation	0.580	1.790	0.000	0.019
32	Cell surface receptor linked signal transduction	0.495	1.789	0.000	0.018
33	Regulation of cell adhesion	0.726	1.789	0.004	0.018
34	Regulation of binding	0.651	1.784	0.002	0.018
35	Apoptosis	0.487	1.783	0.000	0.018
36	Cation homeostasis	0.612	1.781	0.002	0.018
37	Cell proliferation	0.492	1.777	0.000	0.018
38	G protein coupled receptor protein signaling pathway	0.535	1.770	0.000	0.020
39	Cellular cation homeostasis	0.618	1.767	0.000	0.020
40	Programmed cell death	0.487	1.761	0.000	0.021

41	Sarcoidosi pbmc gene signature	0.707	1.757	0.000	0.021
42	Reproductive process	0.587	1.746	0.002	0.024
43	Ion homeostasis	0.579	1.742	0.000	0.024
44	Protein import into nucleus	0.645	1.740	0.006	0.024
45	Cellular homeostasis	0.560	1.733	0.000	0.027
46	Regulation of transcription factor activity	0.668	1.731	0.006	0.027
47	Regulation of transport	0.638	1.726	0.006	0.027
48	Negative regulation of translation	0.751	1.720	0.008	0.029
49	Negative regulation of biosynthetic process	0.714	1.718	0.007	0.028
50	Negative regulation of cellular biosynthetic process	0.729	1.712	0.012	0.030
51	Nuclear import	0.623	1.706	0.008	0.031
52	Response to stress	0.466	1.700	0.000	0.033
53	Cell development	0.458	1.697	0.000	0.033
54	Response to chemical stimulus	0.485	1.675	0.000	0.041
55	Regulation of programmed cell death	0.465	1.645	0.000	0.053
56	Protein import	0.593	1.637	0.000	0.055
57	Regulation of apoptosis	0.465	1.636	0.000	0.055
58	Regulation of biological quality	0.460	1.629	0.000	0.058
59	Wound healing	0.618	1.618	0.012	0.063
60	Regulation of developmental process	0.446	1.618	0.000	0.063
61	Negative regulation of cellular protein metabolic process	0.611	1.605	0.018	0.069
62	Cell cycle arrest	0.581	1.601	0.019	0.070
63	Cellular component disassembly	0.626	1.586	0.024	0.079
64	Negative regulation of protein metabolic process	0.590	1.578	0.018	0.083
65	Cell activation	0.550	1.577	0.017	0.082
66	Negative regulation of cell cycle	0.540	1.568	0.013	0.087
67	Regulation of mitotic cell cycle	0.688	1.557	0.043	0.094
68	Protein amino acid phosphorylation	0.443	1.547	0.000	0.101
69	Blood coagulation	0.595	1.543	0.038	0.103
70	Negative regulation of cell differentiation	0.688	1.536	0.048	0.108
71	Multi organism process	0.490	1.535	0.013	0.106
72	Mono up regulation after vitd	0.451	1.527	0.003	0.112
73	Leukocyte activation	0.554	1.521	0.029	0.115
74	Metal ion transport	0.532	1.515	0.029	0.118
75	Apoptotic program	0.545	1.512	0.029	0.119
76	Phosphorylation	0.431	1.511	0.002	0.119
77	Reproduction	0.468	1.508	0.014	0.120
78	Response to virus	0.568	1.502	0.042	0.124
79	Coagulation	0.595	1.498	0.047	0.125
80	Cytokine production	0.519	1.478	0.048	0.144

Appendix 9. 31-gene sarcoidosis PBMC gene signature.

Alias	Description
CD247	CD247 molecule
CD28	CD28 molecule
CD3D	CD3d molecule, delta (CD3-TCR complex)
CDE	CD3e molecule, epsilon (CD3-TCR complex)
CD3G	CD3g molecule, gamma (CD3-TCR complex)
CD8A	CD8a molecule
CBLB	Cas-Br—M (murine) ecotropic retroviral transforming sequence b
GRAP2	GRB2-related adaptor protein 2
ITK	IL2-inducible T cell kinase
NCK1	NCK adaptor protein
RASGRP1	RAS guanyl releasing protein 1 (calcium and DAG-regulated)
DLG1	Disc, large homologue1 (Drosophila)
ICOS	Inducible T cell co-stimulator
IFNG	Interferon gamma
IL7R	Interleukin 7 receptor
JUN	Jun oncogene
LCK	Lymphocyte-specific protein tyrosine kinase
MAPK9	Mitogen-activated protein kinase 9
MALT1	Mucosa associated lymphoid tissue lymphoma translocation gene 1
NFATC3	Nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 2
NFATC2	Nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 3
PIK3CA	Phosphoinositide-3-kinase, catalytic alpha polypeptide
PLCG1	Phospholipase C, gamma1
AKT3	v-akt murine thymoma viral oncogene homologue 3 (protein kinase B, gamma)
ZAP70	Zeta-chain (TCR) associated protein kinase 70kDa
CCND2	Cyclin D2
IL2RA	Interleukin 2 receptor, alpha
IL2RB	Interleukin 2 receptor, beta
STAT4	Signal transducer and activator of transcription 4
SPRED1	Sprouty-related, EVH1 domain containing 1
SOCS4	Suppressor of cytokine signalling 4

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